MULTIPLEXED ANGIOGENIC BIOMARKER QUANTIFICATION ON SINGLE CELLS

BY

SI CHEN

DISSERTATION

Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Bioengineering in the Graduate College of the University of Illinois at Urbana-Champaign, 2018

Urbana, Illinois

Doctoral Committee:

Associate Professor Princess Imoukhuede, Chair Professor Rohit Bhargava Professor Brendan Harley Professor Catherine Murphy Associate Professor Andrew Smith

ABSTRACT

Clinical and biomedical research seeks single-cell quantification to better understand their roles in a complex, multi-cell environment. Recently, quantification of vascular endothelial growth factor receptors (VEGFRs) provided important insights into endothelial cell (EC) characteristics and response in tumor microenvironments. However, data on other angiogenic receptors, such as platelet derived growth factor receptors (PDGFRs), Tie receptors, are also necessary for the development of an accurate angiogenesis model.

To gain insights on the involvement of these angiogenic receptors in angiogenesis, I develop a method to quantify receptor concentrations as well as the cell-by-cell heterogeneity. I establish protocols to measure cell membrane VEGFR, NRP1, Tie2, and PDGFR concentration on several cell and tissue models including human dermal fibroblasts (HDFs) *in vitro*, a 2D endothelial/fibroblast co-culture model *in vitro*, and a patient-derived xenograft (PDX) model of glioblastoma (GBM). I demonstrate VEGF-A₁₆₅-mediated downregulation of membrane PDGFR α (~25%) and PDGFR β (~30%) on HDFs, following a 24-hour treatment. This supports the idea that VEGF-A₁₆₅ acts independently of VEGFRs to signal through PDGFR α and PDGFR β . I uncover high intratumoral heterogeneity within the GBM PDX model, with tumor EC-like subpopulations having high concentrations of membrane VEGFR1, VEGFR2, EGFR, IGFR, and PDGFRs.

To gain greater insights into cell heterogeneity and examine angiogenic signaling pathways as a whole, I utilize the unique spectral properties of quantum dots (Qdots), and combines Qdots with qFlow cytometry, to dually quantify VEGFR1 and VEGFR2 on human umbilical vein endothelial cells (HUVECs). To enable this quantification, I reduce nonspecific binding between Qdot-conjugated antibodies and cells, identify optimal labeling conditions, and establish that 800 – 20,000 is the dynamic range where accurate Qdot-enabled quantification can be achieved. Through these optimizations we demonstrate measurement of 1,100 VEGFR1 and 6,900 VEGFR2 per HUVEC. 24 h VEGF-A₁₆₅ treatment induce ~90% upregulation of VEGFR1 and ~30% downregulation of VEGFR2 concentration. We further analyze HUVEC heterogeneity and observe that 24 h VEGF-A₁₆₅ treatment induces ~15% decrease in VEGFR2 heterogeneity. Overall, we demonstrate experimental and analysis strategies for quantifying two or more RTKs at single-level using Qdots, which will provide new insights into biological systems.

ii

献给我的父亲和母亲:

陈振祥和龚银钗

ACKNOWLEDGEMENTS

I dedicate this thesis to my parents, Zhenxiang and Yinchai, who have always supported me, near or far, to pursue a life of continuous learning. I owe to them my values, work ethic and courage to explore the unknown. I thank my incredibly supportive boyfriend Chris for being patient and compassionate through the completion of my degree. I am grateful for the guidance and inspiration from my friends, coworkers, and those who have become both, especially Spencer Mamer, Ali Ansari, and Wendy Woods. I also thank Jared Weddell, Brendan Harley, Thien Le, Kareem Al-Qadi, Xinyi Guo, Osazomon Imarenezor, Brendan Mathias, Pavan Gupta, Grace Conard, and James Parkin, who have taken part in the collaborative efforts of this thesis. Finally, I thank my research advisor, Princess Imoukhuede, an inspiring mentor, leader, and a brilliant scientist who has given me tremendous opportunities for the past five and half years to learn a broad range of scientific disciplines, the intellectual freedom to explore beyond my work, the encouragement to work harder than I have ever thought possible, and a role model I can only hope to live up to.

TABLE OF CONTENTS

CHAI	PTER 1 INTRODUCTION1		
1.1	BACKGROUND AND SIGNIFICANCE		
1.2	THESIS GOALS		
1.3	REFERENCES		
CHAPTER 2 THE DEVELOPMENT OF QFLOW CYTOMETRY-BASED			
BIOMARKER SCREENING 11			
2.1	INTRODUCTION12		
2.2	MATERIALS 16		
2.3	METHODS		
2.4	NOTES		
2.5	FIGURES AND FIGURE LEGENDS		
2.6	TABLE		
2.7	REFERENCES		
CHAPTER 3 QUANTIFICATION OF VEGFRS, NRP1, AND PDGFRS ON			
END	OTHELIAL CELLS AND FIBROBLASTS REVEALS SERUM, INTRA-FAMILY		
LIGA	ND, AND CROSS-FAMILY LIGAND REGULATION 46		
3.1	INTRODUCTION		
3.2	MATERIALS AND METHODS 50		
3.3	RESULTS		
3.4	DISCUSSION		
3.5	CONCLUSIONS		
3.6	FIGURES AND FIGURE LEGENDS 70		
3.7	TABLES		
3.8	REFERENCES		

СНА	PTER 4 A 2D ENDOTHELIAL/FIBROBLAST CO-CULTURE MODEL		
4.1	INTRODUCTION		
4.2	MATERIALS AND METHODS		
4.3	RESULTS AND DISCUSSION	100	
4.4	CONCLUSIONS	105	
4.5	FIGURES AND FIGURE LEGENDS	106	
4.6	REFERENCES	115	
СНА	PTER 5 CHARACTERIZING GLIOBLASTOMA HETEROGENEITY V	/IA	
SING	GLE-CELL RECEPTOR QUANTIFICATION	118	
5.1	INTRODUCTION	119	
5.2	RESULTS AND DISCUSSION	123	
5.3	CONCLUSIONS	130	
5.4	FIGURES AND FIGURE LEGENDS	133	
5.5	REFERENCES	135	
CHAPTER 6 MULTIPLEXING ANGIOGENIC RECEPTOR QUANTIFICATION VIA			
QUA	NTUM DOTS		
6.1	INTRODUCTION	148	
6.2	MATERIALS AND METHODS	150	
6.3	RESULTS AND DISCUSSION	156	
6.4	CONCLUSIONS		
6.5	FIGURES AND FIGURE LEGENDS	167	
6.6	REFERENCES	177	
APPENDIX A: CURRENT STATE-OF-THE-ART AND FUTURE DIRECTIONS IN			
SYST	TEMS BIOLOGY		

CHAPTER 1 INTRODUCTION

1.1 BACKGROUND AND SIGNIFICANCE

Over 70 diseases rely on angiogenesis, the process of blood vessels forming from existing ones ¹. For example, abnormal angiogenesis promotes tumor development in several cancers ^{2,3}. Drugs that inhibit vascular endothelial growth factor (VEGF), like bevacizumab ⁴, sequester VEGF but only moderately improve patient survival, and these patients eventually become resistant to these drugs ^{5,6}. Clearly, we must pursue new approaches to understand anti-VEGF resistance in these vascular dysfunctions.

The involvement of other signaling axes potentially explains anti-VEGF failings. After anti-VEGF treatment, several growth factors, including platelet derived growth factors (PDGFs)^{7,8} are upregulated. Under cross-axis signaling, upregulated PDGFs would directly activate VEGFRs and lead to anti-VEGF therapy resistance. PDGFs also contribute to vascular function. PDGF (-AA, -BB, -AB, -CC, and –DD) binding to corresponding PDGFRs (α and β) maintains and stabilizes EC tubes during development ⁹, induces vessel growth ¹⁰ and regeneration ¹¹, and induces reperfusion after arterial blockage¹². The fact that both VEGFs and PDGFs are key regulators of angiogenesis implies that shifting focus from VEGF-family to dual-family and ultimately, to multi-family (other important angiogenesis families) would more accurately represent angiogenesis.

In addition to the complexity of signaling pathways, increasing evidence suggests that intratumoral heterogeneity may contribute to therapeutic resistance ^{13–17}. The emergence of single-cell omics tools has enabled researchers to map individual cell heterogeneity. Using a

patient-derived xenograft model, a single-cell analysis of patient-derived xenografts (PDXs) of GBM39 also found higher heterogeneity in resistant tumors than in responsive tumors ¹⁸. Our lab and others have shown VEGFR1 heterogeneity may be contributing to anti-VEGF resistance in various cancer types ^{13,19–21}. Therefore, it is important to support single-cell technologies that facilitate resolving such heterogeneity with new computational approaches ^{22–25}.

Many single-cell analysis methods are low-throughput and only semi-quantitative. Immunohistochemistry (IHC) is the accepted standard for receptor profiling in tissue ²⁶. While IHC provides relative protein expression mapping, it does not provide cell-by-cell readouts, multiplexing, or absolute receptor quantification needed for modeling. Many studies examine receptor mRNA and total protein levels as a proxy for membrane receptors ^{27–30}. However, correlations across mRNA levels and total protein can be inconsistent ³¹ or protein-specific ³², and correlations between total protein and membrane receptors require insight into trafficking ³³. This reveals a need to quantitatively profile receptors.

Three primary methods are used for receptor quantification, each with advantages and limitations. Radiolabeling, a reliable approach used in early biochemical studies ^{34,35}, may be less accessible to laboratories due to safety considerations ^{36,37}, specialized training requirements ³⁸, and disposal challenges. Mass spectrometry (MS) can be high-throughput; however, stringent technique is required and sensitive to sample preparation artifacts ³⁹. Fluorescence is an ideal technique. Its ease of use makes it more attractive than radiolabeling, and its lower detection limit (<100 molecules/cell vs. ~7,500 molecules/cell MS) makes it more attractive than MS. We have led efforts to fluorescently quantify VEGFRs ^{19,40,41}. We detect VEGFRs via PE-conjugated antibodies and calibrate via commercially available PE beads ^{42,43}. Its brightness, relative

robustness to photobleaching, and 1:1 antibody binding make PE an advantageous fluorophore. However, its broad emission spectrum limits quantification to a single readout (single-color) ^{44,45}. As we shift towards integrating multi-axis signaling, receptor quantification must move towards multiplexing (multi-color).

Quantum dots (Qdots) are ideal tools for multiplexing: they emit within a narrow and largely symmetric range, allowing simultaneous monitoring of multiple Qdot-conjugates ^{46,47}. Moreover, Qdots are brighter and 100x more resistant to photobleaching than organic fluorophores. Qdots have enabled simultaneous imaging of eight biomarkers ⁴⁸ and more sensitive detection of early cancer biomarkers ⁴⁹. Towards quantification, Yezhelyev et al.⁵⁰ pioneered a relative comparison of receptor expression (HER2, ER, PR); however, this study did not provide absolute receptor concentrations. Previously, Wu et al.^{51,52} described difficulties encountered when providing absolute Qdot concentrations. They postulated that Qdot valency (up to eight streptavidin tetrameric units per Qdot) makes biotin polystyrene beads unsuitable for calibration beads and developed a molecular assembly, M2 beads. With the M2 beads, they restricted Qdot-antibody binding to achieve a 1:1 ratio. Still, this system only quantified one receptor: EGFR. Therefore, there remains a need for multiplexed receptor quantification via Qdots.

1.2 THESIS GOALS

This thesis aims to provide the experimental framework and applied engineering strategies for the development of qFlow cytometry for quantitative detection of angiogenic biomarkers and for advancing systems biology. This goal is approached through three directions. First, an experimental framework and data analysis strategy is developed. This is provided by an extensive protocol of qFlow cytometry (Chapter 2), from cell staining to heterogeneity analysis,

as well as notes for troubleshooting. Second, a quantitative understanding of angiogenic biomarkers under normal condition and diseased is developed. This is achieved by applying qFlow cytometry to cell types associated with vessel formation in vitro (Chapter 3), a 2D coculture angiogenesis assay (Chapter 4), as well as by characterizing a panel of angiogenic biomarkers and tumor heterogeneity in a glioblastoma xenograft model (Chapter 5). Finally, the potential of combining quantum dots with qFlow cytometry to achieve multiplexed quantification is tested. This is performed by characterizing the performance of quantum dotconjugated antibodies, with particular emphasis on their specific and nonspecific binding, as well as the impact of their sizes, measuring limit and accuracy when combined with qFlow cytometry (Chapter 6). 1.3 REFERENCES

- P. Carmeliet, "Angiogenesis in life, disease and medicine.," *Nature*, vol. 438, no. 7070, pp. 932–6, Dec. 2005.
- [2] C. Xu, X. Wu, and J. Zhu, "VEGF promotes proliferation of human glioblastoma multiforme stem-like cells through VEGF receptor 2," *ScientificWorldJournal*, vol. 2013, p. 417413, 2013.
- P. Carmeliet and R. K. Jain, "Angiogenesis in cancer and other diseases," *Nature*, vol. 407, no. 6801, pp. 249–257, Sep. 2000.
- [4] N. Ferrara, "Discovery and development of bevacizumab, an anti-VEGF antibody for treating cancer," *Nat Rev Drug Discov*, vol. 3, pp. 391–400, 2004.
- [5] G. Bergers and D. Hanahan, "Modes of resistance to anti-angiogenic therapy.," *Nat. Rev. Cancer*, vol. 8, no. 8, pp. 592–603, Aug. 2008.
- Y. Crawford and N. Ferrara, "Tumor and stromal pathways mediating refractoriness/resistance to anti-angiogenic therapies.," *Trends Pharmacol. Sci.*, vol. 30, no. 12, pp. 624–30, Dec. 2009.
- [7] G. Bergers, S. Song, N. Meyer-Morse, E. Bergsland, and D. Hanahan, "Benefits of targeting both pericytes and endothelial cells in the tumor vasculature with kinase inhibitors," *J. Clin. Invest.*, vol. 111, no. 9, pp. 1287–1295, May 2003.
- [8] R. Erber, A. Thurnher, A. D. Katsen, G. Groth, H. Kerger, H.-P. P. Hammes, M. D. Menger, A. Ullrich, P. Vajkoczy, R. Erber, A. Thurnher, A. D. Katsen, G. Groth, H. Kerger, H.-P. P. Hammes, M. D. Menger, A. Ullrich, and P. Vajkoczy, "Combined inhibition of VEGF and PDGF signaling enforces tumor vessel regression by interfering with pericyte-mediated endothelial cell survival mechanisms.," *FASEB J.*, vol. 18, no. 2, pp. 338–340, Feb. 2004.
- [9] C. Betsholtz, L. Karlsson, and P. Lindahl, "Developmental roles of platelet-derived growth factors.," *Bioessays*, vol. 23, pp. 494–507, 2001.
- [10] D. M. Brown, S. P. Hong, C. L. Farrell, G. F. Pierce, and R. K. Khouri, "Platelet-Derived

Growth Factor BB Induces Functional Vascular Anastomoses In vivo," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 92. National Academy of Sciences, pp. 5920–5924.

- [11] V. Lindner and M. A. Reidy, "Platelet-derived growth factor ligand and receptor expression by large vessel endothelium in vivo.," *Am. J. Pathol.*, vol. 146, no. 6, pp. 1488–97, Jun. 1995.
- [12] R. N. Martins, J. O. Chleboun, P. Sellers, M. Sleigh, and J. Muir, "The Role of PDGF-BB on the Development of the Collateral Circulation after Acute Arterial Occlusion," *Growth Factors*, vol. 10, no. 4, pp. 299–306, Jul. 1994.
- [13] J. C. Weddell and P. I. Imoukhuede, "Quantitative characterization of cellular membrane-receptor heterogeneity through statistical and computational modeling.," *PLoS One*, vol. 9, no. 5, p. e97271, Jan. 2014.
- [14] D. L. Schonberg, D. Lubelski, T. E. Miller, and J. N. Rich, "Brain tumor stem cells: Molecular characteristics and their impact on therapy," *Mol. Aspects Med.*, vol. 39, pp. 82–101, 2014.
- [15] W. Wei, Y. S. Shin, M. Xue, T. Matsutani, K. Masui, H. Yang, S. Ikegami, Y. Gu, K. Herrmann, D. Johnson, X. Ding, K. Hwang, J. Kim, J. Zhou, Y. Su, X. Li, B. Bonetti, R. Chopra, C. D. James, W. K. Cavenee, T. F. Cloughesy, P. S. Mischel, J. R. Heath, and B. Gini, "Single-Cell Phosphoproteomics Resolves Adaptive Signaling Dynamics and Informs Targeted Combination Therapy in Glioblastoma," *Cancer Cell*, vol. 29, no. 4, pp. 563–573, 2016.
- [16] H. P. Ellis, M. Greenslade, B. Powell, I. Spiteri, A. Sottoriva, and K. M. Kurian, "Current Challenges in Glioblastoma: Intratumour Heterogeneity, Residual Disease, and Models to Predict Disease Recurrence.," *Front. Oncol.*, vol. 5, p. 251, 2015.
- [17] S. NA, F. Simpson, T. EW, H. MM, L. Endo-Munoz, G. Leggatt, M. RF, and A. Guminski, "Role of intratumoural heterogeneity in cancer drug resistance: molecular and clinical perspectives," *EMBO Mol Med*, vol. 4, pp. 675–684, 2011.
- [18] Q. Shi, L. Qin, W. Wei, F. Geng, R. Fan, Y. S. Shin, D. Guo, L. Hood, P. S. Mischel, and

J. R. Heath, "Single-cell proteomic chip for profiling intracellular signaling pathways in single tumor cells.," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 109, no. 2, pp. 419–24, Jan. 2012.

- [19] P. I. Imoukhuede and A. S. Popel, "Quantitative fluorescent profiling of VEGFRs reveals tumor cell and endothelial cell heterogeneity in breast cancer xenografts.," *Cancer Med.*, vol. 3, no. 2, pp. 225–44, Apr. 2014.
- [20] D. Lambrechts, H.-J. Lenz, S. de Haas, P. Carmeliet, and S. J. Scherer, "Markers of response for the antiangiogenic agent bevacizumab.," *J. Clin. Oncol.*, vol. 31, no. 9, pp. 1219–30, Mar. 2013.
- [21] A. J. Weickhardt, D. S. Williams, C. K. Lee, F. Chionh, J. Simes, C. Murone, K. Wilson, M. M. Parry, K. Asadi, A. M. Scott, C. J. A. Punt, I. D. Nagtegaal, T. J. Price, J. M. Mariadason, and N. C. Tebbutt, "Vascular endothelial growth factor D expression is a potential biomarker of bevacizumab benefit in colorectal cancer," *Br. J. Cancer*, vol. 113, no. 1, pp. 37–45, Jun. 2015.
- [22] J. R. Heath, A. Ribas, and P. S. Mischel, "Single-cell analysis tools for drug discovery and development.," *Nat. Rev. Drug Discov.*, vol. 15, no. 3, pp. 204–16, Mar. 2016.
- [23] E. D. Amir, K. L. Davis, M. D. Tadmor, E. F. Simonds, J. H. Levine, S. C. Bendall, D. K. Shenfeld, S. Krishnaswamy, G. P. Nolan, and D. Pe'er, "viSNE enables visualization of high dimensional single-cell data and reveals phenotypic heterogeneity of leukemia.," *Nat. Biotechnol.*, vol. 31, no. 6, pp. 545–52, Jun. 2013.
- [24] R. V. Bruggner, B. Bodenmiller, D. L. Dill, R. J. Tibshirani, and G. P. Nolan, "Automated identification of stratifying signatures in cellular subpopulations," *Proc. Natl. Acad. Sci.*, vol. 111, no. 26, pp. E2770–E2777, Jul. 2014.
- [25] D. A. Jaitin, E. Kenigsberg, H. Keren-Shaul, N. Elefant, F. Paul, I. Zaretsky, A. Mildner, N. Cohen, S. Jung, A. Tanay, and I. Amit, "Massively parallel single-cell RNA-seq for marker-free decomposition of tissues into cell types.," *Science*, vol. 343, no. 6172, pp. 776–9, Feb. 2014.
- [26] D. Faratian, J. Christiansen, M. Gustavson, C. Jones, C. Scott, I. Um, and D. J. Harrison,"Heterogeneity mapping of protein expression in tumors using quantitative

immunofluorescence.," J. Vis. Exp., no. 56, p. e3334, Jan. 2011.

- [27] J. Li, L. F. Brown, M. G. Hibberd, J. D. Grossman, J. P. Morgan, and M. Simons, "VEGF, flk-1, and flt-1 expression in a rat myocardial infarction model of angiogenesis.," *Am. J. Physiol.*, vol. 270, no. 5 Pt 2, pp. H1803-11, May 1996.
- [28] M. E. Gerritsen, J. E. Tomlinson, C. Zlot, M. Ziman, and S. Hwang, "Using gene expression profiling to identify the molecular basis of the synergistic actions of hepatocyte growth factor and vascular endothelial growth factor in human endothelial cells.," *Br. J. Pharmacol.*, vol. 140, no. 4, pp. 595–610, Oct. 2003.
- [29] M. Dougher and B. I. Terman, "Autophosphorylation of KDR in the kinase domain is required for maximal VEGF-stimulated kinase activity and receptor internalization.," *Oncogene*, vol. 18, no. 8, pp. 1619–27, Mar. 1999.
- [30] M. Duval, S. Bédard-Goulet, C. Delisle, and J.-P. Gratton, "Vascular endothelial growth factor-dependent down-regulation of Flk-1/KDR involves Cbl-mediated ubiquitination. Consequences on nitric oxide production from endothelial cells.," *J. Biol. Chem.*, vol. 278, no. 22, pp. 20091–7, May 2003.
- [31] Y. Guo, P. Xiao, S. Lei, F. Deng, G. G. Xiao, Y. Liu, X. Chen, L. Li, S. Wu, Y. Chen, H. Jiang, L. Tan, J. Xie, X. Zhu, S. Liang, and H. Deng, "How is mRNA expression predictive for protein expression? A correlation study on human circulating monocytes.," *Acta Biochim. Biophys. Sin. (Shanghai).*, vol. 40, no. 5, pp. 426–36, May 2008.
- [32] R. Bhargava, W. L. Gerald, A. R. Li, Q. Pan, P. Lal, M. Ladanyi, and B. Chen, "EGFR gene amplification in breast cancer: correlation with epidermal growth factor receptor mRNA and protein expression and HER-2 status and absence of EGFR-activating mutations.," *Mod. Pathol.*, vol. 18, no. 8, pp. 1027–33, Aug. 2005.
- [33] J. Xu, H. Chai, K. Ehinger, T. M. Egan, R. Srinivasan, M. Frick, and B. S. Khakh,
 "Imaging P2X4 receptor subcellular distribution, trafficking, and regulation using P2X4pHluorin.," *J. Gen. Physiol.*, vol. 144, no. 1, pp. 81–104, Jul. 2014.
- [34] J. Waltenberger, L. Claesson-Welsh, A. Siegbahn, M. Shibuya, and C. H. Heldin,"Different signal transduction properties of KDR and Flt1, two receptors for vascular

endothelial growth factor.," J. Biol. Chem., vol. 269, no. 43, pp. 26988–95, Oct. 1994.

- [35] S. Soker, H.-Q. Miao, M. Nomi, S. Takashima, and M. Klagsbrun, "VEGF165 mediates formation of complexes containing VEGFR-2 and neuropilin-1 that enhance VEGF165receptor binding.," *J. Cell. Biochem.*, vol. 85, no. 2, pp. 357–68, Jan. 2002.
- [36] A. Bahadori, Personnel protection and safety equipment for the oil and gas industries.
- [37] A. C. Patel and S. R. Matthewson, "Radiolabeling of Peptides and Proteins," in *Molecular Biomethods Handbook*, Totowa, NJ: Humana Press, 1998, pp. 411–411.
- [38] I. Obodovskiy, *Fundamentals of radiation and chemical safety*. Elsevier, 2015.
- [39] M. Breker and M. Schuldiner, "The emergence of proteome-wide technologies: systematic analysis of proteins comes of age," *Nat. Rev. Mol. Cell Biol.*, vol. 15, no. 7, pp. 453–464, Jun. 2014.
- [40] P. I. Imoukhuede and A. S. Popel, "Quantification and cell-to-cell variation of vascular endothelial growth factor receptors," *Exp. Cell Res.*, vol. 317, no. 7, pp. 955–965, 2011.
- [41] P. I. Imoukhuede, A. O. Dokun, B. H. Annex, and A. S. Popel, "Endothelial cell-by-cell profiling reveals temporal dynamics of VEGFR1 and VEGFR2 membrane-localization following murine hindlimb ischemia," *Am J Physiol Hear. Circ Physiol*, vol. 4, no. 8, pp. H1085-93, 2013.
- [42] S. Lyer, J. Bishop, B. Abrams, V. Maino, A. Ward, T. Christian, and K. Davis, *QuantiBRITE: a new standard for PE flourescence quantitation. In: White Paper.* San Jose, CA: Becton Dickinson Immunocytometry Systems, 1997.
- [43] K. K. Pannu, E. T. Joe, and S. B. Iyer, "Performance evaluation of QuantiBRITE phycoerythrin beads.," *Cytometry*, vol. 45, no. 4, pp. 250–8, Dec. 2001.
- [44] L. Wang, F. Abbasi, A. K. Gaigalas, R. F. Vogt, and G. E. Marti, "Comparison of fluorescein and phycoerythrin conjugates for quantifying CD20 expression on normal and leukemic B-cells.," *Cytometry B. Clin. Cytom.*, vol. 70, no. 6, pp. 410–5, Nov. 2006.
- [45] M. N. Kronick and P. D. Grossman, "Immunoassay techniques with fluorescent phycobiliprotein conjugates.," *Clin. Chem.*, vol. 29, no. 9, pp. 1582–6, Sep. 1983.

- [46] R. J. Byers and E. R. Hitchman, "Quantum Dots Brighten Biological Imaging," *Prog. Histochem. Cytochem.*, vol. 45, no. 4, pp. 201–237, 2011.
- [47] Y. Xing, Q. Chaudry, C. Shen, K. Y. Kong, H. E. Zhau, L. W. Chung, J. A. Petros, R. M. O'Regan, M. V Yezhelyev, J. W. Simons, M. D. Wang, and S. Nie, "Bioconjugated quantum dots for multiplexed and quantitative immunohistochemistry," *Nat. Protoc.*, vol. 2, no. 5, pp. 1152–1165, 2007.
- [48] P. K. Chattopadhyay, D. A. Price, T. F. Harper, M. R. Betts, J. Yu, E. Gostick, S. P. Perfetto, P. Goepfert, R. A. Koup, S. C. De Rosa, M. P. Bruchez, and M. Roederer, "Quantum dot semiconductor nanocrystals for immunophenotyping by polychromatic flow cytometry.," *Nat. Med.*, vol. 12, no. 8, pp. 972–7, Aug. 2006.
- [49] T. Y. Rakovich, O. K. Mahfoud, B. M. Mohamed, A. Prina-Mello, K. Crosbie-Staunton, T. Van Den Broeck, L. De Kimpe, A. Sukhanova, D. Baty, A. Rakovich, S. A. Maier, F. Alves, F. Nauwelaers, I. Nabiev, P. Chames, and Y. Volkov, "Highly Sensitive Single Domain Antibody–Quantum Dot Conjugates for Detection of HER2 Biomarker in Lung and Breast Cancer Cells," ACS Nano, vol. 8, no. 6, pp. 5682–5695, Jun. 2014.
- [50] M. V. Yezhelyev, A. Al-Hajj, C. Morris, A. I. Marcus, T. Liu, M. Lewis, C. Cohen, P. Zrazhevskiy, J. W. Simons, A. Rogatko, S. Nie, X. Gao, and R. M. O'Regan, "In Situ Molecular Profiling of Breast Cancer Biomarkers with Multicolor Quantum Dots," *Adv. Mater.*, vol. 19, no. 20, pp. 3146–3151, 2007.
- [51] T. Buranda, Y. Wu, and L. A. Sklar, "Quantam Dots for Quantitative Flow Cytometry," *Methods Mol. Biol.*, vol. 699, pp. 67–84, 2011.
- Y. Wu, G. P. Lopez, L. A. Sklar, and T. Buranda, "Spectroscopic characterization of streptavidin functionalized quantum dots," *Anal. Biochem.*, vol. 364, no. 2, pp. 193–203, 2007.

CHAPTER 2 THE DEVELOPMENT OF QFLOW CYTOMETRY-BASED BIOMARKER SCREENING ¹

Nanosensor-based detection of biomarkers can improve medical diagnosis; however, a critical factor in nanosensor development is deciding which biomarker to target, as most diseases present several biomarkers. Biomarker-targeting decisions can be informed via an understanding of biomarker expression. Currently, immunohistochemistry (IHC) is the accepted standard for profiling biomarker expression. While IHC provides a relative mapping of biomarker expression, it does not provide cell-by-cell readouts of biomarker expression or absolute biomarker quantification. Flow cytometry overcomes both of these IHC challenges by offering biomarker expression on a cell-by-cell basis, and when combined with calibration standards, providing quantitation of biomarker concentrations: this is known as qFlow cytometry. Here, we outline the key components for applying qFlow cytometry to detect biomarkers within the angiogenic vascular endothelial growth factor receptor family. The key aspects of the qFlow cytometry methodology include antibody specificity testing, immunofluorescent cell labeling, saturation analysis, fluorescent microsphere calibration, and quantitative analysis of both ensemble and cell-by-cell data. Together, these methods enable high-throughput quantification of biomarker expression.

¹ This chapter was published in Biomedical Nanotechnology as part of the Methods in Molecular Biology book series (MIMB, volume 1570). PMID: 28238133, DOI: <u>https://doi.org/10.1007/978-1-4939-6840-4_8</u>.

2.1 INTRODUCTION

2.1.1 Nanosensor-based detection of membrane angiogenic receptors can inform drug development

Nanosensors are playing an increasingly important role in biomedicine ^{1–3} with exciting new applications to cardiovascular diseases and cancer. This is due to the fact that nanosensors enable highly-sensitive, early-stage disease detection, which is linked with better clinical outcomes ⁴. For example, the use of RGD-peptide targeted Copper-64 (⁶⁴Cu)-quantum dots (QDs) as nanosensors to provide contrast for Cerenkov luminescence imaging has enabled atherosclerotic plaque detection in a rodent model ⁵. Similarly, $\alpha_{\nu}\beta_{3}$ -targeted Cu-nanoparticles have been coupled with photoacoustic imaging to visualize angiogenesis in a rodent model ⁶, and single-chain cysteine-tagged recombinant vascular endothelial growth factor (VEGF)-121 molecules have been used as nanosensors with near-infrared fluorescence imaging (NIRF) to assess VEGF₁₂₁ uptake in tumor-activated host vasculature in a mouse model ⁷. These examples demonstrate the immense potential of nanosensors in disease management, and highlight a common nanosensor feature: they can target specific biomarkers important to disease progression.

As new nanosensors are developed to guide therapy selection and disease management ⁸, nanosensor development requires considering the biomarker localization. Most diseases present several overexpressed plasma membrane and intracellular biomarkers, typically genes, proteins, or other biomolecules ^{9–11}. Therefore, determining the biomarker target is an important step in nanosensor development. The simplest biomarker targets are plasma membrane proteins: their extracellular residues render them highly-accessible. Conversely, intracellular biomarkers

require manipulating membrane permeability or cell trafficking to enable nanosensor binding. Thus, plasma membrane proteins are promising disease biomarkers.

Nanosensor development also requires considering biomarker abundance; biomarkers expressed at low levels require high-affinity nanosensors, whereas lower-affinity nanosensors may sufficiently target biomarkers expressed at high levels. To measure plasma membrane protein abundance on the cell scale, many studies examine mRNA and total protein expression using quantitative real time polymerase chain reaction (qRTPCR) and Western blot, respectively ^{12–16}. However, identifying plasma membrane protein abundance from these assays requires correlations between DNA or mRNA and translated protein that are inconsistent ¹⁷, protein-specific ¹⁸, or require trafficking insights ¹⁹. To measure plasma membrane protein abundance on the tissue scale, immunohistochemistry (IHC) is the employed standard ²⁰. However, IHC only provides a relative protein expression mapping within an area, including the membrane, and does not provide an absolute protein quantification or cell-by-cell protein expression analysis. Therefore, new high-resolution techniques are necessary for accurate plasma membrane protein quantification.

2.1.2 Rationale for quantitative flow cytometric assays

Traditional flow cytometry directly profiles membrane proteins using an affinity probe conjugated to a fluorophore, providing biomarker expression readout on the plasma membrane. Advantages of traditional flow cytometry are its amenability to live-cell analysis and its inherent multi-dimension data obtained. In particular, both fluorescence intensity and light scattering data are obtained on a cell-by-cell basis, providing cell-subpopulation information. Furthermore, biomarker expression can be dynamically observed in response to experimental parameters, such

as temperature or drug administration ²¹. However, a major disadvantage of traditional flow cytometry is its non-quantitative nature. Traditional flow cytometry provides a fluorescence signal correlating with protein abundance: higher fluorescence intensity indicates higher protein expression. To translate fluorescence intensity to protein abundance, housekeeping proteins (positive control) are used to provide comparative insight to target protein expression levels. This comparative estimation allows trends and differences in samples to be identified; however, these trends and differences in samples can be erroneous if the positive control is not fully established. For example, housekeeping proteins may exhibit unexpected shifts due to internal factors, such as sample conditions, or external factors, such as variation across flow cytometry instruments ^{22–26}. To avoid these erroneous measurements, recent advances have made traditional flow cytometry quantitative by including fluorophore calibration standards ²⁷, a technique termed qFlow cytometry ^{27–29}.

qFlow cytometry advances non-quantitative traditional flow cytometry by converting the arbitrary flow cytometry signal to absolute protein concentration. Absolute protein quantification overcomes the shortcomings inherent to positive control comparisons used in traditional flow cytometry. For example, we have observed changing protein concentrations across slightly over-confluent to under-confluent cell cultures (data not shown), as have others ³⁰. Again, when such changes happen in both a housekeeping protein and a target protein, the relative differences may not be detected or falsely translated using traditional flow cytometry. Since qFlow cytometry reports absolute protein concentrations, it can detect such differences, alerting the researcher to possible problems in their experimental protocol. Thus, qFlow cytometry allows for experimental standardization, allowing researchers to understand experimental variation and easily compare data across labs.

qFlow cytometry offers a promising approach to advance computational modeling, which is widely used to accelerate scientific discovery and optimize therapeutic approaches by delineating the complex behaviors inherent to biological systems. For example, Weddell and Imoukhuede found that anti-VEGF efficacy depends on endothelial VEGFR1 plasma membrane concentration, with high VEGFR1 concentrations resulting in ineffective anti-VEGF treatment, using a whole-body computational model ³¹. Likewise, our lab found that small increases in plasma membrane receptor concentrations (< 1,000 receptors/cell) doubles nuclear-based receptor signaling ³² using an endocytosis computational model. However, such computational models require parameterization with physiological data, including protein concentrations ³³, to accurately represent the biological system. Accurate biomarker concentrations are therefore necessary to develop computational models ^{21,31,33}. qFlow cytometry renders this much needed accuracy to biomarker quantification, ensuring optimal parameterization and physiologically relevant computational models.

Here, we describe the method to successfully quantify membrane-localized biomarkers on a cellby-cell basis. We discuss antibody specificity, establishing saturation conditions, immunofluorescent labeling strategies, live-cell versus fixed cell methods, and cell-by-cell analysis considerations (e.g., background subtraction, quantification, and statistically analyzing protein heterogeneity). We describe this method in the context of quantifying the angiogenesis-related membrane proteins vascular endothelial growth factor receptor 1 (VEGFR1), VEGFR2, VEGFR3, and neuropilin 1 (NRP1) on human umbilical vein endothelial cells (HUVECs), and platelet-derived growth factor receptor alpha (PDGFR α) and PDGFR β on adult human dermal fibroblasts (HDFs). However, the method presented here can be adapted and applied to any cell type and biomarker.

2.2 MATERIALS

2.2.1 Cell culturing

- 1. Human umbilical vein endothelial cells (HUVECs).
- 2. EGMTM-2 BulletKit endothelial cell growth medium.
- 3. Adult human dermal fibroblasts (HDFs).
- 4. FGMTM-2 BulletKit fibroblast growth medium.
- 5. Phosphate-buffered saline (PBS), pH 7.4.
- Sterile Disposable Bottle Top Filters with PES Membrane. The filter membrane pore size should be 0.20 μm.
- 7. TrypLETM Express.
- 2.2.2 Cell harvest and membrane receptor staining for qFlow cytometry
 - 1. PBS, pH 7.4.
 - 2. CellstripperTM (*see* **Note 1**).
 - 3. 5 ml polystyrene round-bottom tubes.
 - Stain buffer: PBS supplemented with 0.2% bovine serum albumin (BSA) and 0.05% NaN₃, pH 7.4.
 - Phycoerythrin (PE)-conjugated antibodies specific for human VEGFR1, VEGFR2, VEGFR3, NRP1, PDGFRα and PDGFRβ (*see* Note 2).
- 2.2.3 Quantitative flow cytometry
 - 1. LSR Fortessa (BD Biosciences) or equivalent flow cytometer.
 - 2. SYTOXTM Blue Dead Cell Stain, for flow cytometry (*see* **Note 3**).
 - QuantiBRITETM PE beads (this protocol uses beads from BD Biosciences, but equivalent beads from other manufacturers could be used).
 - 4. Stain buffer from step 2.2.4.

2.2.4 Data analysis software

- 1. FlowJo (TreeStar) software is used for analyzing and exporting flow cytometry data.
- 2. Excel software is used for performing calculations on exported flow cytometry data.
- 3. The R programming language software is used for mixture modeling.
- 4. MATLAB software is used for statistically describing uni-population data (see Note 4).

2.3 METHODS

Readers are assumed to have knowledge of aseptic cell culture technique and have access to the necessary equipment for growing cells: a biosafety cabinet in which the cell flasks can be opened and culture media can be changed, an incubator in which the cells can be kept at an appropriate temperature for growth, a microscope for cell observation, and pipettes/pipette tips.

2.3.1 Cell culturing

- Culture HUVECs in EGMTM-2 BulletKit medium per standard cell culture protocols
 ^{21,34–37}
- Culture HDFs in FGMTM-2 BulletKit medium per standard cell culture protocols ²¹.
 Plasma membrane receptor concentrations may differ depending on the serum level in the medium (*see* Note 5).
- 3. Remove and discard culture media upon cell passaging.
- 4. Briefly rinse the cell layer with 5 -10 mL PBS to remove all traces of serum that inhibit the action of trypsin.
- Add 3.0 5.0 mL of TrypLETM Express to flask and incubate at 37 °C in a 5% CO₂ humidified incubator for 5 min.
- 6. Remove flask from incubator. Gently tap on the side of flasks and monitor cell release from the bottom of the flask using a microscope.

- 7. Add 3.0 5.0 mL of complete growth media and aspirate cells by gently pipetting.
- Add appropriate aliquots of the cell suspension to new culture flasks. A sub-culture ratio of 1:3 1:5 is recommended, split every 4 5 days for HUVECs and 9 12 days for HDFs.
- 9. Incubate cell cultures at 37 $^{\circ}C/5\%$ CO₂.
- 2.3.2 Cell harvest from T-175 cell culture flasks
 - 1. Harvest cells when they grow to 75 85% confluent (*see* Note 6).
 - 2. Remove culture media from cells with an aspirating pipette. In this and all subsequent steps, be careful not to scrape the cell layer with the tip of the pipette.
 - Gently add 10 mL PBS to the cells and let sit for 5 10 seconds. Remove PBS from the cells.
 - 4. Add 10 mL CellstripperTM to the cell culture flask.
 - 5. Incubate at 37 °C in a 5% CO₂ humidified incubator for 5 min.
 - Remove flask from incubator. Gently tap on the side of flasks and monitor cell release from the bottom of the flask using a microscope. If the cells are not releasing, subject flasks to abrupt mechanical force to dislodge cell adherence (*see* Note 7).
 - Collect the released cells in a 50 mL conical tube, add 10 mL of stain buffer, and keep on ice.
 - 8. Perform a cell count using a hemocytometer or automated cell counter.
 - 9. Centrifuge the cell suspension from step 6 at $500 \times g$ for 5 min at 4 °C to pellet the cells. Remove the supernatant, being careful not to remove any cells from the pellet.
 - 10. Resuspend the cell pellet in stain buffer to a final concentration of 4×10^6 cells/mL based on the cell count determined in step 8, and keep the cells on ice.

- 2.3.3 Cell surface staining with PE-conjugated monoclonal antibodies
 - 1. Prepare and label the 5 mL polystyrene round-bottom tubes. The number of tubes used in one experiment depends on how many samples you have.
 - 2. In a biosafety cabinet, transfer a 25 μ L aliquot of cell suspension (1 × 10⁵ cells) to each 5 mL polystyrene round-bottom tube.
 - Add PE-conjugated monoclonal antibodies to each tube (*see* Note 8). For non-labeled cell samples, do not add antibodies. We recommend having 2 4 replicates for each antibody.
 - 4. Incubate cells with added antibodies for 40 min on ice in the dark.
 - 5. Add 4 mL stain buffer to each tube.
 - 6. Centrifuge at $500 \times g$ for 4 min at 4 °C to form a cell pellet, then remove the supernatant.
 - 7. Repeat washing as described in steps 3.3.5 and 3.3.6.
 - 8. Resuspend cells in 250 μ L of stain buffer, and keep on ice.

2.3.4 Data acquisition using flow cytometry

We describe data acquisition using a LSR Fortessa (BD) Flow cytometer with BD FACSDIVA software. Other flow cytometers and software should also work if the correct lasers and filters are included in the system. When using a LSR Fortessa (BD) Flow cytometer, use the Pacific Blue channel to measure fluorescence intensity of SYTOX Blue Stain, and use the PE channel to measure PE fluorescence intensity. If the reader is using a different flow cytometer, SYTOX Blue can be excited by a violet laser and its fluorescence intensity can be detected with a 450/50 band pass filter; PE can be excited by a yellow-green laser and detected with a 582/15 band pass filter.

- Reconstitute one tube of QuantiBRITETM PE, which contains a lyophilized pellet of beads, with 500 µL of stain buffer and vortex briefly. Each QuantiBRITETM PE tube can be re-used up to 2 − 3 times within a month.
- 2. Place the reconstituted PE beads from step 3.4.1 at the inlet to the flow cytometer and begin analysis following proper protocols for your instrument.
- 3. Adjust the voltage for PE channel or equivalent to ensure all four bead populations are distinctively displayed on a PE histogram (*see* **Note 9**). Collect 10,000 events above the threshold. The geometric mean of each bead population will be used to determine the calibration curve for PE as described in step 3.5.
- 4. Do not adjust the voltage for PE channels or the speed of the flow after acquiring these events.
- 5. Add 5 μg/mL SYTOXTM Blue Dead Cell Stain to a sample tube (from step 3.3.8) and vortex briefly immediately prior to placement in a flow cytometer (*see* **Note 10**).
- 6. Within the flow cytometer software, display a forward scatter area (FSC-A) versus side scatter area (SSC-A) dot plot for cell samples. Adjust voltages for FSC-A and SSC-A to ensure gating on single-cell populations can be achieved (*see* Note 11).
- 7. Display the histogram of the cell samples for Pacific Blue channel or equivalent. Adjust voltages for Pacific Blue channel to ensure that two cell populations are distinctively displayed. Gate the population on the left side (expressing lower SYTOXTM Blue) and collect at least 10,000 gated events in each sample.

2.3.5 qFlow cytometric analysis: ensemble-averaged plasma membrane receptor concentrationsFlowJo (TreeStar) software and Excel are used for data analysis.

- Within FlowJo software, plot FSC-A versus SSC-A for the QuantiBRITE[™] PE calibration beads and gate the single-bead population. Representative gating is shown in Fig. 1A.
- 2. Plot a histogram of PE of the gated single-bead population. Gate the four distinctive peaks respectively. Representative gating is shown in **Fig. 1B**.
- Using Excel, plot a linear regression of log₁₀ PE molecules per bead against log₁₀ PE geometric mean using the equation y=mx + b, where m and b represent the slope and intercept of the linear regression respectively. A representative PE calibration curve is shown in Fig. 1C.
- Using FlowJo, plot histograms of Pacific Blue channel for cell samples stained with SYTOXTM Blue Dead Cell Stain and gate the live-cell populations. Representative gating is shown in Fig. 1D.
- Plot FSC-A versus SSC-A for gated live cells and gate the single-cell populations. Representative gating is shown in Fig. 1E.
- Plot a histogram of PE of the gated live single-cell population for each sample. The PE geometric means of both live cell samples labeled with PE-conjugated antibodies (PE_{labeled} geometric mean) and unlabeled live cell samples (PE_{unlabeled} geometric mean) are then quantified.
- 7. Using Excel, determine the number of PE-conjugated antibodies per cell for both PEstained samples and non-labeled live cell samples using the equations:

(number of PE-antibodies/cell)_{labeled} = $10^{\frac{1}{m}(\log_{10} PE_{labeled} \text{ geometric mean}-b)}$ (number of PE-antibodies/cell)_{unlabeled} = $10^{\frac{1}{m}(\log_{10} PE_{unlabeled} \text{ geometric mean}-b)}$

8. Determine number of receptors per cell using the equation:

number of receptors/cell = (number of PE-antibodies/cell)_{labeled} - (number of PEantibodies/cell)_{unlabeled}.

- Express ensemble-averaged plasma membrane receptor concentration as mean of number of receptors/cell ± standard error from replicates of each antibody.
- 2.3.6 qFlow cytometric analysis: cell-by-cell analysis
 - Export cell-by-cell PE fluorescence intensity from both labeled and unlabeled live cell samples as a CSV file from FlowJo.
 - 2. Open the CSV file exported in step 3.6.1 with Excel. Sum PE fluorescence intensity of all cells labeled with PE-conjugated antibody, $\sum PE_{labeled}$, and divide that sum by the number of labeled cells, $n_{labeled}$.
 - 3. Sum PE fluorescence intensity of all unlabeled cells, $\sum PE_{unlabeled}$, and divide the sum by the number of unlabeled cells, $n_{unlabeled}$.
 - 4. Calculate the actual PE fluorescence intensity by subtracting the background signal, $PE_{subtracted}$, using the equation (*see* Note 12):

$$PE_{subtracted} = PE_{labeled} \cdot \left(1 - \frac{\left(\sum PE_{unlabeled}\right) / n_{unlabeled}}{\left(\sum PE_{labeled}\right) / n_{unlabeled}} \right)$$

- 5. Loop through the cell-by-cell $PE_{subtracted}$ data and calculate the number of receptors/cell for each cell using the equation described in step 3.5.8.
- 6. For each sample, construct a histogram *H* where the number of receptors/cell is contained in equally spaced bins. The histogram is defined by bin centers S, the mean number of receptors/cell defined by each bin, and frequency *W*, the fraction of total

cells contained in each bin. After constructing the histogram, eliminate outliers 31 , store the bincenters as vector S, and store the frequency as vector W.

- 2.3.7 qFlow cytometric analysis: mixture modeling for statistically describing subpopulationsR programming software is used for this data analysis.
 - Import the number of receptors/cell, with outliers removed as described in step 3.6.6, as vector v₁ into the R programming language software. Note this will need to be done individually for each sample.
 - 2. Take the natural logarithm of v_1 using the 'log' command and store into a second vector v_2 .
 - 3. Use the 'normalmixEM' command in the 'mixtools' package to fit vector v_2 to a logarithm mixture model with 2 subpopulations. Store the mixture model fit as a new variable $L_{fit,2}$. The logarithm mixture model for any number of subpopulations n is defined by:

$$L(v_2) = \sum_{i=1}^n p_i l(\mu_i, \sigma_i)$$

where L is the lognormal mixture and l defines the lognormal subpopulation with index i, mean μ_i , standard deviation σ_i , and density p_i .

- 4. Repeat step 3.7.3, this time fitting to a logarithm mixture model with 3 subpopulations, storing as a new variable $L_{fit.3}$.
- 5. Create the 2 subpopulation mixture model:

$$L_2(s) = p_1 l(s \mid \mu_1, \sigma_1) + p_2 l(s \mid \mu_2, \sigma_2)$$

where *S* defines the bincenter positions (number of receptors/cell), as described in step 3.6.6, and the densities, means, and standard deviations are given by $L_{fit,2}$.

6. Create the 3 subpopulation mixture model:

$$L_3(s) = p_1 l(s \mid \mu_1, \sigma_1) + p_2 l(s \mid \mu_2, \sigma_2) + p_3 l(s \mid \mu_3, \sigma_3)$$

where the densities, means, and standard deviations are given by $L_{fit,3}$.

7. Calculate the sum of squared error between the 2 subpopulation mixture model SSE_2 and the number of receptors/cell data by:

$$SSE_2 = \sum_{i=1}^{nbins} (L_2(s_i) - w_i)^2$$

where i is the bin index with bincenter s_i , *nbins* is the number of bins, $L_2(s_i)$ is the 2 subpopulation mixture model value at bincenter s_i , and W_i is the frequency of cells contained within i as described in step 3.6.6.

8. Calculate the sum of squared error between the 3 subpopulation mixture model SSE_3 and the number of receptors/cell data by:

$$SSE_3 = \sum_{i=1}^{nbins} (L_3(s_i) - w_i)^2$$

where $L_3(s_i)$ is the 3 subpopulation mixture model value at bincenter s_i .

- 9. If SSE_2 (step 3.7.7) is less than SSE_3 (step 3.7.8), define the best fit mixture model $L_B(s_i)$ as $L_2(s_i)$. Otherwise, define $L_B(s_i)$ as $L_3(s_i)$.
- 10. Express $L_B(s_i)$ as the subpopulation means μ_B , standard deviations σ_B , and densities P_B .

- 11. For graphical representation, plot *S* versus $L_B(s_i)$ alone, or with the histogram *H* from step 3.6.6. An example of mixture modeling is given in **Note 10** and **Fig. 4B**.
- 2.3.8 qFlow cytometric analysis: non-normality and diversity analysis for statistically describing uni-population data
 - MATLAB software is used for this data analysis.
 - For each sample, import the number of receptors/cell bincenters as S and frequency as W, as described in step 3.6.6, into MATLAB software.
 - 2. Import the corresponding number of receptors/cell for each sample, as described in step 3.5.8, into MATLAB software as a vector v_1 .
 - 3. Determine the Gaussian mean μ and standard deviation σ of v_1 using the MATLAB commands 'mean (v_1) ' and 'std (v_1) ', respectively.
 - 4. Generate a reference Gaussian distribution using the command 'g=normpdf(s, μ, σ)', as given by the equation:

$$g(s \mid \mu, \sigma) = \frac{1}{\sigma\sqrt{2\pi}} \exp\left\{-\frac{(s-\mu)^2}{2\sigma^2}\right\}$$

- 5. Generate the probability distribution of $g(s | \mu, \sigma)$, P_{ref} , using the command 'Pref = g./sum(g)', where sum(g) is the sum of all elements in vector $g(s | \mu, \sigma)$.
- 6. Compute scalar statistic K-S values p_{KS} for each sample using the command 'Pks = kstest2(*w*, Pref)' (*see* Note 13).
- 7. Compute the quadratic entropy *QE* (see Note 13) as given by the equation:

$$QE = \sum_{j=1}^{nbins-1} \sum_{i=j+1}^{nbins} (s_i - s_j) \cdot w_i \cdot w_j, \ 1 \le j < i \le nbins$$

where s_i and s_j are centers of the bins with indices i and j respectively, and W_i and W_j are the frequencies of the bins with indices i and j respectively.

2.4 NOTES

1. Trypsin-based cell dissociation using solutions such as TrypLETM involves cleaving peptide bonds on the C-terminal sides of lysine and arginine ³⁸. This action may cleave cell surface receptors 35 or stimulate receptor shedding $^{39-42}$, and either mechanism would lead to invalid qFlow cytometry results. Therefore, non-enzymatic dissociation solution such as CellstripperTM is recommended for preserving cell surface receptors when performing qFlow cytometry. For example, we have previously observed TrypLETM-mediated decreases in NRP1 on HUVECs, while cell surface VEGFR1 and VEGFR2 remained relatively unchanged ³⁶. In this chapter, we observe similar results when we extend the methods to VEGFR3 and Tie2 on HUVECs (Fig. 2A). VEGFR2 and VEGFR3 plasma membrane concentrations on HUVECs are consistent with a previous report, while VEGFR1 is \sim 30% higher (p < (0.01), which may be attributed to donor-specific differences ²¹. Interestingly, TrypLETM treatment results in a ~90% increase in PDGFRβ plasma membrane concentrations on HDF surface (p < 0.001), while PDGFRa concentrations remain unchanged (Fig. 1B). Furthermore, the previously reported NRP1 decrease is not specific to HUVECs; we also observe a two order of magnitude decrease in NRP1 plasma membrane concentrations on HDFs following the TrypLE treatment (Fig. 2B). Altogether, our data and previous reports indicate that if the researcher prefers to use an enzymatic cell dissociation solution, that they check how the solution affects plasma membrane receptor concentrations.

- 2. Antibody specificity is required for accurate receptor quantification. We labeled mouse 3T3 fibroblasts with either human or mouse-specific antibodies to determine antibody binding specificity (Fig. 3). Our positive and negative controls give ~5,800 mVEGFR1 and ~300 mVEGFR2 per mouse 3T3, respectively (Fig. 3). These trends are in line with prior studies reporting high mVEGFR1 plasma membrane concentration and little to no mVEGFR2 ²⁹. As expected, the human antibodies showed low binding to the mouse 3T3s. We observe less than 600 hVEGFR1, hVEGFR2, hPDGFRα or hPDGFRβ per mouse 3T3 fibroblast, which is similar in value to our negative controls (Fig. 3). Therefore, the cross-reactivity of the human antibody to mouse receptors is very low. When analyzing multiple biomarkers, the researcher should use similar methods, as we have outlined, to ensure that their antibodies have good specificity.
- 3. SYTOX Blue can be excited with a solid-state laser (407 nm) and its emission collected using a 450/50 band-pass filter. SYTOX Blue live/dead stain is preferable to Propidium Iodide (PI) or 7-AAD because SYTOX Blue emission has little overlap with PE. Live-dead cell staining should always be performed, as we noted above, and if the researcher chooses to use a stain other than SYTOX Blue, they should check the spectral spillover to ensure accurate receptor quantification.
- 4. This protocol is defined to use the software explicitly listed in the "Methods" section, however, alternative software can be used, based on the user preferences. An alternative flow cytometry software option that we have had good experience with is FCS Express (De Novo Software). The Purdue University Cytometry Laboratory (PUCL) offers a comprehensive listing of free flow cytometry software ⁴³.

Alternatives to Excel for data calculations and storage include any spreadsheet software capable of basic algebraic functions and data storage, such as Accel Spreadsheet. Alternatives to R for performing mixture modeling include any programming language capable of conducting mathematical operations, such as C/C++ or Python. Likewise, other mathematical programming languages such as C/C++ or Python, or statistical analysis software, such as Minitab or SPSS, can be used instead of MATLAB for conducting statistical analyses. Overall, we advise researchers to choose the software based on experience, preference, and availability.

- 5. A commonly used fibroblast culture medium is Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% of Penicillin Streptomycin (Pen Strep)^{21,44-46}. However, we would like to point out to readers that plasma membrane concentrations can be affected by serum levels in the cell culture medium. We previously found that PDGFRs and NRP1 plasma membrane concentrations decreased when HDFs were cultured in DMEM supplemented with 10% FBS compared to the standard FGMTM-2 fibroblast growth medium ²¹, and the new data shown in Figs 2-4 are consistent with our previous results. Given the inverse serum concentration-receptor concentration relationship that we consistently observe, we recommend that researchers consider or assay these effects when performing qFlow cytometry.
- 6. Others and we find that plasma membrane receptor concentrations in HUVECs may change if they reach 100% confluence. Indeed, , Napione et al. also found that long-confluent HUVECs express 2-fold higher VEGFR2 than in sparse cells, and proposed the theory that increased cell concentrations are linked with the presence of mature

cell junctions, which regulate receptor trafficking ³⁰. Therefore, we recommend researchers harvest HUVECs when they reach 80-85% confluence to avoid receptor changes, which made lead to inconsistent data.

- 7. We find that cell dissociation choice comes with advantages and disadvantages. An important disadvantage of TrypLE as noted above, some receptors are affected by enzymatic treatment. However, a noteworthy advantage is that cells are easily dissociated from flasks with TrypLE. Whereas, cell dissociation may be incomplete when using Cellstripper. Sometimes abrupt mechanical force is required to completely dissociate Cellstripper-treated cells. Interestingly, we have not observed significant plasma membrane receptor concentration changes when using abrupt force. However, we do not recommend readers apply the force more than once. Some alternatives are to tap gently on the side of flasks or to place cells in a 37 $^{\circ}C/5\%$ CO₂ incubator with Cellstripper for an additional 1-2 min. However, >2 additional min in the incubator is not recommended, as we have observed unpredictable changes in receptor concentrations with significant incubator-Cellstripper treatment. Taken together, we recommend the use of a non-enzymatic cell dissociation solution as a default; if cells do not lift completely --- one may employ abrupt force or an additional, short incubation step.
- 8. Non-labeled receptors will invalidate qFlow results. Therefore, a receptor saturation study is necessary for accurate qFlow cytometry profiling. We have previously determined the optimal concentrations of PE-conjugated monoclonal antibodies for staining each sample (1×10^5 cells): 14 µg/mL for VEGFR1 and VEGFR2, 7.1 µg/mL for NRP, and 9.4 µg/mL for PDGFRs ^{21,35}. Readers should determine the

optimal antibody concentrations for their respective markers by staining cell samples with a series of increasing antibody concentrations and quantify the biomarker levels using qFlow cytometry. The biomarker levels should reach a plateau when the optimal antibody concentration is applied. Therefore, we advise researchers to perform a saturation study to determine the least amount of antibodies needed to achieve consistent and accurate receptor quantification.

9. The photo-physical properties of PE make it an ideal choice for receptor quantification. Its high extinction coefficient lowers error due to photobleach, and its large size imparts the advantageous 1:1 antibody to fluorophore ratio needed to accurately quantify receptors ⁴⁷. Several studies have established the use of QuantiBRITETM PE beads for receptor quantification ^{21,27,29,35,36}, so the depth of research available further assists the researcher in troubleshooting and optimizing for their application. Important application notes for QuantiBRITETM PE beads described here. They comprise four groups of polystyrene beads conjugated with different PE densities: low (474 PE molecules/bead), medium-low (5,359 PE molecules/bead), medium-high (23,843 PE molecules/bead), and high (62,336 PE molecules/bead). The exact PE number may differ from batch to batch and can be found on the flyer in the kit. A representative figure of all four bead-populations distinctively displayed on a PE histogram can be seen in Figure 1B. Other options for qFlow cytometry include Quantum MESF and Quantum Simply Cellular microspheres, which also offer FITCbased quantitative tools. However, the fluorophore sensitivity to photobleach, buffer, pH, etc. should be considered when choosing a fluorescent bead (e.g., FITC vs. PE). Overall, our approach has been optimized for applying QuantiBRITE-PE for
quantifying angiogenic receptors; however other tools exist and can be translated to qFlow cytometry via optimization.

- 10. Live/dead cell staining or a reliable way to exclude dead cells is necessary for accurate receptor quantification. We observe that there is no significant difference in receptor quantitation when all cells are analyzed (live + dead cells) versus when only live cells are analyzed (via SYTOXTM Blue staining) for the following receptors: VEGFR1, VEGFR2, NRP1, Tie2, and PDGFR α (Fig. 4A). However, we do observe significant changes in VEGFR3 plasma membrane concentrations on HUVECs and PDGFR β on HDFs when dead cells are not excluded (Fig. 4A). To further examine VEGFR3 plasma membrane expression on live and dead cells, cell-by-cell analysis was applied as described in step 3.6. We observe that the live-cell population and live + dead mixture population both exhibit twoVEGFR3 subpopulations (Fig. 4B). Twocomponent lognormal mixture modeling indicates that 97% of live HUVECs have an average of ~1,900 VEGFR3/cell, while 3% display an average of ~65,000 VEGFR3/cell (Table 1). Conversely, the high-VEGFR3 subpopulation has greater density in the live + dead HUVEC population: 33% display ~21,000 VEGFR3/cell and 67% display ~1,700 VEGFR3/cell (Table 1). Increased density of the high-VEGFR3 subpopulation when dead cells are included suggests that the high-VEGFR3 subpopulation is comprised of dead and apoptotic cells. This underlies the necessity to apply live/dead staining for accurate receptor profiling.
- 11. Cells are distributed based on their sizes on a FSC-A vs SSC-A dot plot (Fig. 1E). The bigger the cells are, the higher their FSC-A is; the greater the cell granularity, the higher their SSC-A. When cells are prepared carefully, minimizing aggregation and

cell lysis (e.g., kept on ice, titrated and/or strained prior to imaging, solutions are buffered, solutions are isotonic, solutions do not include Ca or Mg), cell populations primarily resolve as singlets ^{34,35,48–50}. We observe that higher order cell clusters (e.g., doublets, triplets, etc.) are few and can be distinguished linearly in the FSC-A vs SSC-A dot plot. Overall, observing best-practices in handling enables easy gating of single-cell populations.

- 12. Accounting for cell autofluorescence in ensemble qFlow cytometry analysis can be as simple as subtracting the average cell fluorescence of non-labeled cells. However, when performing cell-by-cell analysis accounting for autofluorescence may incorporate error. Indeed, the background subtraction method that we present may result in some negative PE_{real} values, indicating that the noise is larger than the signal. For simplicity, we set the negative values to zero. Figure 5 shows a comparison between cell-by-cell VEGFR1 histogram before and after background subtraction. Overall, the autofluorescence method presented here allows researchers to account for background noise such as cell autofluorescence by shifting the fluorescence signal based on a derived signal to noise ratio ²¹.
- 13. The high-throughput cell-by-cell data obtained by flow cytometry renders it an ideal tool for studying cell heterogeneity. The quantitative nature of qFlow cytometry adds the additional dimension of quantifying cell heterogeneity, which can be useful for several areas of research, most pressingly cancer medicine ³⁴. Towards these goals, K-S and QE values are good analytical tools to statistically characterize cellular heterogeneity. The K-S test compares two populations and statistically determines whether they are drawn from the same continuous distribution. In this protocol, the '

Pks = kstest2(w, Pref), command in MATLAB tests the null hypothesis that the number of receptors/cell population (W) is drawn from a Gaussian distribution, provided by the reference Gaussian distribution Pref. Pks will either equal 0 or 1; Pks = 1 indicates that the null hypothesis is rejected at the 5% significance level (the number of receptors/cell population is not Gaussian), whereas Pks = 0 fails to reject the null hypothesis. Therefore, K-S testing offers a statistical method for reporting differences in gFlow cytometry data that is analyzed on a cell-by-cell level. Traditional diversity measurement assumes all differences between groups are equal, whereas QE, introduced by Rao, accounts for the probability differences among those groups 51-54. The measurement of QE bins a group of cells into a finite number of subsets based on membrane receptors concentrations, and calculates the average dissimilarities between two randomly drawn subsets from the group of cells. QE has been shown to provide a quantitative measure of the diversity of cellular phenotypes in cancer tissue sections for diagnostic applications ⁵⁵. It has also been applied to characterize cellular heterogeneity in response to drug treatment ⁵⁶. As researchers extract quantitative data from qFlow cytometry studies, we recommend K-S and QE values as good approaches for characterizing non-normality and diversity in heterogeneous cell populations ^{21,56}.



Figure 1. Gating on the HDFs for qFlow cytometric analysis using FlowJo. (A) Gate single-bead population on a FSC-A vs. SSC-A dot plot. (B) Gate each population of beads conjugated with different numbers of PE on a histogram of PE-A. (C) PE calibration curve (red) obtained by fitting log₁₀ PE geometric mean against log₁₀ PE molecules/bead. (D) Gate live-cell and dead-cell population on a histogram of Pacific Blue-A. (E) Gate single-cell population from live cells on a FSC-A vs SSC-A dot plot.



Figure 2. Effects of TrypLETM comparing to CellstripperTM on receptor quantification of (A) HUVECs and (B) HDFs. Significance tests were conducted using two-sample t-test where *** indicates p < 0.001.



Figure 3. Quantification of binding sites for antibodies on mouse 3T3, HUVECs and HDFs.



Figure 4. Mixture modeling can be applied to unmix VEGFR3 expression on live and dead HUVECs. (A) Comparison of plasma membrane receptor concentration between live single cells and live + dead single cells. Significance tests are conducted using two-sample t-test where * indicates p < 0.05 and *** indicates p < 0.001. (B) Gaussian mixture models of VEGFR3 subpopulations on HUVECs. Dashed lines represent subpopulations having a higher density and dotted lines represent subpopulations having a smaller density.



Figure 5. Corresponding VEGFR1 cell-by-cell distribution computed from PE fluorescence of labeled HUVECs before (red dotted line) and after background subtraction (grey filled area).

2.6 TABLE

Sample	Mean		Standard deviation		Density	
	μ_1	μ2	σ_1	σ_2	π_1	π_2
Live	1,900	65,000	3.19	2.49	0.97	0.03
Live + Dead	1,700	21,000	2.54	2.88	0.67	0.33

Table 1. Gaussian mixture model parameters for HUVECs labeled with anti-VEGFR3-PE.

2.7 REFERENCES

- B. Y. S. Kim, J. T. Rutka, and W. C. W. Chan, "Nanomedicine.," *N. Engl. J. Med.*, vol. 363, no. 25, pp. 2434–43, Dec. 2010.
- [2] S. Agrawal and R. Prajapati, "Nanosensors and their pharmaceutical applications: a review," *Int. J. Pharm. Sci. Technol.*, vol. 4, pp. 1528–35, 2012.
- [3] M. Swierczewska, G. Liu, S. Lee, and X. Chen, "High-sensitivity nanosensors for biomarker detection.," *Chem. Soc. Rev.*, vol. 41, no. 7, pp. 2641–55, Apr. 2012.
- [4] R. Etzioni, N. Urban, S. Ramsey, M. McIntosh, S. Schwartz, B. Reid, J. Radich, G. Anderson, and L. Hartwell, "The case for early detection.," *Nat. Rev. Cancer*, vol. 3, no. 4, pp. 243–52, Apr. 2003.
- [5] J. Li, L. W. Dobrucki, M. Marjanovic, E. J. Chaney, K. S. Suslick, and S. A. Boppart, "Enhancement and wavelength-shifted emission of Cerenkov luminescence using multifunctional microspheres.," *Phys. Med. Biol.*, vol. 60, no. 2, pp. 727–39, Jan. 2015.
- [6] R. Zhang, D. Pan, X. Cai, X. Yang, A. Senpan, J. S. Allen, G. M. Lanza, and L. V Wang, "alphaVbeta3-targeted copper nanoparticles incorporating an Sn 2 lipase-labile fumagillin prodrug for photoacoustic neovascular imaging and treatment.," *Theranostics*, vol. 5, no. 2, pp. 124–33, Jan. 2015.
- M. V Backer, Z. Levashova, V. Patel, B. T. Jehning, K. Claffey, F. G. Blankenberg, and J. M. Backer, "Molecular imaging of VEGF receptors in angiogenic vasculature with single-chain VEGF-based probes," *Nat Med*, vol. 13, no. 4, pp. 504–509, 2007.
- [8] J. A. Ludwig and J. N. Weinstein, "Biomarkers in cancer staging, prognosis and treatment selection.," *Nat. Rev. Cancer*, vol. 5, no. 11, pp. 845–56, Nov. 2005.
- [9] S. M. Weis and D. A. Cheresh, "Tumor angiogenesis: molecular pathways and therapeutic targets," *Nat Med*, vol. 17, no. 11, pp. 1359–1370, 2011.
- [10] S. J. Harper and D. O. Bates, "VEGF-A splicing: the key to anti-angiogenic therapeutics?," *Nat. Rev. Cancer*, vol. 8, no. 11, pp. 880–7, Nov. 2008.
- [11] T. Arao, K. Matsumoto, K. Furuta, K. Kudo, H. Kaneda, T. Nagai, K. Sakai, Y. Fujita, D. Tamura, K. Aomatsu, F. Koizumi, and K. Nishio, "Acquired drug resistance to vascular

endothelial growth factor receptor 2 tyrosine kinase inhibitor in human vascular endothelial cells.," *Anticancer Res.*, vol. 31, no. 9, pp. 2787–96, Sep. 2011.

- [12] J. Li, L. F. Brown, M. G. Hibberd, J. D. Grossman, J. P. Morgan, and M. Simons, "VEGF, flk-1, and flt-1 expression in a rat myocardial infarction model of angiogenesis.," *Am. J. Physiol.*, vol. 270, no. 5 Pt 2, pp. H1803-11, May 1996.
- [13] L. F. Brown, B. Berse, R. W. Jackman, K. Tognazzi, A. J. Guidi, H. F. Dvorak, D. R. Senger, J. L. Connolly, and S. J. Schnitt, "Expression of vascular permeability factor (vascular endothelial growth factor) and its receptors in breast cancer.," *Hum. Pathol.*, vol. 26, no. 1, pp. 86–91, Jan. 1995.
- [14] M. E. Gerritsen, J. E. Tomlinson, C. Zlot, M. Ziman, and S. Hwang, "Using gene expression profiling to identify the molecular basis of the synergistic actions of hepatocyte growth factor and vascular endothelial growth factor in human endothelial cells.," *Br. J. Pharmacol.*, vol. 140, no. 4, pp. 595–610, Oct. 2003.
- [15] M. Dougher and B. I. Terman, "Autophosphorylation of KDR in the kinase domain is required for maximal VEGF-stimulated kinase activity and receptor internalization.," *Oncogene*, vol. 18, no. 8, pp. 1619–27, Mar. 1999.
- [16] M. Duval, S. Bédard-Goulet, C. Delisle, and J.-P. Gratton, "Vascular endothelial growth factor-dependent down-regulation of Flk-1/KDR involves Cbl-mediated ubiquitination. Consequences on nitric oxide production from endothelial cells.," *J. Biol. Chem.*, vol. 278, no. 22, pp. 20091–7, May 2003.
- [17] Y. Guo, P. Xiao, S. Lei, F. Deng, G. G. Xiao, Y. Liu, X. Chen, L. Li, S. Wu, Y. Chen, H. Jiang, L. Tan, J. Xie, X. Zhu, S. Liang, and H. Deng, "How is mRNA expression predictive for protein expression? A correlation study on human circulating monocytes.," *Acta Biochim. Biophys. Sin. (Shanghai).*, vol. 40, no. 5, pp. 426–36, May 2008.
- [18] R. Bhargava, W. L. Gerald, A. R. Li, Q. Pan, P. Lal, M. Ladanyi, and B. Chen, "EGFR gene amplification in breast cancer: correlation with epidermal growth factor receptor mRNA and protein expression and HER-2 status and absence of EGFR-activating mutations.," *Mod. Pathol.*, vol. 18, no. 8, pp. 1027–33, Aug. 2005.
- [19] J. Xu, H. Chai, K. Ehinger, T. M. Egan, R. Srinivasan, M. Frick, and B. S. Khakh, "Imaging P2X4 receptor subcellular distribution, trafficking, and regulation using P2X4pHluorin.," *J. Gen. Physiol.*, vol. 144, no. 1, pp. 81–104, Jul. 2014.

- [20] D. Faratian, J. Christiansen, M. Gustavson, C. Jones, C. Scott, I. Um, and D. J. Harrison, "Heterogeneity mapping of protein expression in tumors using quantitative immunofluorescence.," J. Vis. Exp., no. 56, p. e3334, 2011.
- [21] S. Chen, X. Guo, O. Imarenezor, and P. I. Imoukhuede, "Quantification of VEGFRs, NRP1, and PDGFRs on Endothelial Cells and Fibroblasts Reveals Serum, Intra-Family Ligand, and Cross-Family Ligand Regulation," *Cell. Mol. Bioeng.*, vol. 8, no. 3, pp. 383– 403, Aug. 2015.
- [22] M. Rocha-Martins, B. Njaine, and M. S. Silveira, "Avoiding pitfalls of internal controls: validation of reference genes for analysis by qRT-PCR and Western blot throughout rat retinal development.," *PLoS One*, vol. 7, no. 8, p. e43028, 2012.
- [23] A. Vigelsø, R. Dybboe, C. N. Hansen, F. Dela, J. W. Helge, and A. Guadalupe Grau,
 "GAPDH and β-actin protein decreases with aging, making Stain-Free technology a superior loading control in Western blotting of human skeletal muscle.," *J. Appl. Physiol.*, vol. 118, no. 3, pp. 386–94, Feb. 2015.
- [24] R. Baumgartner, E. Umlauf, M. Veitinger, S. Guterres, E. Rappold, R. Babeluk, G. Mitulović, R. Oehler, and M. Zellner, "Identification and validation of platelet low biological variation proteins, superior to GAPDH, actin and tubulin, as tools in clinical proteomics.," *J. Proteomics*, vol. 94, pp. 540–51, Dec. 2013.
- [25] R. Nguyen, S. Perfetto, Y. D. Mahnke, P. Chattopadhyay, and M. Roederer, "Quantifying spillover spreading for comparing instrument performance and aiding in multicolor panel design.," *Cytometry. A*, vol. 83, no. 3, pp. 306–15, Mar. 2013.
- [26] L. L. Wheeless, J. S. Coon, C. Cox, A. D. Deitch, R. W. Devere White, L. G. Koss, M. R. Melamed, M. J. O'Connell, J. E. Reeder, R. S. Weinstein, and R. P. Wersto, "Measurement variability in DNA flow cytometry of replicate samples," *Cytometry*, vol. 10, no. 6, pp. 731–738, Nov. 1989.
- [27] K. K. Pannu, E. T. Joe, and S. B. Iyer, "Performance evaluation of quantiBRITE phycoerythrin beads," *Cytometry*, vol. 45, no. 4, pp. 250–258, 2001.
- [28] L. Wang, F. Abbasi, A. K. Gaigalas, R. F. Vogt, and G. E. Marti, "Comparison of fluorescein and phycoerythrin conjugates for quantifying CD20 expression on normal and leukemic B-cells.," *Cytometry B. Clin. Cytom.*, vol. 70, no. 6, pp. 410–5, Nov. 2006.

- [29] P. I. Imoukhuede, A. O. Dokun, B. H. Annex, and A. S. Popel, "Endothelial cell-by-cell profiling reveals the temporal dynamics of VEGFR1 and VEGFR2 membrane localization after murine hindlimb ischemia," *Am J Physiol Hear. Circ Physiol*, vol. 304, no. 8, pp. H1085–H1093, 2013.
- [30] L. Napione, S. Pavan, A. Veglio, A. Picco, G. Boffetta, A. Celani, G. Seano, L. Primo, A. Gamba, and F. Bussolino, "Unraveling the influence of endothelial cell density on VEGF-A signaling.," *Blood*, vol. 119, no. 23, pp. 5599–607, Jun. 2012.
- [31] J. C. Weddell and P. I. Imoukhuede, "Quantitative characterization of cellular membrane-receptor heterogeneity through statistical and computational modeling.," *PLoS One*, vol. 9, no. 5, p. e97271, Jan. 2014.
- [32] J. C. Weddell and P. I. Imoukhuede, "Integrative meta-modeling identifies endocytic vesicles, late endosome and the nucleus as the cellular compartments primarily directing RTK signaling," *Integr. Biol.*, 2017.
- [33] S. Chen, A. Ansari, W. Sterrett, K. Hurley, J. Kemball, J. C. Weddell, and P. I. Imoukhuede, "Current State-of-the-Art and Future Directions in Systems Biology," *Progress and Communication in Sciences*, vol. 1, no. 1. pp. 12–26, 29-Oct-2014.
- [34] P. I. Imoukhuede and A. S. Popel, "Quantitative fluorescent profiling of VEGFRs reveals tumor cell and endothelial cell heterogeneity in breast cancer xenografts.," *Cancer Med.*, vol. 3, no. 2, pp. 225–44, Apr. 2014.
- [35] P. I. Imoukhuede and A. S. Popel, "Quantification and cell-to-cell variation of vascular endothelial growth factor receptors," *Exp. Cell Res.*, vol. 317, no. 7, pp. 955–965, 2011.
- [36] P. I. Imoukhuede and A. S. Popel, "Expression of VEGF receptors on endothelial cells in mouse skeletal muscle.," *PLoS One*, vol. 7, no. 9, p. e44791, Jan. 2012.
- [37] B. J. Roxworthy, M. T. Johnston, F. T. Lee-Montiel, R. H. Ewoldt, P. I. Imoukhuede, and K. C. Toussaint, "Plasmonic optical trapping in biologically relevant media.," *PLoS One*, vol. 9, no. 4, p. e93929, Jan. 2014.
- [38] "TrypLETM Express Enzyme (1X), phenol red Life Technologies." [Online]. Available: https://www.lifetechnologies.com/order/catalog/product/12605036. [Accessed: 07-Jan-2015].

- [39] M. A. Miller, A. S. Meyer, M. T. Beste, Z. Lasisi, S. Reddy, K. W. Jeng, C.-H. Chen, J. Han, K. Isaacson, L. G. Griffith, and D. A. Lauffenburger, "ADAM-10 and -17 regulate endometriotic cell migration via concerted ligand and receptor shedding feedback on kinase signaling.," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 110, no. 22, pp. E2074-83, May 2013.
- [40] V. H. Guaiquil, S. Swendeman, W. Zhou, P. Guaiquil, G. Weskamp, J. W. Bartsch, and C. P. Blobel, "ADAM8 is a negative regulator of retinal neovascularization and of the growth of heterotopically injected tumor cells in mice.," *J. Mol. Med. (Berl).*, vol. 88, no. 5, pp. 497–505, May 2010.
- [41] G. Weskamp, K. Mendelson, S. Swendeman, S. Le Gall, Y. Ma, S. Lyman, A. Hinoki, S. Eguchi, V. Guaiquil, K. Horiuchi, and C. P. Blobel, "Pathological neovascularization is reduced by inactivation of ADAM17 in endothelial cells but not in pericytes.," *Circ. Res.*, vol. 106, no. 5, pp. 932–40, Mar. 2010.
- [42] F. A. Delano, A. Y. Chen, K.-I. S. Wu, E. D. Tran, S. F. Rodrigues, and G. W. Schmid-Schönbein, "The autodigestion and receptor cleavage in diabetes and hypertension.," *Drug Discov. Today. Dis. Models*, vol. 8, no. 1, pp. 37–46, Jan. 2011.
- [43] Purdue University Cytometry Laboratories, "Catalog of Free Flow Cytometry Software." [Online]. Available: http://www.cyto.purdue.edu/flowcyt/software/Catalog.htm.
- [44] S. E. Holton, M. J. Walsh, and R. Bhargava, "Subcellular localization of early biochemical transformations in cancer-activated fibroblasts using infrared spectroscopic imaging," *Analyst*, vol. 136, no. 14, p. 2953, 2011.
- [45] V. Chan, P. Zorlutuna, J. H. Jeong, H. Kong, and R. Bashir, "Three-dimensional photopatterning of hydrogels using stereolithography for long-term cell encapsulation," *Lab Chip*, vol. 10, no. 16, p. 2062, 2010.
- [46] M. A. Qayyum, J. T. Kwak, and M. F. Insana, "Stromal-epithelial responses to fractionated radiotherapy in a breast cancer microenvironment," *Cancer Cell Int.*, vol. 15, no. 1, p. 67, Dec. 2015.
- [47] S. Lyer, J. Bishop, B. Abrams, V. Maino, A. Ward, T. Christian, and K. Davis, *QuantiBRITE: a new standard for PE flourescence quantitation. In: White Paper.* San Jose, CA: Becton Dickinson Immunocytometry Systems, 1997.

- [48] B. Houtz, J. Trotter, and D. Sasaki, "Tips on Cell Preparation for Flow Cytometric Analysis and Sorting," *BD FACService Technotes*, vol. 4, pp. 3–4, 2004.
- [49] M. G. Ormerod and P. R. Imrie, "Flow Cytometry," in *Animal Cell Culture*, New Jersey: Humana Press, pp. 543–558.
- [50] I. Schmid, C. H. Uittenbogaart, and J. V. Giorgi, "Sensitive method for measuring apoptosis and cell surface phenotype in human thymocytes by flow cytometry," *Cytometry*, vol. 15, no. 1, pp. 12–20, Jan. 1994.
- [51] C. R. Rao, "Diversity: Its measurement, decomposition, apportionment and analysis," *Sankhya Indian J. Stat.*, vol. A44, pp. 1–22.
- [52] C. R. Rao, "Diversity and dissimilarity coefficients: A unified approach," *Theor. Popul. Biol.*, vol. 21, no. 1, pp. 24–43, Feb. 1982.
- [53] C. R. Rao, "Quadratic entropy and analysis of diversity," *Sankhya A*, vol. 72, no. 1, pp. 70–80, Feb. 2010.
- [54] Z. Botta-Dukát, "Rao's quadratic entropy as a measure of functional diversity based on multiple traits," *J. Veg. Sci.*, vol. 16, no. 5, pp. 533–540, Oct. 2005.
- [55] S. J. Potts, J. S. Krueger, N. D. Landis, D. A. Eberhard, G. D. Young, S. C. Schmechel, and H. Lange, "Evaluating tumor heterogeneity in immunohistochemistry-stained breast cancer tissue.," *Lab. Invest.*, vol. 92, no. 9, pp. 1342–57, Sep. 2012.
- [56] A. H. Gough, N. Chen, T. Y. Shun, T. R. Lezon, R. C. Boltz, C. E. Reese, J. Wagner, L. A. Vernetti, J. R. Grandis, A. V Lee, A. M. Stern, M. E. Schurdak, and D. L. Taylor, "Identifying and quantifying heterogeneity in high content analysis: application of heterogeneity indices to drug discovery.," *PLoS One*, vol. 9, no. 7, p. e102678, Jan. 2014.

CHAPTER 3 QUANTIFICATION OF VEGFRS, NRP1, AND PDGFRS ON ENDOTHELIAL CELLS AND FIBROBLASTS REVEALS SERUM, INTRA-FAMILY LIGAND, AND CROSS-FAMILY LIGAND REGULATION¹

Computational modeling of angiogenesis is limited by a lack of experimental data on angiogenic receptor levels. Recent receptor profiling quantified vascular endothelial growth factor receptors (VEGFRs); however, data on other angiogenic receptors, such as platelet derived growth factor receptors (PDGFRs), are also necessary for the development of an accurate angiogenesis model. Here, we establish conditions for membrane PDGFR quantification. Additionally, we determine how several environmental conditions control membrane PDGFR levels on human dermal fibroblasts. We demonstrate that membrane PDGFR^β concentrations are negatively correlated with both media serum concentration and cell growth rate, in vitro. We also show VEGF-A₁₆₅mediated downregulation of membrane PDGFRa (~25%) and PDGFRβ (~30%), following a 24hour treatment. This supports the idea that VEGF-A₁₆₅ acts independently of VEGFRs to signal through PDGFR α and PDGFR β . We observe that PDGF-AA and PDGF-AB downregulate membrane PDGFRa by up to 55% and 75%, respectively, while having little to no effect on PDGFRβ or NRP1. We observe that PDGF-BB effects both PDGFRs and NRP1: membrane PDGFR α and PDGFR β were downregulated by up to 70% and 90%, respectively, whereas membrane NRP1 was upregulated by up to 40%. These data provide the necessary insight to accurately represent PDGFRs in angiogenesis models, while offering new insight into the regulation of membrane PDGFRs.

¹ This chapter was published in Cellular and Molecular Bioengineering (2015) Volume 8, Issue 3, pp 383-403. DOI: <u>https://doi.org/10.1007/s12195-015-0411-x</u>.

3.1 INTRODUCTION

Membrane proteins are the initial transducers of cytokine signaling towards intracellular response; their expression defines pathologies and treatment regimens. As such, insights into membrane protein localization and activation can advance our understanding of complex biological processes, such as angiogenesis. Towards this aim, VEGFRs have been extensively profiled ^{1–4} and modeled ^{5–8}. These studies revealed several VEGFR regulatory roles including the following: endothelial tip-cell versus stalk-cell selection ^{9–11}, endothelial proliferation and migration to hypoxic regions ^{12–15}, and vascular anastomoses and ultimate vascular stability ^{16–21}.

While these VEGFR contributions are significant, there are several other signaling axes that are critical to the process of angiogenesis: PDGFRs, which regulate vascular stability ^{22–24}; fibroblast growth factor receptors (FGFRs), which influence cellular proliferation and increase motility ²⁵; Tie receptors, which control vascular quiescence, vascular maintenance, and angiogenesis ^{26–29}; and transforming growth factor receptors (TGFRs), which affect cellular proliferation ⁸. Of these, the PDGF-PDGFR axis offers considerable insight into VEGFR-angiogenic signaling, because of its independent contributions to vascular signaling and its crosstalk with the VEGF axis.

PDGF ligands are within the family of disulfide-bonded homodimers, which include AA-, BB-, CC- and DD- polypeptide chains, as well as the heterodimer PDGF-AB $^{30-33}$ (Fig. 6). The four dimeric isoforms, PDGF-AA, PDGF-AB, PDGF-BB, and PDGFCC bind to PDGFR α ; PDGF-BB and PDGF-DD bind to PDGFR β ; and PDGF-AB, PDGF-BB, and PDGF-CC can bind to heterodimeric, PDGFR $\alpha\beta$ complexes.

PDGF-mediated activation of PDGFRs regulates embryonic development, maintains tissue stability, and serves reparative functions. During development, PDGF signaling enables the

necessary pericyte support of endothelial tubes ^{22,34}. Indeed, PDGF B^{-/-} mice develop defective kidneys and abnormal microvessels that do not recruit pericytes to the vessel wall^{35,36}; and PDGF A^{-/-} mice exhibit defective development of alveoli in the lung with an emphysema-like phenotype^{35,37}. As for its role in tissue stability, PDGF is a potent regulator of cells comprising the connective tissue (e.g., fibroblasts and smooth muscle cells)^{38–39}. PDGF signaling therein helps enable cell proliferation, migration, and maintenance of the interstitial fluid pressure⁴⁰. PDGF signaling also has a reparative function. It stimulates wound-healing^{41–43}, is upregulated following ischemic injury in endothelial and mural cell regions⁴⁴, and induces vascular growth and reperfusion⁴⁵ in animal ischemia models.

While PDGF can induce vascular growth, it is also indicative of VEGF-mediated angiogenic signaling, owing to recently established cross-talk between the families. VEGFR2 and PDGFR β can form complexes on the pericyte-like 10T1/2 cell line ^{46,47}; VEGF-A can bind to both PDGFR α and PDGFR β , mediating migration on human dermal fibroblasts ⁴⁸. Furthermore, VEGF-PDGFR and PDGF-VEGFR interactions have recently been discovered and systematically quantified ⁴⁹. Therefore, the coordinated analysis of VEGF and PDGF signaling would advance our knowledge for ligand-receptor interactions, while uncovering novel approaches for controlling angiogenesis. However, these signaling axes have not been integrated into a comprehensive model.

Systems biology offers promising approaches for integrating VEGF and PDGF signaling. Computational models have characterized VEGF-VEGFR binding in both healthy and diseased tissue^{50,51}, VEGF spatial distribution in skeletal muscle ^{52–54}, angiogenic sprouting in skeletal muscle ^{55,56}, and VEGF gradients in peripheral artery disease (PAD) ⁵⁷. PDGF models have also been developed, which examined PI3K and MAPK pathways in fibroblasts ^{58,59}. In order to integrate and understand VEGF-PDGF cross-talk, deterministic models are necessary. Such models require data on receptor concentrations. However, plasma membrane receptor quantities can be cell-specific⁴ and are not well quantified for PDGFRs.

Quantitative flow (qFlow) cytometry is a useful approach for determining receptor quantities on plasma membranes. Here, immunofluorescent labeling of cells is examined alongside fluorescent microsphere calibration using flow cytometry to provide a high-throughput method of quantifying receptors on a cell-by-cell basis^{1,60,61}. In order to establish this approach for VEGFRs, we previously confirmed VEGFR antibody specificity and determined the conditions in which VEGFR antibody binding were saturating³. Additionally, the VEGFR response to environmental stimuli, such as VEGF ligands and enzymes was fully characterized³⁴. Extending qFlow cytometry to PDGFRs requires similar experimental optimization. However, an understanding of how PDGFRs are regulated by environmental stimuli has yet to be established.

Several studies have shown PDGFRs to be highly responsive to stimuli, including: PDGF⁶², VEGF-A ^{48,63}, and serum concentrations of the culture media ⁶⁴. Ball et al. have shown that VEGF-A can induce proliferation and migration of MSCs via signaling through PDGFR α and PDGFR β ⁴⁸. Furthermore, Battegay et al. observed expression of PDGFR β on angiogenic endothelial cells, of which the proliferation and tube formation was dependent on the presence of serum in the culture media. They further demonstrated that it was PDGF-BB, not PDGF-AA that drove tube/cord formation of the angiogenic endothelial cells ⁶⁴. Therefore, mapping how cells respond to these changes will offer insight into their cell-membrane regulation, while providing the accurate data needed for computational model development.

Here, we examine the regulation of membrane PDGFRs with comparison to VEGFRs and the VEGFR co-receptor, NRP1 on fibroblasts and endothelial cells. We examine how ligands and environmental stimuli affect membrane receptor concentrations. We also examine the heterogeneity in cell response by performing cell-by-cell analysis of receptor concentrations. Altogether, we establish methods for membrane PDGFR quantification, give insight into membrane receptor regulation, and provide data necessary for computational models.

3.2 MATERIALS AND METHODS

3.2.1 Cell culture

Human dermal fibroblasts (HDFs), derived from a normal adult, were purchased (Lonza, Allendale, NJ), expanded, and cryopreserved at a final concentration of 5×10^5 cells/mL. Freezing media, used for cryopreservation, included 80% Fibroblast Growth Medium-2 (FGM-2), 10% Fetal Bovine Serum (FBS), and 10% dimethyl sulfoxide (DMSO). The fibroblasts were cultured on flasks and maintained in one of three types of media: (1) FGM-2 containing 2% (v/v) FBS, 0.1% (v/v) insulin, 0.1% (v/v) human recombinant basic FGF-B (rhFGF-B), and 0.1% (v/v) gentamicin/amphotericin at a 1:1000 ratio (GA-1000). (2) High-serum media: DMEM with 4.5 g/L glucose, L-glutamine, and sodium pyruvate (Cellgro, Inc.) and supplemented with 10% (v/v) FBS and 1% penicillin/streptomycin (Invitrogen). (3) Low-serum media: DMEM with 4.5 g/L glucose, L-glutamine, and sodium pyruvate (Cellgro, Inc.), supplemented with 5% (v/v) FBS and 1% (v/v) penicillin/streptomycin. The media was filtered using NalgeneTM Rapid-FlowTM Sterile Disposable Bottle Top Filters containing a polyethersulfone (PES) Membrane (Nalge Nunc International Corp., Rochester, NY). The pore size of the filter is 0.20 µm. The media was stored at 4 °C and warmed to 37 °C upon usage. Human umbilical vein endothelial cells (HUVECs) were acquired from individual donors (Lonza and Invitrogen). The endothelial cells were

maintained in Endothelial Cell Growth Medium-2 (EGM-2) supplemented by the EGM-2 Single Quot Kit, which contains growth factors and fetal bovine serum (Lonza), as previously described ^{3,65}. All cells were maintained at 37°C in 95% air, 5% CO₂. All cells were grown to confluence before dissociating and were used only up to passage 6.

3.2.2 Media comparison

HDFs were seeded at equal densities in each of the 3 media. All media were changed every two days. HUVECs were seeded at equal densities in supplemented EGM-2 or Media 200 (Table 2) and media were changed every other day. Cells were imaged daily on an inverted EVOS FL microscope (AMG, Mill Creek, WA), using a 10X Plan PH2 0.25 NA air objective. Image brightness, contrast, tone and color were adjusted using both Adobe Photoshop and Image J (Fig. 8A and 9A). Cell confluency was automatically quantified in Image J, as follows: images were converted to 16-bit greyscale (Fig. S1A) and thresholded (Fig. S1B). Particles between 100-1000 μ m² were counted from three different regions of a T-175 flask (Fig. S1C) and normalized to the maximum cell count observed.

3.2.3 Growth factor application

The homodimeric proteins, PDGF-AA and PDGF-BB, the heterodimeric protein PDGF-AB (R&D Systems, Minneapolis, MN) and the recombinant hVEGF-A₁₆₅ (Shenandoah Biotechnology, Warmack, PA) were reconstituted with $1 \times$ Dulbecco's Phosphate-Buffered Saline (PBS) at concentrations of 350 µg/mL for PDGF-AA, PDGFAB, PDGF-BB, and at 100 µg/mL for hVEGF-A₁₆₅ The ligands were frozen, and stored at -20° C. Ligand concentrations were confirmed by a NanoDrop 2000c UV-Vis Spectrophotometer (Thermo Fisher Scientific, Waltham, MA). Each ligand was incubated with cells for 20–24 h.

3.2.4 Cell dissociation

For routine cell culture, cells were detached from flasks using TrypLE Express (Invitrogen); however, serine proteases (e.g., trypsin), can significantly affect cell quantification results³. Therefore, when performing receptor quantification, the non-enzymatic cell dissociation (Millipore, Billerica, MA) was applied for 4-7 min at 37°C, and flasks were subjected to abrupt mechanical force to dislodge cell adherence. Cells were re-suspended in stain buffer (1× PBS, bovine serum albumin, sodium azide)^{3,66}, centrifuged at 500 x g for 5 minutes, supernatant was aspirated, and cells were re-suspended to a final concentration of 4 x 10⁶ cells/mL in stain buffer.

3.2.5 Cell staining

25 μ L aliquots of cells (1 x 10⁵ cells) were added to 5 ml polystyrene round-bottom tubes (BD Biosciences, New Jersey) and labeled with phycoerythrin (PE)-conjugated monoclonal antibodies at the optimal concentrations: 14 μ g/mL for VEGFR1, VEGFR2, 7.1 μ g/mL for NRP1³, and 9.4 μ g/mL for PDGFRs (Fig. 7). VEGFRs were quantified on HUVECs for comparison and not on HDFs, because HDFs express little to no VEGFRs⁴⁸. NRP1, a VEGFR co-receptor, was quantified on HUVECs and HDFs as a comparison. PDGFRs were quantified on human dermal fibroblasts, because fibroblasts are known to express these receptors^{38,48}; whereas, HUVECs have shown little to no PDGFR expression⁶⁷. Tubes were protected from light and incubated for 40 minutes on ice. Cells were washed, centrifuged at 500 ×g twice with 4 mL stain buffer, and resuspended in 300 μ L stain buffer. The precision and accuracy of qFlow cytometry profiling has been rigorously tested ^{68–71}. We chose the PE fluorophore as the basis of our quantitative fluorescence measurements, because its high extinction coefficient reduces error due to photobleaching, its fluorescence is not quenched by common biomolecules (e.g.,

antibodies), its fluorescence is independent of pH, and its size minimizes the possibility of multiple fluorophores conjugated to an antibody ^{72,73}.

3.2.6 Quantitative flow cytometry

Flow cytometry was performed on a LSR Fortessa (BD) Flow cytometer; BD FACSDIVA software was used for data acquisition, and FlowJo (TreeStar) software was used for data analysis. Tubes were vortexed immediately prior to placement in the flow cytometer. 8,000 - 10,000 live cells were collected from each tube. 5 μ g/mL Sytox Blue (Invitrogen) was added to all samples to distinguish between live and dead cells. Sytox blue was excited with a solid-state laser (407 nm) and its emission was collected using a 450/50 band-pass filter. We plotted histograms of Sytox Blue fluorescence to identify the live cell population. Cells exhibiting little to no Sytox Blue fluorescence are gated as live cells. These gated cells were then examined in a plot of forward scatter area (FSC-A) versus side scatter area (SSC-A) to gate the single-cell population.

3.2.7 Statistical analysis: ensemble-averaged data

Quantibrite PE beads (BD) were collected and analyzed under the same compensation and voltage settings as cell fluorescence data. Quantibrite PE beads comprise a combination of polystyrene beads conjugated with different density of PE molecules: low (474 PE molecules/bead), medium-low (5,359 PE molecules/bead), medium-high (23,843 PE molecules/bead), and high (62,336 PE molecules/bead). A calibration curve that translated PE geometric mean to the number of bound molecules was determined using linear regression: y = mx+b, where $x=log_{10}^{Number of PE molecules per bead}$, y represented $log_{10}^{PE geometric mean per bead}$, and m and b represented the slope and intercept of the linear regression, respectively. For each experiment we

collected 2 – 4 biological replicates per condition. The number of independent samples and cells analyzed included in this manuscript for each receptor is shown in Table 3. Here, a "sample" is defined as a vial containing ~4×10⁶ cells stained with antibody. The vials were composed of a cell suspension cultured on T-175 or T-75 flasks. The number of cells in Table 3 represents the number of gated, live, single cells that were analyzed. Ensemble averaged data were expressed as mean \pm standard error of the mean. Unless otherwise noted, p < 0.05 was considered statistically significant using the Fisher test of variance (ANOVA) and is indicated with *, 0.001 is indicated with **, and p < 0.001 is indicated with ***.

3.2.8 Cell-by-cell background subtraction

In order to accurately quantify cell-by-cell receptor levels, the background signal from cell autofluorescence must be subtracted from the fluorescence signal. To subtract the background signal present in a single emission channel for all noise magnitudes, we used a weighted integral approach First, we obtained cell-by-cell fluorescence data from labeled and unlabeled cells using flow cytometry, using the same excitation and emission spectra for all cells. We then took the weighted integral of the labeled and unlabeled cells by summing the fluorescence values and dividing by the number of values. We derived a signal to noise ratio by dividing the weighted integral of the labeled cell signal with the unlabeled cell signal. We weighted the labeled cell signal using this signal to noise ratio, shifting the signal and effectively removing the background. Mathematically:

$$PE_{subtracted} = PE_{labeled} \cdot \left(1 - \frac{\left(\sum PE_{unlabeled}\right) / n_{unlabeled}}{\left(\sum PE_{labeled}\right) / n_{unlabeled}}\right)$$
(Equation 1)

where $PE_{subtracted}$ is the number of receptors per cell obtained after background subtraction, $PE_{labeled}$ is the fluorescence from labeled cells, $PE_{unlabeled}$ is the fluorescence from unlabeled cells, and *n* is the number of cells in a dataset. This subtraction method does result in some negative $PE_{subtracted}$ values, indicating that the noise is larger than the signal; negative values are set to zero. Thus, the background subtraction method presented here allows us to accurately quantify fluorescent levels within the channel of interest by shifting the signal to remove noise from cell autofluorescence.

3.2.9 Statistical analysis: non-normality and diversity

A two sample Kolmogorov–Smirnov (K–S) test was performed on cell-by-cell distributions and a reference normal distribution in Matlab as described by previous study⁷⁴. The p-value from the K-S test is used to quantify the receptor-specific non-normality of the cell distribution. Samples having p > 0.05 were considered to have a significant non-normal distribution and may suggest subpopulations of cells responding differently. Quadratic entropy was measured using a previous method⁷⁴ on cell-by-cell distributions in Matlab to compare the cell diversity between receptors and different treatment (Fig. S3).

3.3 RESULTS

3.3.1 Antibody saturation affected qFlow cytometry accuracy

Non-labeled receptors will invalidate qFlow results. Therefore, a receptor saturation study is necessary for accurate qFlow cytometry profiling. The saturation regime was previously established for VEGFR antibodies (14 μ g/mL for VEGFR1, VEGFR2, 7.1 μ g/mL for NRP1)³. Here, we observed that membrane PDGFR α (Fig. 7A) and PDGFR β (Fig. 7B) saturated at 9.4 μ g

antibody/mL. This represented a 75% higher labeling concentration compared to that suggested by the manufacturer. Using our regression analysis, we estimated that the manufacturer suggested labeling of 2.3 μ g/mL would result in labeling only 40% of membrane PDGFRs, versus ~90% of receptors at 9.38 μ g/mL. Therefore, verifying antibody saturation is an important first step to ensure accurate quantification of cell membrane receptors.

3.3.2 HUVEC media did not significantly affect membrane VEGFR1/2 & -NRP1 levels.

We have previously shown that VEGF-A₁₆₅ can regulate membrane VEGFR concentrations³. Since endothelial media contains several supplements (Table 2), we examined whether two commonly used endothelial cell growth media result in differential membrane VEGFR presentation on HUVECs. The ensemble averaged data showed that neither media exerted a significant effect on the membrane VEGFR1 or VEGFR2 concentrations (Fig. 8C). The cell-bycell analysis confirmed this finding, with membrane VEGFR1 (Fig. 8D) and VEGFR2 (Fig. 8E) giving similar distributions regardless of growth media. However, we observed a higher variability of VEGFR1 and VEGFR2 cell-by-cell distributions between different trials of experiments (Fig. S2).

Interestingly, we observed significantly higher (ANOVA, p<0.001), ~50%, membrane VEGFR3 on HUVECs grown in Media 200 compared with EGM-2 (Fig. 8C). This difference is best understood when examining the population data (Fig. 8F). Here, the cell-by-cell analysis revealed a significant difference in HUVEC membrane VEGFR3 levels between media types (K-S test, p < 0.01) (Fig. 8F). Moreover, we observed that membrane VEGFR3 resolves into 2 sub-populations. Along with this observation, it also suggests that Media 200 may not provide the

ideal environment for reducing cell death. Indeed, we observed significantly slower cell growth with longer Media 200 culture times (ANOVA, p<0.05, Fig. 8A-B).

3.3.3 Serum downregulated fibroblast membrane PDGFRβ and membrane NRP1 levels and increased PDGFRβ non-normality.

We similarly examined the effect of media on the growth rate of HDFs (Fig. 9A-B) and the concentration of membrane PDGFRs and membrane NRP1. The HDF growth progression did not reflect a statistically significant difference between FGM-2 and DMEM + 5%FBS (Fig. 9B). However, the slope of the linear regression reflected that HDFs grown in DMEM+10%FBS grew significantly faster compared to FGM-2 (p < 0.05, ANOVA), which resulted in these cells reaching confluence ~1-2 days sooner than cells grown in the other two media (Fig. 9A).

Membrane PDGFRs and membrane NPR1 were quantified on confluent cells with FGM-2 reflecting control conditions. Here, HDFs grown in DMEM +10% FBS showed a ~40% decrease in membrane PDGFR β concentrations (p < 0.001) and a ~40% decrease in membrane NRP1 concentrations (p < 0.001) (Fig. 9C). Similarly, DMEM+5% FBS downregulated membrane PDGFR β and membrane NRP1 by ~ 20% (p < 0.05). These serum-induced decreases in membrane PDGFR β and membrane NRP1 were visualized in the cell-by-cell histograms as increased numbers of cells presenting < 10,000 receptors (Fig. 9E & F). Serum did not significantly affect membrane PDGFR α : the ensemble averaged data showed no significant differences across media. However, a K-S test on the cell-by-cell analysis showed that the non-normality in membrane PDGFR α distributions were proportional to serum concentrations (Fig. 9D & G).

3.3.4 PDGF did not affect HUVEC membrane VEGFR1/2 levels.

Given the recently discovered VEGF-A signaling via PDGFR β^{48} , we examined whether PDGFs could regulate plasma membrane levels of VEGFRs and the co-receptor, NRP1 (Fig. 10A). 100 ng/mL PDGF-AA, -AB and -BB treatment for 20-24 hours affected neither membrane VEGFR1 (Fig. 10B) nor membrane VEGFR2 (Fig. 10C). Membrane VEGFR3 was unaffected by PDGF-AB; however, its levels were increased by ~60% in the presence of 100 ng/mL PDGF-AA, and it was upregulated by ~50% in the presence of 100 ng/mL PDGF-BB (p < 0.01, Fig. 10A & D). As with the Media 200-mediated VEGFR3 increase, PDGF-AA and PDGF-BB presented increased numbers of the high- VEGFR3 subpopulation. Although NRP1 has not been shown to bind PDGFs, its membrane levels were upregulated by ~10% upon treatment of 100 ng/mL of PDGF-AB and PDGF-BB while its concentrations remained unaffected by 100 ng/mL of PDGF-AA (Fig. 9A & E).

3.3.5 VEGF-A₁₆₅ downregulated fibroblast membrane PDGFRα levels.

The cross-family dose-response relationship was obtained for HDFs using concentrations of VEGF-A₁₆₅ ranging from 25 ng/mL to 100 ng/mL (Fig. 11A). Membrane PDGFR α levels remained constant with increasing VEGF-A₁₆₅ concentration from 25 ng/mL to 50 ng/mL. Membrane PDGFR α levels were decreased by ~25%, at 100 ng/mL VEGF-A₁₆₅. Membrane PDGFR β levels were similarly decreased at this dose (p < 0.05). These VEGF-A₁₆₅-induced decreases in PDGFR α and PDGFR β plasma membrane levels were also reflected in leftward shifts in their cell-by-cell distributions (Fig. 11B-C). The downregulation in membrane PDGFR α can be quantitatively described as a ~65% decrease in the number of HDFs expressing > 5,000 membrane PDGFR α /cell and a ~50% increase in the number of HDFs expressing 1,400 – 5,000

membrane PDGFR α /cell. Similarly, 100 ng/mL VEGF-A₁₆₅ treated HDFs present a ~45% increase in the number of cells expressing < 80,000 membrane PDGFR β /cell. While NRP1 has been shown to bind VEGF-A₁₆₅, membrane NRP1 levels remained constant at ~ 119,230 ± 3,410 NRP1/HDF as the concentration of VEGF-A₁₆₅ increased (Fig. 11A & D). Furthermore, a K-S test on cell-by-cell distributions revealed that the non-normality of the membrane PDGFR β and NRP1 distributions increased with increasing VEGF-A₁₆₅ concentration from 25 ng/mL to 100 ng/mL (Fig. 11E).

3.3.6 PDGF-AA downregulated fibroblast membrane PDGFR α levels and did not affect membrane PDGFR β .

PDGF ligands regulate mural cell proliferation and migration through selective PDGFR activation. In particular, PDGF-AA signals exclusively through PDGFR α , so we treated HDFs with PDGF-AA to determine if it exerts a regulatory effect on membrane PDGFRs (Fig. 12A). We observed that as PDGF-AA concentrations increased, PDGFR α membrane levels significantly decreased by up to 55% (p < 0.05 for PDGF-AA concentrations \geq 5 ng/mL, Fig. 12A-B). This PDGF-AA-mediated decrease of membrane PDGFR α was further observed as a significant leftward shift in the cell-by-cell distributions (Fig. 12B). Membrane PDGFR β , which does not bind PDGF-AA, was unaffected by PDGF-AA (Fig. 12A & 12C). In contrast to VEGF-A₁₆₅ treated HDFs, a K-S test on the PDGF-AA treated HDFs reveals that high concentrations of PDGF-AA correlates with increased normality in PDGFR β distributions (Fig. 12D). 3.3.7 PDGF-AB downregulated fibroblast membrane PDGFRα levels and did not affect membrane NRP1.

PDGF-AB is a binding partner of PDGFRα homodimers and PDGFRαβ heterodimers, and its signaling through these receptors stimulates angiogenesis in solid tumors and malignancies ⁷⁵. We therefore looked at the comparative regulatory effect of PDGF-AB on membrane PDGFRs and membrane NRP1 (Fig. 13A). Following a 20-24 h treatment, PDGFRα membrane levels significantly decreased on HDFs, up to 76% (p < 0.001 for PDGF-AB concentrations \geq 5 ng/mL). This aligned with our observations of a shift in PDGFRα cell-by-cell distributions upon treatment with 50 ng/mL PDGF-AA (Fig. 12B). PDGFRβ membrane levels treated with 50 ng/mL PDGF-AB treated PDGFRβ cell-by-cell distributions did reflect this 20% decrease as a leftward distribution shift (Fig. 13C). As expected, NRP1, which is not known to bind PDGF-AB, was not affected by PDGF-AB. Membrane NRP1 concentrations remained stable at 123,970 ± 7,670 (Fig. 13A & 13D), which is unlike our observations on HUVECs (Fig. 10A). Although unaffected in receptor levels, a K-S test on HDF cell-by-cell distributions shows that NRP1 has increasing normality with increasing PDGF-AB concentration (Fig. 13E).

3.3.8 PDGF-BB regulated both fibroblast PDGFRα and PDGFRβ.

PDGF-BB binds both PDGFRs. Moreover, previous studies have suggested that PDGF-BB released from endothelial cells promotes recruitment and proliferation of adjacent mural cell progenitors via signaling through PDGFR β ⁷⁶. Therefore, in order to determine if PDGF-BB exerts a regulatory effect on PDGFRs we tested PDGF-BB concentrations ranging from 5 ng/mL to 50 ng/mL on HDFs for 20 – 24 hr (Fig. 14A). Both membrane PDGFR α and PDGFR β were

significantly affected by the addition of PDGF-BB. Membrane PDGFR α levels increased by ~30 -40% as PDGF-BB concentration increased from 0 to 5 ng/mL (p < 0.01 at 0.5 ng/mL and p < 0.001 at 5 ng/mL). Membrane PDGFRa was downregulated by 50 ng/mL and 100 ng/mL PDGF-BB treatment by ~30% (p < 0.05) and ~70% (p < 0.001), respectively. Similarly, membrane PDGFR^β concentrations were significantly upregulated by PDGF-BB at concentrations of 0.5 ng/mL and 5 ng/mL by ~60% and ~50%, respectively. However, as PDGF-BB concentrations increased to 50 ng/mL and 100 ng/mL, membrane PDGFR β levels decreased ~90% compared to control (p < 0.001). The downregulation of PDGFR α and PDGFR β membrane levels by 100 ng/mL PDGF-BB, observed in the ensemble averaged data, was qualitatively observed in the cell-by-cell analysis by the shifting of receptor distributions to the left (Fig. 14B & C). The nonnormality test does not show a PDGF-BB correlation with normality (Fig. 14E). Interestingly, HDF membrane NRP1 showed a similarly delayed dose response as PDGFR α : a 15 – 40% increase in ensemble average as PDGF-BB concentration increased from 0 - 50 ng/mL (p < 0.001 at 50 ng/mL PDGF-BB) and a ~30% decrease when PDGF-BB reached 100 ng/mL. As with the other PDGFs, PDGF-BB has not been shown to bind NRP1. Contrary to increased membrane NRP1 levels upon treatment of 100 ng/mL PDGF-BB on HUVECs, HDF membrane NRP1 was downregulated by 100 ng/mL of PDGF-BB.

3.4 DISCUSSION

PDGFRs are important regulators of vascular development and this role underlies a need to better understand their cell-surface distribution. Towards this goal, we successfully: (1) quantified PDGFR surface-levels on fibroblasts and (2) examined their regulation by serum and angiogenic growth factors.

3.4.1 PDGFR α versus PDGFR β membrane levels.

We observed significant presentation of PDGFRs on adult human dermal fibroblasts, with PDGFR β present at levels two orders of magnitude higher than PDGFR α . These findings are consistent with prior radiolabeling studies. In particular, newborn foreskin fibroblasts (AG1523) were reported to present ~2,000-5,000 PDGFR α and ~150,000 PDGFR β , and ~12,000 PDGFR α and ~275,000 PDGFR β on adult fibroblasts (SK5) ⁷⁷. Another study observed human fibroblasts presenting 50,000 PDGFRs on the plasma membrane³⁸ with 5-10 times more PDGFR β than PDGFR α . The consistently higher PDGFR β relative to PDGFR α is important in the context of cross-family signaling, because radiolabeling studies would not discern the presence of any additional receptors (e.g. VEGFRs) with similar binding affinities as the PDGFRs. While we have not observed VEGFRs on the HDFs (data not shown), VEGFRs might be present on the cell types tested in those previous reports. Indeed, both VEGFRs and PDGFRs have been quantified on human dermal microvascular endothelial cells (HDMECs), at ~1,900 VEGFR1, ~7,200 VEGFR2 and ~30,000 total PDGFRs ^{3,38,64}.

There are a few studies quantifying PDGFR-levels, these include: radiolabeling analysis of PDGF binding to 32D mouse hematopoietic cells engineered to express PDGFR α (32D α R), and Chinese hamster ovary (CHO) fibroblasts engineered to express PDGFR β (HR5 β R cells)⁷⁸. They reported that 32D α R and HR5 β R cells express ~50,000 PDGFR α /cell ⁷⁹ and ~55,000 PDGFR β /cell ⁸⁰, respectively. Since these were overexpression models, the number of receptors might not accurately reflect stromal cell PDGFR-levels. However, the characteristic expression pattern of lower PDGFR α and higher PDGFR β indicated a unique regulation of these receptors that was preserved even under induction. Indeed, only one study has reported higher PDGFR α

than PDGFR β : a human sarcoma cell line, MG-63, where ~60,000 PDGFR α and ~50,000 PDGFR β were present ⁷⁷.

3.4.2 Why is there differential PDGFR expression?

An explanation may lay in the specific roles of the PDGFRs. One study suggested that low PDGFRa is a mechanism of modulating PDGF-AA-mediated mitogenic activity, without changing PDGF-AA potency, given that MG-63 cells, which expressed similar level of PDGFRa and PDGFR β^{77} , presented similar mitogenic activity stimulated by PDGF-AA and PDGF-BB. In another study, PDGF-AA was shown to significantly stimulate cardiac fibroblast growth, and these cells expressed an unusually high level of PDGFRa (15,300 PDGFRa and 24,800 PDGFRβ per cell)⁸¹. These findings suggested a mitogenic role of PDGFRa in which membrane PDGFRa levels regulate the mitogenic activity of ligands such as PDGF-AA and PDGF-AB. Other studies also supported the theory of differential PDGFR roles ^{82,83}. For example, PDGFRa activation inhibited fibroblast and smooth muscle cell chemotaxis, while PDGFRß stimulated fibroblast chemotaxis^{84,85}. Indeed, these differential roles also extend to pathological conditions. For instance, PDGFR^β is highly expressed in cancer-associated fibroblasts in human breast cancer samples, while PDGFR α expression is not as frequently observed on tumor fibroblasts ^{86,87}. In addition, immunohistology and confocal microscopy analysis have revealed the specific upregulation of PDGFRβ on blood vessels of an ischemic mouse model ⁸⁸. Together, these prior studies and our data offer new insight into the differential presentation of PDGFRs, and these PDGFR α and PDGFR β signaling contributions can be better unmixed through computational modeling.

3.4.3 Cell growth conditions: Media

Examining cell growth conditions and mapping these to changes in protein levels is critical towards understanding and ultimately controlling the environmental conditions directing angiogenesis. A recent study showed that VEGFR2 protein levels are dependent on cell density, with twice as many surface VEGFR2 receptors in long confluent cells, those with cell-to-cell junctions, compared to sparse cells, those lacking cellular contacts ⁸⁹. Although we found that HUVECs maintained in EGM-2 exhibited growth to confluence in a shorter time than HUVECs maintained in M200 media, once confluence is reached, we saw no appreciable differences in averaged receptor levels of VEGFR1 or VEGFR2 (Fig. 8B). These studies also brought into question endothelial response to growth factor, because of the differences in media supplementation. In comparison to Media 200, EGM-2 is supplemented with VEGF and IGF (Table 2), which are both known regulators of endothelial cell survival and migration^{90–92}. Here we observed that such supplementation does increase growth rate; however, cells lacking these supplements converged to the same receptor-based steady state as cells treated with these supplements.

3.4.4 Cell growth conditions: FBS

FBS is a common supplement to in vitro culture media. Some common components of serum include growth factors such as fibroblast growth factor (FGF) and platelet-derived growth factor (PDGF), hormones, enzymes, serum proteins, fatty acids, amino acids, and carbohydrates ^{93,94}. Our results showed that this combination of biomolecules can selectively downregulate PDGFRβ and NRP1, while increasing cell growth rate. A previous study has reported that DMEM+10%FBS cultured fibroblasts showed tumor cell-like growth, while human fibroblasts

in living dermis rarely proliferated and maintained homeostasis of the dermis through collagen production ⁹⁵. Based on our studies, we recommend using media supplemented with lower level of serum when culturing HDFs in order to preserve surface receptor levels and to reflect a more normalized fibroblast growth rate.

3.4.5 VEGF-mediated changes in receptor levels confirms cross-talk

The VEGF-A₁₆₅-induced decreases in PDGFRα and PDGFRβ indicate that VEGF acts independently of VEGFRs to regulate PDGFR levels, possibly through internalization of signaling receptors. This is a significant finding, because it indicates that under conditions of high-VEGF, VEGF signaling through PDGFRs and regulation of PDGFR concentrations could play a significant signaling role. Furthermore, the fact that VEGF can downregulate PDGFRs means that VEGF can modulate PDGFR sensitivity to its native-ligand: PDGF. In particular, the downregulation of PDGFRs following VEGF application can lead to lower sensitivity of PDGFRs to their native ligands: PDGFs, thus possibly reducing PDGF-mediated signaling. Indeed, we have found one study suggesting VEGF-A inhibition of PDGF signaling; wherein VEGF inhibited PDGF-induced mesenchymal stem cell (MSC) migration⁴⁸.

3.4.6 High-ligand concentrations offer insight into local signaling

The concentrations tested in this study are higher than currently reported serum ligand levels⁹⁶. However, there are opportunities for local spikes in ligand concentration to the nM level, which can be mediated by extracellular matrix-ligand binding and its proteolytic release⁵². This mechanism for producing high-local VEGF-concentrations has been predicted computationally ⁹⁷, and together with our data suggests a mechanism for VEGF-mediated regulation of PDGFRs under the proteolytic states observed in cardiovascular disease⁹⁸ and pathological vascularization^{99,100}. In contrast, VEGF regulation of PDGFR may be inhibited by VEGF sequestration by heparin sulfate proteoglycans (HSPGs) on the cell-surface. Computational modeling of FGF-HSPG binding has shown that high levels of HSPG can significantly increase FGF-receptor ligation¹⁰¹. Similar studies of VEGF-A₁₆₅ binding to HSPGs¹⁰² indicated a similar role; however, a recent study suggested that HSPG-mediated VEGF ligation decreased VEGFR internalization¹⁰³. As such, HSPGs should be explored towards decreasing VEGF-PDGFR regulation, while proteases should be explored towards increasing VEGF-mediated regulation of PDGFRs. Conversely, it is important to note that we did not observe a VEGF-A₁₆₅-induced decrease in NRP1, the VEGFR co-receptor, on HDFs. Nonetheless, we have previously reported VEGF-A induced downregulation of NRP1 on HUVECs³. This suggested that VEGF-NRP1 downregulation requires VEGFRs. Therefore, our receptor quantification outlines an expanded regulatory role of VEGF signaling.

3.4.7 Regulation of PDGFRs by PDGF

Our data showing significant downregulation of PDGFR α confirmed that PDGFR α binds to all three PDGF ligands with high affinity, and aligned with previous studies^{104,105}. Furthermore, our observation of significant downregulation of PDGFR β by PDGF-BB but not PDGF-AB or PDGF-AA indicated that PDGFR β binds to PDGF-BB with the highest affinity. Indeed, previous research showed that PDGF-BB binds to PDGFR β with a K_d of ~0.5 nM, whereas PDGF-AB binds to a small number of PDGFR β s with a K_d of ~0.8 nM and the majority of PDGFR β s with a K_d of ~6 nM^{104,106–108}. Interestingly, we observed that the ensemble average of membrane PDGFR β concentration was not significantly affected by the application of PDGF-AB, while our cell-by-cell analysis showed that the receptor distribution shifted to the left when
treated with 50 ng/mL PDGF-AB. The small population shift may map to the PDGFRβs with differing PDGF-AB affinity: a theory that can be better resolved via computational modeling.

Other explanations for the small PDGF-AB-mediated population shift may lay in the dimerization state of PDGFRs. Although heterodimeric receptor PDGFR $\alpha\beta$ can form and be activated when treated with PDGF-AB, Hammacher et al. suggested that in the absence of PDGFR α , PDGF-AB acts as a PDGFR β antagonist because it binds to PDGFR β without activating them^{32,109}. It is possible that the small number of PDGFR β downregulated upon treatment of PDGF-AB existed in the form of a heterodimer, which were bound to PDGF-AB with higher affinity. In addition, the unchanged membrane levels of PDGFR β on PDGF-AA treated HDFs was consistent with previous studies where PDGFR β failed to bind PDGF-AA with any appreciable affinity^{104,108}. Again, these PDGF concepts can be clarified by examining cell populations with different PDGF-PDGFR binding affinities.

3.4.8 Fibroblasts and PDGF signaling

It has become increasingly appreciated that stromal cells, such as myofibroblasts and cancerassociated fibroblasts (CAFs) play an important role in tumorigenesis^{76,110,111}. CAFs are heterogeneous and may derive from tissue fibroblasts, bone-marrow-derived progenitor cells or transdifferentiating epithelial cells^{30,112}. Indeed, CAFs express overlapping markers as fibroblasts such as NG-2, α -SMA, SM22- α and PDGFR ^{86,112,113}. CAFs have been demonstrated to enhance tumor cell proliferation, angiogenesis, invasion and metastasis by secreting multiple growth factors and cytokines¹¹⁰. Recent studies have demonstrated that tumor cell-secreted PDGF-AA promoted recruitment of PDGFR α -positive fibroblasts, which in turn produced VEGF-A and enhanced tumor angiogenesis¹¹⁴. In another study, tumor-derived PDGF-BB was found to

promote stroma formation and tumor growth of melanoma¹¹⁵. Therefore, quantifying angiogenic receptors such as PDGFR β on HDFs provides a better understanding of the roles of stromal cells in tumorigenesis; modeling inter-family and cross-family signaling between PDGF and VEGF will also offer new insights into anti-angiogenic therapy.

3.4.9 Cell-by-cell heterogeneity

As a main component of drug resistance, cellular heterogeneity presents a grand challenge in understanding drug response and predicting therapeutic outcome^{5,74}. In our study, we characterized cell-by-cell heterogeneity by its non-normality and diversity through the K-S test. This method was defined by Gough et al. in a previous study⁷⁴. One of the interesting findings in our study is that increasing the amount of serum in HDF culture media increased the nonnormality HDFs presenting membrane PDGFR β . That indicated an increase in cellular heterogeneity, as defined by membrane PDGFR β . As described before, serum is an important and commonly used supplement to in vitro culture media. Some common components of serum include growth factors, hormones, enzymes, etc⁶⁶. This result suggests that in a growth factor/enzyme-rich environment, such as tumors and wounds, we may observe increased cellular heterogeneity, defined by PDGFR β .

3.5 CONCLUSIONS

PDGFR, VEGFR, and NRP1 are critical to angiogenic signaling in several diseases, including prostate cancer, lung cancer, and endometrial cancers. Therefore understanding their regulation by their ligands offers new insight into pathologies where increased ligand is present. Cells were treated with several ligands, and we observed significant decreases in the corresponding PDGFR binding partners. The VEGF-A-induced changes in PDGFRs also advocate the angiogenic

functionality of VEGF-PDGF signaling network under high-VEGF conditions. Media components were also varied in our study and it revealed how the presence of serum can increase cell growth while decreasing membrane receptors. Overall, the membrane receptor changes that we observed have shed light onto cross-family regulation. We have helped to establish conditions for qFlow profiling of tyrosine kinase receptors, while providing the data necessary for advancing computational models of PDGF-VEGF cross-family signaling.

3.6 FIGURES AND FIGURE LEGENDS



Figure 6. Schematic of cross-family binding between VEGF, PDGF family and NRP1. * NRP1 is shown as a co-receptor to VEGFR dimmers R and R'.



Figure 7. Saturation of antibody labeling for surface receptors on HDFs. (A) PDGFR α and (B) PDGFR β both saturate at 9.4 µg/mL on HDFs with respect to an exponential fitting of normalized receptor density with increasing labeling concentration.



Figure 8. HUVEC media comparison. (A) Representative pictures of HUVECS cultured in each media on day 1-4. (B) Normalized HUVEC confluence. (C) Ensemble averages of VEGFR levels AND Cell-by-cell analysis (D) VEGFR1, (E) VEGFR2, and (F) VEGFR3. Significance tests were conducted using ANOVA where * indicates p < 0.05 and *** indicates p < 0.001.



Figure 9. HDF media comparison. (A) Representative pictures of HDFs cultured in each media on day 1 – day 9. Scale bar is 250 μ m. (B) Normalized HDF confluency given number of days cultured of each media. (C) Ensemble averages of HDF receptor levels cultured in different media. Cell-by-cell analysis was performed on HDF receptor levels grown in each media: (D) PDGFR α , (E) PDGFR β , and (F) NRP1 were analyzed. (G) p-value from K-S test of PDGFR α , PDGFR β and NRP1 on HDFs cultured in different media. Significance tests were conducted using ANOVA where * indicates p < 0.05 and *** indicates p < 0.001.



Figure 10. PDGF regulation of HUVECs receptor levels. (A) Ensemble averages of HUVEC receptorlevels regulated by 100 ng/mL (4 nM) of PDGFAA, PDGFAB, PDGFBB. Cell-by-cell analysis of (B) VEGFR1, (C) VEGFR2, (D) VEGFR3 and (E) NRP1 responses induced by PDGF ligands. Significance tests conducted using ANOVA where * indicates p < 0.05, ** indicates p < 0.01 and *** indicates p < 0.001.



Figure 11. Response of PDGFR α , PDGFR β and NRP1 to 20–24 h application of 0 - 100 ng/mL (2.6 nM) VEGF-A₁₆₅. (A) VEGFA dose-receptor response and cell-by-cell analysis of control (FGM-2) and highest VEGF dose for (B) PDGFR α , (C) PDGFR β and (D) NRP1. (E) p-value from K-S test of PDGFR α , PDGFR β and NRP1 on HDFs treated with VEGF was also analyzed. Significance tests were conducted using ANOVA where ** indicates p < 0.01 and *** indicates p < 0.001.



Figure 12. Response of PDGFR α , PDGFR β to 20–24 h application of 0 – 50 ng/mL (0 – 1.75 nM) PDGFAA. (A) PDGFAA dose-receptor response and cell-by-cell analysis of control (FGM-2) and highest PDGFAA dose for (B) PDGFR α and (C) PDGFR β . (D) p-value from K-S test of PDGFR α and PDGFR β on HDFs treated with PDGFAA was also analyzed. Significance tests were conducted using ANOVA where ** indicates p < 0.01 and *** indicates p < 0.001.



Figure 13. Response of PDGFR α , PDGFR β and NRP1 to 20–24 h application of 0 – 50 ng/mL (0 – 2 nM) PDGFAB. (A) PDGFAB dose-receptor response and cell-by-cell analysis of control (FGM-2) and highest PDGFAB dose for (B) PDGFR α , (C) PDGFR β and (D) NRP1. (E) p-value from K-S test of PDGFR α , PDGFR β and NRP1 on HDFs treated with PDGFAB was also analyzed. Significance tests were conducted using ANOVA where ** indicates p < 0.01 and *** indicates p < 0.001.



Figure 14. Response of PDGFRa, PDGFR β and NRP1 to 20–24 h application of 0 – 100 ng/mL (0 – 4 nM) PDGFBB. (A) PDGFBB dose-receptor response and cell-by-cell analysis of control (FGM-2) and highest PDGFBB dose for (B) PDGFRa, (C) PDGFR β and (D) NRP1. (E) p-value from K-S test of PDGFRa, PDGFR β and NRP1 on HDFs treated with PDGFBB was also analyzed. Significance tests were conducted using ANOVA where ** indicates p < 0.01 and *** indicates p < 0.001.



Supplemental Figure 1. Image processing for cell concluency quantification. A image of HUVECs was converted to 16-bit greyscale using Adobe Photoshop (A) and thresholded using imageJ (B). Particles between $100 - 1000 \ \mu\text{m}^2$ were gated out using imageJ and the number of particles was recorded as cell number.



Supplemental Figure 2. Cell by cell analysis of VEGFR1 distribution on HUVECs grown in (A) M200 media and (B) EGM-2 under different trials. We observed a higher variability of receptor distribution within trials in HUVECs cultured in M200 compared to EGM-2.



Supplemental Figure 3. Quadratic entropy of receptors responding to different media or ligand treatment.

3.7 TABLES

Table 2. HUVEC media supplement components

Component	Media-200 (Life Technologies)	EGM-2 (Lonza)
2% Fetal Bovine Serum	2%	2%
Hydrocortisone	1 µg/mL	0.2 μg/mL
hEGF	10 ng/mL	10 ng/ml
bFGF	3 ng/mL	4 ng/ml
heparin	10 µg/mL	1 ng/mL
GA-1000 (Gentamicin, Amphotericin-B)		3 μg/mL
VEGF		2 ng/mL
R3-IGF-1		5 ng/ml
Ascorbic Acid		75 mg/ml

		Ensemble averaging					Cell-by-cell analysis					
		n	Mear	ו ± S	EM	Number of	Median	IQR	Skewness	K-S test		
						cells						
VEGFR1						HUVECs						
	Control	8	990	±	50	82,195	940	1,110	65	1.29 × 10 ⁻¹⁵		
	100 ng/mL	4	990	±	40	40,342	1,000	1,010	60	1.68 × 10 ⁻¹⁵		
	PDGFAA											
	100 ng/mL	3	830	±	20	33,730	840	930	71	1.24 × 10 ⁻¹⁴		
	PDGFAB											
	100 ng/mL	8	1,000	±	40	58,879	980	1,150	64	5.31 × 10 ⁻¹⁵		
	PDGFBB											
VEGFR2						HUVECs						
	Control	7	1,890	±	110	71,066	1,960	2,160	82	9.15 × 10 ⁻¹⁴		
	100 ng/mL	4	1,780	±	160	42,824	1,850	2,000	106	1.21 × 10 ⁻¹⁵		
	PDGFAA											
	100 ng/mL	3	1,650	±	90	34,149	1,800	1,950	70	1.17 × 10 ⁻¹³		
	PDGFAB											
	100 ng/mL	8	2,080	±	80	63,562	2,120	2,310	76	1.42 × 10 ⁻¹³		
	PDGFBB											
VEGFR3						HUVECs						
	Control	6	1,840	±	200	77,745	1,920	300%	3,864	56		
	100 ng/mL	4	2,440	±	80	40,424	2,630	380%	2,401	47		
	PDGFAA											
	100 ng/mL	3	1,480	±	20	32,350	1,380	350%	3,060	51		
	PDGFAB											
	100 ng/mL	4	2,310	±	70	38,245	2,410	440%	1,917	42		
	PDGFBB											

Table 3. Receptor statistics

PDGFRα						HDFs				
	FGM-2	51	4,580	±	120	233,831	4,740	160%	1,543	25
	DMEM + 5% FBS	14	4,680	±	430	45,137	7,030	140%	268	10
	DMEM + 10% FBS	4	5.110	±	920	28.775	7.180	90%	1.265	21
	100 pg/mL VEGEA	4	3 030	+	40	19 795	2 530	200%	887	23
		4	2,030	-	100	14 206	2,000	230%	1 280	20
		7	1,070	-	20	17.910	1 200	770%	2 2 2 2	40
		3	1,070	Ŧ	20	17,010	1,300	04.00/	3,230	49
	100 ng/mL PDGFBB	3	1,330	±	70	4,679	1,430	810%	2,351	40
PDGFRß	HDFs									
	FGM-2	55	93,290	±	3,230	220,970	95,150	60%	63	4
	DMEM +	16	77,060	±	4,250	51,206	91,710	60%	84	5
	5% FBS									
	DMEM + 10% FBS	7	53,440	±	710	34,382	70,250	60%	31	3
	100 ng/mL VEGFA	4	69,320	±	1,260	19,440	74,380	80%	164	9
	50 ng/mL PDGFAA	4	107,640	±	1,600	26,695	121,870	40%	38	3
	50 ng/mL PDGFAB	3	71,330	±	1,460	17,636	69,670	50%	5	1
	100 ng/mL PDGFBB	3	6,660	±	300	5,084	68,740	460%	1,272	34
NRP1						HUVECs				
	Control	5	44,090	±	330	50,386	47,750	40%	156	4
	100 ng/mL PDGFAA	5	47,400	±	1,900	74,593	50,690	40%	128	4
	100 ng/mL PDGFAB	3	48,400	±	1,130	57,957	51,180	50%	320	10
	100 ng/mL PDGFBB	4	47,440	±	340	67,189	50,010	40%	330	9
						HDFs				
	FGM-2	36	120,470	±	2,730	147,633	113,200	60%	13	1
	DMEM +	11	100,020	±	7,970	24,818	112,060	60%	33	3
	5% FBS									
	DMEM + 10% FBS	8	69,620	+	4,210	10 450	79,060	50%	11	1
		1	108 210	÷	1 890	20 813	114 420	50%	12	1
		-+	122 400	T	2 220	10.440	120.050	50%	12	2
		3	132,180	±	2,230	19,449	138,850	50%	43	3
	100 ng/mL PDGFBB	3	100,830	±	230	4,527	125,630	40%	7	1

Table 3. Receptor statistics (continued)

3.8 REFERENCES

- 1. Imoukhuede, P. I., Dokun, A. O., Annex, B. H. & Popel, A. S. Endothelial cell-by-cell profiling reveals the temporal dynamics of VEGFR1 and VEGFR2 membrane localization after murine hindlimb ischemia. *Am J Physiol Hear. Circ Physiol* **304**, H1085–H1093 (2013).
- Imoukhuede, P. I. & Popel, A. S. Quantitative fluorescent profiling of VEGFRs reveals tumor cell and endothelial cell heterogeneity in breast cancer xenografts. *Cancer Med.* 3, 225–44 (2014).
- 3. Imoukhuede, P. I. & Popel, A. S. Quantification and cell-to-cell variation of vascular endothelial growth factor receptors. *Exp. Cell Res.* **317**, 955–965 (2011).
- 4. Imoukhuede, P. I. & Popel, A. S. Expression of VEGF receptors on endothelial cells in mouse skeletal muscle. *PLoS One* **7**, e44791 (2012).
- 5. Weddell, J. C. & Imoukhuede, P. I. Quantitative characterization of cellular membranereceptor heterogeneity through statistical and computational modeling. *PLoS One* **9**, e97271 (2014).
- 6. Finley, S. D. E.-S. M. O., Imoukhuede, P. I. & Popel, A. S. Pharmacokinetics and pharmacodynamics of VEGF-neutralizing antibodies. *BMC Syst Biol* **5**, (2011).
- 7. Gabhann, F. Mac & Popel, A. S. Systems Biology of Vascular Endothelial Growth Factors. *Microcirculation* **15**, 715–738 (2008).
- 8. Finley, S. D., Chu, L.-H. & Popel, A. S. Computational systems biology approaches to anti-angiogenic cancer therapeutics. *Drug Discov. Today* **00**, (2014).
- 9. Bentley, K., Gerhardt, H. & Bates, P. A. Agent-based simulation of notch-mediated tip cell selection in angiogenic sprout initalisation. *J Theor Biol* **250**, 25–36 (2008).
- Bentley, K., Mariggi, G., Gerhardt, H. & Bates, P. A. Tipping the balance: robustness of tip cell selection, migration and fusion in angiogenesis. *PLoS Comput. Biol.* 5, e1000549 (2009).
- 11. Jakobsson, L. *et al.* Endothelial cells dynamically compete for the tip cell position during angiogenic sprouting. *Nat Cell Biol* **12**, 943–953 (2010).

- 12. Shweiki, D., Itin, A., Soffer, D. & Keshet, E. Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. *Nature* **359**, 843–5 (1992).
- 13. Neufeld, G., Cohen, T., Gengrinovitch, S. & Poltorak, Z. Vascular endothelial growth factor (VEGF) and its receptors. *FASEB J* **13**, 9–22 (1999).
- 14. Yoshida, A., Anand-Apte, B. & Zetter, B. R. Differential endothelial migration and proliferation to basic fibroblast growth factor and vascular endothelial growth factor. *Growth Factors* **13**, 57–64 (1996).
- 15. Barleon, B. *et al.* Migration of human monocytes in response to vascular endothelial growth factor (VEGF) is mediated via the VEGF receptor flt-1. *Blood* **87**, 3336–43 (1996).
- 16. Fantin, A. *et al.* Tissue macrophages act as cellular chaperones for vascular anastomosis downstream of VEGF-mediated endothelial tip cell induction. *Blood* **116**, 829–40 (2010).
- Takahashi, Y., Kitadai, Y., Bucana, C. D., Cleary, K. R. & Ellis, L. M. Expression of Vascular Endothelial Growth Factor and Its Receptor, KDR, Correlates with Vascularity, Metastasis, and Proliferation of Human Colon Cancer. *Cancer Res.* 55, 3964–3968 (1995).
- Von Tell, D., Armulik, A. & Betsholtz, C. Pericytes and vascular stability. *Exp. Cell Res.* 312, 623–629 (2006).
- Lin, S.-L. *et al.* Targeting endothelium-pericyte cross talk by inhibiting VEGF receptor signaling attenuates kidney microvascular rarefaction and fibrosis. *Am. J. Pathol.* 178, 911–23 (2011).
- 20. Benjamin, L. E., Golijanin, D., Itin, A., Pode, D. & Keshet, E. Selective ablation of immature blood vessels in established human tumors follows vascular endothelial growth factor withdrawal. *J. Clin. Invest.* **103**, 159–65 (1999).
- 21. Benjamin, L. E., Hemo, I. & Keshet, E. A plasticity window for blood vessel remodelling is defined by pericyte coverage of the preformed endothelial network and is regulated by PDGF-B and VEGF. *Development* **125**, 1591–8 (1998).
- 22. Betsholtz, C., Karlsson, L. & Lindahl, P. Developmental roles of platelet-derived growth factors. *Bioessays* 23, 494–507 (2001).

- 23. Zhang, J. *et al.* Differential roles of PDGFR-alpha and PDGFR-beta in angiogenesis and vessel stability. *FASEB J.* **23**, 153–63 (2009).
- 24. Carmeliet, P. Mechanisms of angiogenesis and arteriogenesis. *Nat. Med.* **6**, 389–95 (2000).
- 25. Lieu, C., Heymach, J., Overman, M., Tran, H. & Kopetz, S. Beyond VEGF: inhibition of the fibroblast growth factor pathway and antiangiogenesis. *Clin. Cancer Res.* **17**, 6130–6139 (2011).
- Augustin, H. G., Koh, G. Y., Thurston, G. & Alitalo, K. Control of vascular morphogenesis and homeostasis through the angiopoietin-Tie system. *Nat. Rev. Mol. Cell Biol.* 10, 165–77 (2009).
- 27. Thurston, G. *et al.* Angiopoietin-1 protects the adult vasculature against plasma leakage. *Nat. Med.* **6**, 460–3 (2000).
- 28. Arai, F. *et al.* Tie2/angiopoietin-1 signaling regulates hematopoietic stem cell quiescence in the bone marrow niche. *Cell* **118**, 149–61 (2004).
- 29. Scharpfenecker, M., Fiedler, U., Reiss, Y. & Augustin, H. G. The Tie-2 ligand angiopoietin-2 destabilizes quiescent endothelium through an internal autocrine loop mechanism. *J. Cell Sci.* **118**, 771–80 (2005).
- 30. Heldin, C.-H. Targeting the PDGF signaling pathway in tumor treatment. *Cell Commun. Signal.* **11**, 97 (2013).
- 31. Fredriksson, L., Li, H. & Eriksson, U. The PDGF family: four gene products form five dimeric isoforms. *Cytokine Growth Factor Rev.* **15**, 197–204 (2004).
- 32. Heldin, C. H. & Westermark, B. Platelet-derived growth factor: mechanism of action and possible in vivo function. *Cell Regul.* **1**, 555–66 (1990).
- 33. Li, X. *et al.* PDGF-C is a new protease-activated ligand for the PDGF alpha-receptor. *Nat. Cell Biol.* **2**, 302–9 (2000).
- 34. Carmeliet, P. & Collen, D. Vascular development and disorders: molecular analysis and pathogenic insights. *Kidney Int.* **53**, 1519–1549 (1998).

- 35. Heldin, C.-H., Östman, A. & Rönnstrand, L. Signal transduction via platelet-derived growth factor receptors. *Biochim. Biophys. Acta Rev. Cancer* **1378**, F79–F113 (1998).
- 36. Levéen, P. *et al.* Mice deficient for PDGF B show renal, cardiovascular, and hematological abnormalities. *Genes Dev.* **8**, 1875–87 (1994).
- 37. Boström, H. *et al.* PDGF-A signaling is a critical event in lung alveolar myofibroblast development and alveogenesis. *Cell* **85**, 863–73 (1996).
- Beitz, J. G., Kim, I. S., Calabresi, P. & Frackelton, A. R. Human microvascular endothelial cells express receptors for platelet-derived growth factor. *Proc. Natl. Acad. Sci. U. S. A.* 88, 2021–5 (1991).
- 39. Ostman, A. & Heldin, C. H. Involvement of platelet-derived growth factor in disease: development of specific antagonists. *Adv. Cancer Res.* **80**, 1–38 (2001).
- 40. Robson, M. C., Phillips, L. G., Thomason, A., Robson, L. E. & Pierce, G. F. Plateletderived growth factor BB for the treatment of chronic pressure ulcers. *Lancet* **339**, 23–5 (1992).
- 41. Lynch, S. E., Nixon, J. C., Colvin, R. B. & Antoniades, H. N. Role of platelet-derived growth factor in wound healing: synergistic effects with other growth factors. *Proc. Natl. Acad. Sci.* **84**, 7696–7700 (1987).
- 42. Greenhalgh, D. G., Sprugel, K. H., Murray, M. J. & Ross, R. PDGF and FGF stimulate wound healing in the genetically diabetic mouse. *Am. J. Pathol.* **136**, 1235–46 (1990).
- 43. Pierce, G. F., Mustoe, T. A., Altrock, B. W., Deuel, T. F. & Thomason, A. Role of platelet-derived growth factor in wound healing. *J. Cell. Biochem.* **45**, 319–65 (1991).
- 44. Lindner, V. & Reidy, M. A. Platelet-derived growth factor ligand and receptor expression by large vessel endothelium in vivo. *Am. J. Pathol.* **146**, 1488–97 (1995).
- 45. Brown, L. F. *et al.* Expression of vascular permeability factor (vascular endothelial growth factor) and its receptors in breast cancer. *Hum. Pathol.* **26**, 86–91 (1995).
- 46. Greenberg, J. I. & Cheresh, D. A. VEGF as an inhibitor of tumor vessel maturation: implications for cancer therapy. *Expert Opin. Biol. Ther.* **9**, 1347–56 (2009).

- 47. Greenberg, J. I. *et al.* A role for VEGF as a negative regulator of pericyte function and vessel maturation. *Nature* **456**, 809–13 (2008).
- 48. Ball, S. G., Shuttleworth, C. A. & Kielty, C. M. Vascular endothelial growth factor can signal through platelet-derived growth factor receptors. *J. Cell Biol.* **177**, 489–500 (2007).
- 49. Mamer, S. B. *et al.* Discovery of High-Affinity PDGF-VEGFR Interactions: Redefining RTK Dynamics. *Sci. Rep.* **7**, 16439 (2017).
- 50. Qutub, A. A., Mac Gabhann, F., Karagiannis, E. D., Vempati, P. & Popel, A. S. Multiscale models of angiogenesis. *IEEE Eng. Med. Biol. Mag.* **28**, 14–31
- Mac Gabhann, F., Qutub, A. A., Annex, B. H. & Popel, A. S. Systems biology of proangiogenic therapies targeting the VEGF system. *Wiley Interdiscip. Rev. Syst. Biol. Med.* 2, 694–707 (2010).
- 52. Vempati, P., *et al.* Formation of VEGF isoform-specific spatial distributions governing angiogenesis: computational analysis. *BMC Syst. Biol.* **5**, 59 (2011).
- 53. Vempati, P., *et al.* Quantifying the proteolytic release of extracellular matrix-sequestered VEGF with a computational model. *PLoS One* **5**, e11860 (2010).
- 54. Mac Gabhann, F., Ji, J. W. & Popel, A. S. VEGF gradients, receptor activation, and sprout guidance in resting and exercising skeletal muscle. *J. Appl. Physiol.* **102**, 722–34 (2007).
- 55. Liu, G., *et al.* Module-based multiscale simulation of angiogenesis in skeletal muscle. *Theor. Biol. Med. Model.* **8**, 6 (2011).
- 56. Qutub, A. A. & Popel, A. S. Elongation, proliferation & migration differentiate endothelial cell phenotypes and determine capillary sprouting. *BMC Syst. Biol.* **3**, 13 (2009).
- 57. Ji, J. W., Mac Gabhann, F. & Popel, A. S. Skeletal muscle VEGF gradients in peripheral arterial disease: simulations of rest and exercise. *Am. J. Physiol. Heart Circ. Physiol.* **293**, H3740-9 (2007).
- 58. Cirit, M. & Haugh, J. M. Data-driven modelling of receptor tyrosine kinase signalling networks quantifies receptor-specific potencies of PI3K- and Ras-dependent ERK activation. *Biochem. J.* **441**, 77–85 (2012).

- 59. Park, C. S., Schneider, I. C. & Haugh, J. M. Kinetic analysis of platelet-derived growth factor receptor/phosphoinositide 3-kinase/Akt signaling in fibroblasts. *J. Biol. Chem.* **278**, 37064–37072 (2003).
- 60. Pannu, K. K., Joe, E. T. & Iyer, S. B. Performance evaluation of quantiBRITE phycoerythrin beads. *Cytometry* **45**, 250–258 (2001).
- 61. Wang, L., Abbasi, F., Gaigalas, A. K., Vogt, R. F. & Marti, G. E. Comparison of fluorescein and phycoerythrin conjugates for quantifying CD20 expression on normal and leukemic B-cells. *Cytometry B. Clin. Cytom.* **70**, 410–5 (2006).
- 62. Ichiki, Y., Smith, E., LeRoy, E. C. & Trojanowska, M. Different effects of basic fibroblast growth factor and transforming growth factor-beta on the two platelet-derived growth factor receptors' expression in scleroderma and healthy human dermal fibroblasts. *J. Invest. Dermatol.* **104**, 124–7 (1995).
- 63. Pennock, S. & Kazlauskas, A. Vascular endothelial growth factor A competitively inhibits platelet-derived growth factor (PDGF)-dependent activation of PDGF receptor and subsequent signaling events and cellular responses. *Mol. Cell. Biol.* **32**, 1955–66 (2012).
- Battegay, E. J., Rupp, J., Iruela-Arispe, L., Sage, E. H. & Pech, M. PDGF-BB modulates endothelial proliferation and angiogenesis in vitro via PDGF beta-receptors. *J. Cell Biol.* 125, 917–28 (1994).
- 65. Chen, S. *et al.* Towards clinical application: Establishing in vitro standards for qFlow cytometry-based profiling of receptors. In revision
- 66. Roxworthy, B. J. *et al.* Plasmonic optical trapping in biologically relevant media. *PLoS One* **9**, e93929 (2014).
- Heldin, C. H., Westermark, B. & Wasteson, a. Specific receptors for platelet-derived growth factor on cells derived from connective tissue and glia. *Proc. Natl. Acad. Sci. U. S. A.* 78, 3664–8 (1981).
- 68. Willett, C. G. *et al. Direct evidence that the VEGF-specific antibody bevacizumab has antivascular effects in human rectal cancer. Nature medicine* **10**, (2004).
- 69. Bergers, G., Song, S., Meyer-Morse, N., Bergsland, E. & Hanahan, D. Benefits of targeting both pericytes and endothelial cells in the tumor vasculature with kinase

inhibitors. J. Clin. Invest. 111, 1287–1295 (2003).

- Erber, R. *et al.* Combined inhibition of VEGF and PDGF signaling enforces tumor vessel regression by interfering with pericyte-mediated endothelial cell survival mechanisms. *FASEB J.* 18, 338–40 (2004).
- Casanovas, O., Hicklin, D. J., Bergers, G. & Hanahan, D. Drug resistance by evasion of antiangiogenic targeting of VEGF signaling in late-stage pancreatic islet tumors. *Cancer Cell* 8, 299–309 (2005).
- 72. He, B. *et al.* Grand challenges in interfacing engineering with life sciences and medicine. *IEEE Trans. Biomed. Eng.* **60**, 589–98 (2013).
- 73. Burrell, R. a, McGranahan, N., Bartek, J. & Swanton, C. The causes and consequences of genetic heterogeneity in cancer evolution. *Nature* **501**, 338–45 (2013).
- 74. Gough, A. H. *et al.* Identifying and quantifying heterogeneity in high content analysis: application of heterogeneity indices to drug discovery. *PLoS One* **9**, e102678 (2014).
- Tsirakis, G. *et al.* Role of platelet-derived growth factor-AB in tumour growth and angiogenesis in relation with other angiogenic cytokines in multiple myeloma. *Hematol. Oncol.* **30**, 131–6 (2012).
- 76. Andrae, J., Gallini, R. & Betsholtz, C. Role of platelet-derived growth factors in physiology and medicine. *Genes Dev.* **22**, 1276–312 (2008).
- 77. Seifert, R. A. *et al.* Two different subunits associate to create isoform-specific plateletderived growth factor receptors. *J. Biol. Chem.* **264**, 8771–8 (1989).
- 78. Escobedo, J. A. *et al.* A common PDGF receptor is activated by homodimeric A and B forms of PDGF. *Science* **240**, 1532–4 (1988).
- 79. Matsui, T. *et al.* Independent expression of human alpha or beta platelet-derived growth factor receptor cDNAs in a naive hematopoietic cell leads to functional coupling with mitogenic and chemotactic signaling pathways. *Proc. Natl. Acad. Sci.* **86**, 8314–8 (1989).
- 80. Lokker, N. A. *et al.* Functional importance of platelet-derived growth factor (PDGF) receptor extracellular immunoglobulin-like domains. Identification of PDGF binding site and neutralizing monoclonal antibodies. *J. Biol. Chem.* **272**, 33037–44 (1997).

- 81. Simm, A., Nestler, M. & Hoppe, V. PDGF-AA, a potent mitogen for cardiac fibroblasts from adult rats. *J. Mol. Cell. Cardiol.* **29**, 357–68 (1997).
- Rosenkranz, S. & Kazlauskas, A. Evidence for distinct signaling properties and biological responses induced by the PDGF receptor alpha and beta subtypes. *Growth Factors* 16, 201–16 (1999).
- 83. Donovan, J., Shiwen, X., Norman, J. & Abraham, D. Platelet-derived growth factor alpha and beta receptors have overlapping functional activities towards fibroblasts. *Fibrogenesis Tissue Repair* **6**, 10 (2013).
- 84. Heldin, C. H., Wasteson, A. & Westermark, B. Platelet-derived growth factor. *Mol. Cell. Endocrinol.* **39**, 169–87 (1985).
- 85. Heldin, C. H. & Westermark, B. Mechanism of action and in vivo role of platelet-derived growth factor. *Physiol. Rev.* **79**, 1283–316 (1999).
- 86. Paulsson, J. *et al.* Prognostic significance of stromal platelet-derived growth factor betareceptor expression in human breast cancer. *Am. J. Pathol.* **175**, 334–41 (2009).
- 87. Räsänen, K. & Vaheri, A. Activation of fibroblasts in cancer stroma. *Exp. Cell Res.* **316**, 2713–22 (2010).
- Renner, O. *et al.* Time- and cell type-specific induction of platelet-derived growth factor receptor-β during cerebral ischemia. *Mol. Brain Res.* 113, 44–51 (2003).
- 89. Napione, L. *et al.* Unraveling the influence of endothelial cell density on VEGF-A signaling. *Blood* **119**, 5599–607 (2012).
- 90. Dejana, E. Endothelial cell-cell junctions: happy together. *Nat. Rev. Mol. Cell Biol.* **5**, 261–70 (2004).
- 91. Bar, R. S. INTERACTIONS OF INSULIN AND INSULIN-LIKE GROWTH FACTORS (IGF) WITH ENDOTHELIAL CELLS. *Ann. N. Y. Acad. Sci.* **401**, 150–162 (1982).
- 92. Shigematsu, S. *et al.* IGF-1 regulates migration and angiogenesis of human endothelial cells. *Endocr. J.* **46 Suppl**, S59-62 (1999).
- 93. Brunner, D. et al. Serum-free cell culture: the serum-free media interactive online

database. ALTEX 27, 53–62 (2010).

- 94. Bowen-Pope, D. F., Hart, C. E. & Seifert, R. A. Sera and conditioned media contain different isoforms of platelet-derived growth factor (PDGF) which bind to different classes of PDGF receptor. *J. Biol. Chem.* **264**, 2502–8 (1989).
- 95. Ejiri, H. *et al.* Use of synthetic serum-free medium for culture of human dermal fibroblasts to establish an experimental system similar to living dermis. *Cytotechnology* (2014). doi:10.1007/s10616-014-9709-0
- 96. Kut, C., Mac Gabhann, F. & Popel, A. S. Where is VEGF in the body? A meta-analysis of VEGF distribution in cancer. *Br. J. Cancer* **97**, 978–85 (2007).
- 97. Vempati, P., Popel, A. S. & Mac Gabhann, F. Extracellular regulation of VEGF: isoforms, proteolysis, and vascular patterning. *Cytokine Growth Factor Rev.* **25**, 1–19 (2014).
- 98. Keegan, P. M., Wilder, C. L. & Platt, M. O. Tumor necrosis factor alpha stimulates cathepsin K and V activity via juxtacrine monocyte-endothelial cell signaling and JNK activation. *Mol. Cell. Biochem.* **367**, 65–72 (2012).
- 99. Weskamp, G. *et al.* Pathological neovascularization is reduced by inactivation of ADAM17 in endothelial cells but not in pericytes. *Circ. Res.* **106**, 932–40 (2010).
- Guaiquil, V. H. *et al.* ADAM8 is a negative regulator of retinal neovascularization and of the growth of heterotopically injected tumor cells in mice. *J. Mol. Med.* 88, 497–505 (2010).
- Shen, W., Zhang, C., Fannon, M. W., Forsten-Williams, K. & Zhang, J. A computational model of FGF-2 binding and HSPG regulation under flow. *IEEE Trans. Biomed. Eng.* 56, 2147–55 (2009).
- 102. Yamazaki, Y. & Morita, T. Molecular and functional diversity of vascular endothelial growth factors. *Mol. Divers.* **10**, 515–27 (2006).
- 103. Anderson, S. M. *et al.* VEGF internalization is not required for VEGFR-2 phosphorylation in bioengineered surfaces with covalently linked VEGF. *Integr. Biol.* **3**, 887–96 (2011).
- Claesson-Welsh, L. Signal transduction by the PDGF receptors. *Prog. Growth Factor Res.* 5, 37–54 (1994).

- 105. Hart, C. E. *et al.* Two classes of PDGF receptor recognize different isoforms of PDGF. *Science* **240**, 1529–31 (1988).
- Severinsson, L., Claesson-Welsh, L. & Heldin, C. H. A B-type PDGF receptor lacking most of the intracellular domain escapes degradation after ligand binding. *Eur. J. Biochem.* 182, 679–86 (1989).
- Sorkin, A., Westermark, B., Heldin, C. H. & Claesson-Welsh, L. Effect of receptor kinase inactivation on the rate of internalization and degradation of PDGF and the PDGF betareceptor. *J. Cell Biol.* **112**, 469–78 (1991).
- 108. Hart, C. E. *et al.* Two classes of PDGF receptor recognize different isoforms of PDGF. *Science* **240**, 1529–31 (1988).
- 109. Hammacher, A., Mellström, K., Heldin, C. H. & Westermark, B. Isoform-specific induction of actin reorganization by platelet-derived growth factor suggests that the functionally active receptor is a dimer. *EMBO J.* **8**, 2489–95 (1989).
- 110. Hanahan, D. & Weinberg, R. A. Hallmarks of Cancer: The Next Generation. *Cell* **144**, 646–674 (2011).
- 111. Micke, P. & Ostman, A. Tumour-stroma interaction: cancer-associated fibroblasts as novel targets in anti-cancer therapy? *Lung Cancer* **45 Suppl 2**, S163-75 (2004).
- 112. Sugimoto, H., Mundel, T. M., Kieran, M. W. & Kalluri, R. Identification of fibroblast heterogeneity in the tumor microenvironment. *Cancer Biol. Ther.* **5**, 1640–6 (2006).
- 113. Morikawa, S. *et al.* Abnormalities in pericytes on blood vessels and endothelial sprouts in tumors. *Am. J. Pathol.* **160**, 985–1000 (2002).
- 114. Dong, J. *et al.* VEGF-null cells require PDGFR alpha signaling-mediated stromal fibroblast recruitment for tumorigenesis. *EMBO J.* **23**, 2800–10 (2004).
- 115. Forsberg, K., Valyi-Nagy, I., Heldin, C. H., Herlyn, M. & Westermark, B. Platelet-derived growth factor (PDGF) in oncogenesis: development of a vascular connective tissue stroma in xenotransplanted human melanoma producing PDGF-BB. *Proc. Natl. Acad. Sci.* **90**, 393–397 (1993).

CHAPTER 4 A 2D ENDOTHELIAL/FIBROBLAST CO-CULTURE MODEL¹

Angiogenesis research often applies in vitro models, such as 2D culture, wherein the vascular structure and microenvironment are not fully present. However, current co-culture analysis is usually limited to structural examinations, such as: tubule length, number of tubules and branch points. As co-culture gains expanded use, it must be further probed to determine whether it displays other important characteristics of native vasculature, namely endothelial heterogeneity. An improved in vitro model involves co-culture of endothelial cells and mural cells that support vessel formation, in combination with quantitative analysis not only on macro features of the tissue model, but also characteristics at molecular level. In this chapter, we first present the culturing logistics important for developing a tube formation assay using a 2D HUVEC/fibroblast co-culture model. Then, we quantitatively characterize the endothelial networks formed in the span of tube formation (24 hours -17 days). We show measurements of both bulk-level and cell-by-cell concentration of a panel of receptors involved in angiogenesis: VEGFR1, VEGFR2, PDGFR α , PDGFR β , Tie2, and NRP1. We examine the cellular heterogeneity within each cell population and how it changes as endothelial tubes form. This combined tube formation assay with qFlow cytometry analysis can be applied to study a number of vascular-based tissue dysfunction and cancer.

¹ This chapter was modified from a manuscript being prepared for publication.

4.1 INTRODUCTION

One of the most powerful tools to study cellular crosstalk is the co-culture assay. Ronald et al. have discussed several considerations when setting up a co-culture system including: choice of cells, choice of cultured media, phenotypic characterization of co-cultured cells, seeding logistics (seeding cell numbers and the order of seeding), static or dynamic, and two- or three-dimensional $(2D \text{ or } 3D)^1$. In particular, a tubule formation assay commonly involves the co-culture of ECs with stromal cells (i.e. fibroblasts, smooth muscle cells, pericytes)^{2,3}. When human fibroblasts are co-cultured with endothelial cells, the fibroblast secretes matrix components leading ECs to the formation of micro-capillaries or tubules in 2D cell culture flasks, which closely resemble the capillary bed in vivo^{2,3}. However, this assay is time consuming (12–21 days) and poorly characterized due to undefined matrix components secreted by fibroblasts. In terms of media, endothelial growth media (EGM) is the most commonly used media for culturing ECs with fibroblasts^{2,4–6}; normal HDF standard media FGM-2 has also been used in some assays⁷. Kirkpatrick et al. suggest that in a co-culture system the more sensitive cell type will usually have the greater weight in the formulation of the final media to be used¹. While a 3D assay is likely to be more representative of angiogenesis in vivo than the 2D assays, there are technical challenges in both setting up the assays and in fully analyzing them. In addition, analysis of such co-culture assay is usually limited to tubule length, number of tubules and branch points⁸. Certainly, our focus on regulation and heterogeneity of receptor-levels will be a novel approach for evaluating not only the influences of co-culture on both cell types, but also the relativity of a co-culture assay to an injury-induced tissue environment in vivo.

As the challenge with selecting the appropriate assays for angiogenesis research remains, it is essential to assess not only the macro features such as tube formation of each assay, but also the

micro features such as its molecular characteristics. Here, we performed qFlow cytometry to quantify plasma membrane receptors that are involved in angiogenic signaling pathways.

4.2 MATERIALS AND METHODS

Fibroblasts/EC co-culture. Multilayer co-cultures will be prepared by seeding mixtures of single-cell suspensions from HDFs (1×104) and HUVECs (1×104, 5×104 or 2×103 according to three different ratio of HDFs to HUVECs) on to 96-well plates (BD Biosciences/ Falcon, Heidelberg, Germany), modified from previous investigations⁷. Another approach of the co-culture is to seed HDFs first onto the plate and seed HUVECs when HDFs are confluent (usually 3 days later). In parallel, co-culture systems with corresponding relative seeding cell number, seeding order, media condition will be cultured on T75 flasks in order to have enough cells for the quantitative flow cytometric studies (see below). The media (see below) in the experimental setup will be routinely renewed every other day. Multilayers will be grown at 37°C in 95% air, 5% CO2. HDFs will be used only up to passage 12 and HUVECs will be used only up to passage 6⁹. The formation of tubular structures will be visualized using an Olympus IX51 inverted microscope after Immunofluorescent staining on day 3, 7 and 14 of culturing.

Media comparison. In order to evaluate the effect of different media on tubule formation and key angiogenic receptor levels, co-cultures will be maintained in two different media: (1) EGM-2, and (2) DMEM supplemented with 5% FBS.

Immunofluorescent imaging. Immunofluorescent staining of fibroblast/EC co-cultures in 96well plates will be performed according to an established protocol^{2,3}. In brief, multilayer cocultures will be fixed with ice-cold 4% PFA for 10 min at room temperature and blocked in PBS supplemented with 6% BSA for 1 hr. Then multilayers will be labelled with 1:1000 diluted FITC conjugated mouse anti-human CD31 antibody for 45 min at 37 °C and 1:1000 diluted DAPI for 5 min. After washing in PBS, multilayers can be stored at 4 °C for future imaging. For documentation and analysis, three images per well will be taken at defined locations with a magnification of \times 4, \times 10, and \times 20 with an Olympus IX51 inverted microscope (Olympus, Inc.). Images will be processed using ImageJ in order to increase contrast and brightness.

Angiogenesis analysis. The Angiogenesis Analyzer was installed as an plugin in ImageJ, and it detects and measures cellular networks from microscope images. It differentiates networks into four main components (Fig. S4): junctions, which are where a tube branches; segments, tubes that link two junctions together; branches, tubes that only connect to one junction; and meshes, which are regions of the image that are completely enclosed by the network. The plugin generates a map of the network which is overlaid onto the original image. In addition, it creates a separate window containing the number of junctions, branches, segments, meshes, and the total length of all branches and segments (in pixels), as well as several other statistics that are not as relevant (e.g. number of isolated elements, total area of enclosed meshes, etc.).

Cell dissociation. After 24 hours, 6 days, 11 days, or 17 days of culturing, EC/fibroblast cocultures were washed twice with $1 \times PBS$, followed by 5 – 7-minute incubation in Cell Stripper at 37 °C. Lifted co-culture tissues were transferred to a petri dish, where HBSS with 2mM EDTA and 0.2% BSA was added to the tissues as they were minced into 2mm × 2mm squares. Minced tissues were then incubated with 0.2% collagenase IV for 5 minutes at 4°C. After the incubation, the collagenase-treated tissues were vortexed for 3 – 5 minute with 1-minute interval to achieve single-cell suspension. The suspension was then kept on ice until antibody staining. HUVECs and HDFs monocultures were harvested and dissociated as described previously (Chapter 2, section 2.3.2).

Cell staining. 25 μ L aliquots of cells (1 x 10⁵ cells) were added to 5 ml polystyrene roundbottom tubes (BD Biosciences, New Jersey). FITC-anti-hCD31 or APC-anti-hCD34 at recommended concentration by their manufacturers were added to each sample tube to differentiate HUVECs from HDFs. Then, phycoerythrin (PE)-conjugated monoclonal antibodies were added at their respective concentrations: 14 µg/mL for VEGFR1, VEGFR2, 7.1 µg/mL for NRP1¹⁰, and 9.4 µg/mL for PDGFRs. Sample tubes were protected from light and incubated for 40 minutes on ice. Cells were washed, centrifuged at 500 ×g twice with 4 mL stain buffer, and resuspended in 300 µL stain buffer. The precision and accuracy of qFlow cytometry profiling has been rigorously tested ^{9,11–13}.

Quantitative flow cytometry. Flow cytometry was performed on a LSR Fortessa (BD) Flow cytometer; BD FACSDIVA software was used for data acquisition, and FlowJo (TreeStar) software was used for data analysis. Tubes were vortexed immediately prior to placement in the flow cytometer. 8,000 - 10,000 live cells were collected from each tube. 5 μ g/mL Sytox Blue (Invitrogen) was added to all samples to distinguish between live and dead cells. Sytox blue was excited with a solid-state laser (407 nm) and its emission was collected using a 450/50 band-pass filter. We plotted histograms of Sytox Blue fluorescence to identify the live cell population. Cells exhibiting little to no Sytox Blue fluorescence are gated as live cells. These gated cells were then examined in a plot of forward scatter area (FSC-A) versus side scatter area (SSC-A) to gate the single-cell population.

Statistical analysis: To examine the average receptor concentrations in all cells, ensembleaverages were calculated as described in Chapter 3 (section 3.2.7). To examine variation of receptor concentration within cell populations, cell-by-cell analysis and mixture modeling was conducted as described in Chapter 2 (section 2.3.6 and 2.3.7). Finally, quadratic entropy (QE) was calculated for each receptor distribution as a quantitative measure of cell heterogeneity defined by receptor concentration. Please refer to Chapter 2 (section 2.3.8) for a detailed protocol.

4.3 RESULTS AND DISCUSSION

4.3.1 HUVECs supported by fibroblasts form capillary-like structures in vitro

To establish a co-culture system with HUVECs and HDFs, the following culturing logistics were assessed (Fig. 15): media, HUVEC:HDF seeding ratio, and seeding order. When cultured alone, HUVECs and HDFs organized into typical cell monolayers (Fig. 15a, e), whereas they showed variability in cell behavior and organization when cultured together. Co-cultures with 1:5 HDFs to HUVECs seeding ratio did not form tubules up to day 17, and displayed HUVEC overgrowth regardless of the culturing order of cells (Fig. 15b, g). Co-cultures with 5:1 HDFs to HUVECs seeding ratio presented more EC clusters and fewer tubules comparing to 1:1 HDFs to HUVECs (Fig. 15c, d). HDFs were able to proliferate in DMEM supplemented with 10% FBS, however, DMEM media was not able to support growth of HUVECs in co-cultures (Fig. 15f). To test if ECMs secreted by culturing HDFs first were sufficient to support tubule formation, we seeded HDFs first, following by HUVECs 3 days later. Regardless of HDFs being cultured first or at the same time as HUVECs, we did not observe tubule structures up to 17 days (Fig. 15h).

When HUVEC and HUVECs were incubated together at a 1:1 seeding ratio $(3 \times 10^4 \text{ cells/cm}^2)$ in standard growth media for endothelial cells (EGM-2), and the media was changed every other day, both cell types proliferated until the culture reached confluence. After 24 hours, HUVECs were seen to form clusters, with HDFs dispersed amongst them (Fig. S5a). As HDFs grew more confluent, tubule structures were seen to sprout from HUVEC clusters, usually by day 6 (Fig. S5b). These HUVEC clusters and sprouts continued to sprout, lengthen, and organized into capillary-like structures by day 11 (Fig. S5c). By day 17, most of the development of HUVECs into tubule-like structures occurred (Fig. S5d). To quantitatively characterize the tubule growth for the established HUVEC/fibroblast co-culturing system, the average number of junctions and branches and was determined, and their length (Fig. 16). An increase in the number of junctions and branches was observed between day 6 and 17 and accompanied by a significant increase in tubule length (p<0.01).

4.3.2 Co-culturing induced a rapid increase of VEGFR1 on HDFs within 24 hours

VEGFRs are key regulators of angiogenesis; however, the exact role of VEGFR1 has been controversial. As such, VEGFR1 quantification can offer insights into how they support vessel growth. We found that HUVECs in co-cultures presented fewer than 800 VEGFR1s/cell up to 17 days, whereas HDFs showed a higher VEGFR1 concentration (~2,700 receptors/cell) within 24 hours (Fig. 17A). Cell-by-cell analysis confirmed this rapid increase through a right-handed shift of VEGFR1 distribution comparing to HDF monoculture (Fig. 18D). By day 6, VEGFR1 concentration on HDF plasma membranes reduced to ~200 receptors/cell and remained lower than ~500 receptors/cell. To ensure plasma membrane VEGFR1 on HDFs following 24-hr co-culturing was not induced by collagenase, we examined the effect of collagenase on receptors tested in this study and found no significant change in receptor concentrations (Fig. S6). These

findings for the first time showed quantitative proof that HDFs, which do not natively express VEFGR1, can express VEGFR1 on their plasma membranes when co-cultured with HUVECs.

4.3.3 Co-cultured HUVECs showed higher VEGFR2 concentration than monoculture throughout tubule development

VEGF-VEGFR2 signaling has been widely studied and signaling via VEGFR2 is considered to be pro-angiogenic. In line with its pro-angiogenic role, we observed 20% - 140% increase in plasma membrane VEGFR2 concentrations on co-cultured HUVECs as tubules develop---this increase is when compared to monocultures. Although, both co-cultured and mono-cultured HUVECs showed 3,300-8,100 VEGFR2s/cell, co-cultured HDFs had lower VEGFR2 [390 -1,100 receptors/cell] (Fig. 17B). Cell-by-cell analysis suggest subpopulations of varying VEGFR2 concentrations among co-cultured HUVECs (Fig. 18B). The subpopulations within VEGFR2 cell-by-cell distribution and their characteristics were further determined by Gaussian mixture modeling ^{14,15}, a method we employed to identify log-normal sub-populations described by their mean, standard deviation, and density. Particularly, a 3-component mixture model showed 25% of the HUVECs from 24-hr co-cultures had 3,100 VEGFR2s/cell on average, 46% had an average of 8,400 VEGFR2s/cell, and 29% had an average of 17,000 VEGFR2s/cell (Fig. S7A). This high heterogeneity was also captured by quadratic entropy (QE), a measure we employed to quantify diversity of cellular phenotypes ^{16–18}, as QE of 24-hr VEGFR2 was ~2-fold higher than day 6-17 (Fig. S8A). These quantitative results showed that RTK signaling through VEGFR2 played an active role throughout tubule formation in a HUVEC/fibroblast co-culture system.
4.3.4 Tie2 concentration on HUVECs reached highest during late-stage co-cultures

Tie2 maintains the vascular integrity of mature vessels and is thought to mediate quiescence of blood vessels through Ang-Tie2 signaling ¹⁹. Although we did not observe significant changes in Tie2 concentration ensembles throughout tubule development [1,500 - 2,300 Tie2s/cell] when compared to HUVEC monocultures [2,100 Tie2s/cell], ensemble averages showed a 50% increase of Tie2 concentration from day 11 to day 17 (Fig. 17C). Cell-by-cell analysis confirmed this increase by a right-handed shift of Tie2 distribution on day 17 when compared to co-cultures at earlier stage (Fig. 18C).

4.3.5 NRP1 concentration decreased on HUVEC/fibroblast co-cultured cells within 11 days NRP1, a VEGFR co-receptor, is highly expressed on both endothelial cells (ECs) and fibroblasts, where we have measured 35,000 – 73,000 NRP1s/EC and 70,000 – 120,500 NRP1s/fibroblast *in vitro* ^{10,20}. We observed a downregulation of NRP1 on co-cultured HUVECs from the initial HUVEC-HDF contacts to day 11 (Fig. 17D). Quantitatively, NRP1 concentration on co-cultured HUVECs decreased from 47,000 NRP1/cell to 28,900 NRP1/cell after 11 days, and remained steady until day 17. However, we did not observe any significant difference in NRP1 concentration between HUVEC monocultures and co-cultures. In contrast, co-cultured HDFs present significantly lower number of NRP1s on their plasma membrane throughout tubule development when compared to mono-cultured HDFs decreased from 73,300 NRP1s/cell to 52,400 NRP1s/cell within 6 days, and bounced back to 77,800 NRP1s/cell on day 17.

Cell-by-cell analysis of NRP1 concentration revealed a highly heterogeneous HUVEC population on day 17 (Fig. 18G), which was confirmed by a high QE of 0.6 (Fig. S8A). A 3-

component mixture model identified a low-NRP1 subpopulation (9,000 NRP1s/cell, 67%) and a high-NRP1 subpopulation (49,300 NRP1s/cell, 29%) within co-cultured HUVECs on day 17, as shown in Fig. S7B. For co-cultured HDFs, cell-by-cell analysis confirmed the changes in NRP1 plasma membrane concentration (Fig. 18J), while QE remained lower than 0.35 except for day 6 (Fig. S8B). A further analysis of co-cultured HDFs on day 6 revealed a low-NRP1 subpopulation (5,500 NRP1s/cell, 12%) and a high-NRP1 subpopulation (58,500 NRP1s/cell, 88%) using a 2-component mixture model (Fig. S7C).

4.3.6 PDGFR α and -R β showed a steady increase on co-cultured HDFs from day 6

PDGFRs, typically expressed by perivascular cells including pericytes and fibroblasts, serve important roles in supporting vasculature and tissue repair $^{20-24}$. We have previously quantified 4,600 PDGFR α s/cell and 93,300 PDGFR β s/cell on confluent HDF monocultures, and how growth factors regulate the receptors 20 . While PDGFR α concentration on co-cultured HDFs remained 2 – 6-fold lower than mono-cultured HDFs, we observed a steady increase of PDGFR α from 1,000 receptors/cell on day 6 to 4,500 receptors/cell on day 17 (Fig. 17E). Similarly, as tubule started to form, PDGFR β concentration increased from 34,800 receptors/cell on day 6 to 103,300 receptors/cell on day 17 (Fig. 17F). Cell-by-cell analysis of co-cultured HDFs confirmed this increase in both PDGF receptors throughout tubule development (Fig. 18K-L).

4.3.7 PDGFR β observed on co-cultured HUVECs but not PDGFR α

ECs do not natively express PDGF receptors when cultured alone ²⁵, however, multiple studies have reported PDGFR expression on ECs in angiogenic environment ^{26,27}. Aligned with these findings, we observed consistent PDGFR β expression (~600 – 2,900 PDGFR β /cell) on co-cultured HUVEC plasma membrane throughout tubule development (Fig. 17F). In contrast to

the steady increase of PDGFRs on HDFs from day 6, PDGFR β concentration on HUVECs were higher during the initial HUVEC-HDF contacts (1,900 PDGFR β s/HUVEC after 24 hours) and cluster formation (2,900 PDGFR β s/HUVEC on day 6), but decreased as tubules develop. Cellby-cell analysis confirmed the increase in PDGFR β concentration by day 6, evidenced by a righthanded shift of histograms (Fig. 18I). Although ensemble average of PDGFR β concentration was low on day 17, high-PDGFR β subpopulation was observed among co-cultured HUVECs. A 2component mixture model determined a low-PDGFR β subpopulation (700 receptors/cell, 90%) and a high-PDGFR β subpopulation (8,800 receptors/cell, 10%) within co-cultured HUVECs on day 17 (Fig. S7B). This heterogeneity was also captured by a high QE of 0.55 as well as a 2-fold increase from day 11 to day 17 (Fig. S8A). These quantitative findings showed HUVECs, when interacting with fibroblasts, may express transmembrane PDGF receptors.

4.4 CONCLUSIONS

In summary, we have established a simple 2D tube formation assay by co-culturing HUVECs with HDFs and examined concentrations and heterogeneity of a panel of angiogenic receptors up to 17 days. Particularly, we optimized and established the seeding logistics so that HUVECs supported by fibroblasts form capillary-like structures during the course of 17 days. Within 24 h co-culturing, we observed ~2,700 VEGFR1 per cell on HDFs, which do not natively express VEGFR when cultured alone. In line with the pro-angiogenic role of VEGFR2, we observed 20% - 140% increase in plasma membrane VEGFR2 concentrations [4,000—8,100 receptors per cell] on co-cultured HUVECs as tubules develop when compared to monocultures. We observed a ~50% increase in Tie2 concentration on HUVECs during late-stage tube formation, which aligns with the theory that Ang-Tie2 signaling mediates quiescence of blood vessels. We showed

105

steady increase in PDGFRs on HDFs, and PDGFR β s were found on HUVECs during the first 6 hours of co-culturing [1,900 – 2,900 PDGFR β s per cell].



4.5 FIGURES AND FIGURE LEGENDS

Figure 15. HUVEC/fibroblast co-culture tubule formation dependent on seeding ratio and culturing media. Representative images of monocultures and co-cultures of HUVECs and HDFs on day 17. HUVECs were stained with PE-anti-hCD31 (green) and cell nucleus were stained with DAPI (blue).



Figure 16. Comparison of the mean (\pm SEM) number of junctions, branches, and length of tubules formed in fibroblast/HUVEC co-cultures on day 6 and day 17. Two representative images from each day were analyzed using Angiogenesis Analyzer ²⁰⁵ in ImageJ. Mean (\pm SEM) were compared using ANOVA Tukey test, where ** indicates p<0.01.



Figure 17. Quantification of angiogenic receptor concentrations on HUVECs and HDFs following 24hour, 6-day, 11-day, and 17-day co-culturing. Dashed lines represent receptor concentrations of collagenase-treated monocultures (Red: HUVECs; Blue: HDFs). Mean \pm SEM of receptor concentrations in co-cultures were compared to monocultures and ANOVA Tukey test was performed to determine the significant differences (* indicates p<0.05, ** indicates p<0.01, and *** indicates p<0.001).



Figure 18. Cell-by-cell analysis of fibroblast/HUVEC co-culture up to 17 days. We showed receptor distributions of (A & D) VEGFR1, (B &E) VEGFR2, (C & F) Tie2, (G & J) NRP1, (H &K) PDGFRα, and (I & L) PDGFRβ on HUVECs and HDFs.



Supplemental Figure 4. Representative images of analyzed fibroblast/HUVEC co-cultures on day 6 and day 17 using Angiogenesis Analyzer in ImageJ. Red dots: joints, blue lines: branches.



Supplemental Figure 5. Representative images of fibroblast/HUVEC co-cultures incubated in EGM-2 at 3 x 10⁴ cells/cm² seeding density with a 1:1 seeding ratio after (a) 24 hours, (b) 6 days, (c) 11 days, and (d) 17 days. Cells were stained with FITC-conjugated CD31 antibodies and DAPI, and fluorescent images were taken using a 4x objective and a 20x objective.



Supplemental Figure 6. Comparison of receptor concentrations on cells dissociated using Cell Stripper and cells dissociated using 0.2% collagenase type IV with intermittent vortexing. Mean \pm SEM of replicates were compared using ANOVA Tukey test and no significant changes were observed (p > 0.05).



Supplemental Figure 7. Gaussian mixture models of (A) VEGFR2 on 24-hr co-cultured HUVECs, (B) NRP1 on day-17 co-cultured HUVECs, (C) NRP1 on day-6 co-cultured HDFs, and (D) PDGFRβ on day-17 co-cultured HUVECs.



Supplemental Figure 8. Quadratic entropy (QE) of co-cultured (A) HUVECs and (B) HDFs up to 17 days.

4.6 REFERENCES

- 1. Kirkpatrick, C. J., Fuchs, S. & Unger, R. E. Co-culture systems for vascularizationlearning from nature. *Adv. Drug Deliv. Rev.* **63**, 291–9 (2011).
- 2. Bishop, E. T. *et al.* An in vitro model of angiogenesis: basic features. *Angiogenesis* **3**, 335–44 (1999).
- Donovan, D., Brown, N. J., Bishop, E. T. & Lewis, C. E. Comparison of three in vitro human 'angiogenesis' assays with capillaries formed in vivo. *Angiogenesis* 4, 113–121 (2001).
- 4. Beilmann, M., Birk, G. & Lenter, M. C. Human primary co-culture angiogenesis assay reveals additive stimulation and different angiogenic properties of VEGF and HGF. *Cytokine* **26**, 178–85 (2004).
- 5. Eckermann, C. W., Lehle, K., Schmid, S. A., Wheatley, D. N. & Kunz-Schughart, L. A. Characterization and modulation of fibroblast/endothelial cell co-cultures for the in vitro preformation of three-dimensional tubular networks. *Cell Biol. Int.* **35**, 1097–110 (2011).
- 6. Schmid, S. A. *et al.* Lactate adversely affects the in vitro formation of endothelial cell tubular structures through the action of TGF-beta1. *Exp. Cell Res.* **313**, 2531–49 (2007).
- FRIIS, T., KJAER SORENSEN, B., ENGEL, A.-M., RYGAARD, J. & HOUEN, G. A quantitative ELISA-based co-culture angiogenesis and cell proliferation assay. *APMIS* 111, 658–668 (2003).
- 8. Staton, C. A., Reed, M. W. R. & Brown, N. J. A critical analysis of current in vitro and in vivo angiogenesis assays. *Int. J. Exp. Pathol.* **90**, 195–221 (2009).
- 9. Willett, C. G. et al. Direct evidence that the VEGF-specific antibody bevacizumab has antivascular effects in human rectal cancer. Nature medicine **10**, (2004).
- 10. Imoukhuede, P. I. & Popel, A. S. Quantification and cell-to-cell variation of vascular endothelial growth factor receptors. *Exp. Cell Res.* **317**, 955–965 (2011).
- 11. Bergers, G., Song, S., Meyer-Morse, N., Bergsland, E. & Hanahan, D. Benefits of targeting both pericytes and endothelial cells in the tumor vasculature with kinase inhibitors. *J. Clin. Invest.* **111**, 1287–1295 (2003).

- Erber, R. *et al.* Combined inhibition of VEGF and PDGF signaling enforces tumor vessel regression by interfering with pericyte-mediated endothelial cell survival mechanisms. *FASEB J.* 18, 338–40 (2004).
- Casanovas, O., Hicklin, D. J., Bergers, G. & Hanahan, D. Drug resistance by evasion of antiangiogenic targeting of VEGF signaling in late-stage pancreatic islet tumors. *Cancer Cell* 8, 299–309 (2005).
- 14. Weddell, J. C. & Imoukhuede, P. I. Quantitative characterization of cellular membranereceptor heterogeneity through statistical and computational modeling. *PLoS One* **9**, e97271 (2014).
- Imoukhuede, P. I. & Popel, A. S. Quantitative fluorescent profiling of VEGFRs reveals tumor cell and endothelial cell heterogeneity in breast cancer xenografts. *Cancer Med.* 3, 225–44 (2014).
- Rao, C. R. Diversity and dissimilarity coefficients: A unified approach. *Theor. Popul. Biol.* 21, 24–43 (1982).
- 17. Pavoine, S. & Dolédec, S. The apportionment of quadratic entropy: a useful alternative for partitioning diversity in ecological data. *Environ. Ecol. Stat.* **12**, 125–138 (2005).
- 18. Botta-Dukát, Z. Rao's quadratic entropy as a measure of functional diversity based on multiple traits. *J. Veg. Sci.* **16**, 533–540 (2005).
- 19. Fukuhara, S. *et al.* Differential function of Tie2 at cell–cell contacts and cell–substratum contacts regulated by angiopoietin-1. *Nat. Cell Biol.* **10**, 513–526 (2008).
- 20. Chen, S., Guo, X., Imarenezor, O. & Imoukhuede, P. I. Quantification of VEGFRs, NRP1, and PDGFRs on Endothelial Cells and Fibroblasts Reveals Serum, Intra-Family Ligand, and Cross-Family Ligand Regulation. *Cell. Mol. Bioeng.* **8**, 383–403 (2015).
- Gaengel, K., Genové, G., Armulik, A. & Betsholtz, C. Endothelial-mural cell signaling in vascular development and angiogenesis. *Arterioscler. Thromb. Vasc. Biol.* 29, 630–8 (2009).
- 22. Hellström, M., Kalén, M., Lindahl, P., Abramsson, A. & Betsholtz, C. Role of PDGF-B and PDGFR-beta in recruitment of vascular smooth muscle cells and pericytes during embryonic blood vessel formation in the mouse. *Development* **126**, 3047–55 (1999).

- 23. Zhang, J. *et al.* Differential roles of PDGFR-alpha and PDGFR-beta in angiogenesis and vessel stability. *FASEB J.* **23**, 153–63 (2009).
- 24. Andrae, J., Gallini, R. & Betsholtz, C. Role of platelet-derived growth factors in physiology and medicine. *Genes Dev.* **22**, 1276–312 (2008).
- 25. Chen, S. *et al.* qFlow cytometry-based receptoromic screening: a high-throughput quantification approach informing biomarker selection and nanosensor development. in *Methods Mol Biol* (eds. Hurst Petrosko, S. & S. Day, E.) 117–138 (Springer New York, 2017).
- Battegay, E. J., Rupp, J., Iruela-Arispe, L., Sage, E. H. & Pech, M. PDGF-BB modulates endothelial proliferation and angiogenesis in vitro via PDGF beta-receptors. *J. Cell Biol.* 125, 917–28 (1994).
- 27. Ball, S. G., Shuttleworth, C. A. & Kielty, C. M. Vascular endothelial growth factor can signal through platelet-derived growth factor receptors. *J. Cell Biol.* **177**, 489–500 (2007).

CHAPTER 5 CHARACTERIZING GLIOBLASTOMA HETEROGENEITY VIA SINGLE-CELL RECEPTOR QUANTIFICATION

Dysregulation of tyrosine kinase receptor (RTK) signaling pathways play important roles in glioblastoma (GBM). However, therapies targeting these signaling pathways have not been successful, partially because of drug resistance. Increasing evidence suggests that tumor heterogeneity, more specifically, GBM-associated stem and endothelial cell heterogeneity, may contribute to drug resistance. In this perspective article, we introduce a high-throughput, quantitative approach to profile plasma membrane RTKs on single cells. First, we review the roles of RTKs in cancer. Then, we discuss the sources of cell heterogeneity in GBM, providing context to the key cells directing resistance to drugs. Finally, we present our provisionally patented qFlow cytometry approach, and report results of a "proof of concept" patient-derived xenograft GBM study.

¹ This chapter was published in Frontier Bioeng Biotechnol. 2018; 6: 92. PMID: 30050899. DOI: <u>https://doi.org/10.3389/fbioe.2018.00092</u>.

5.1 INTRODUCTION

GBMs are the most frequent and lethal malignant primary adult brain tumor ¹, which presents a critical need to develop new therapeutics. Addressing the dysregulation of RTK signaling pathways offers promise in overcoming GBM lethality ^{2–5}. RTK dysfunction has been observed in GBM, where these pathways are correlated with tumor cell proliferation ^{6,7}, angiogenesis ^{8,9}, tumor invasiveness ^{10,11}, and resistance to therapy ^{12–14}. Moreover, these pathways are popular targets for small-molecule inhibitors ^{15,16}. Unfortunately, the clinical benefit of these targeted therapies is limited by drug resistance ^{17,18}.

Increasing evidence suggests that drug resistance may be attributed to tumor heterogeneity (variations within an individual tumor) ^{7,19,20}. For example, a landmark study identified tumor subpopulations resistant to therapy prior to treatment by sequencing 4,645 single cells from 19 melanoma patients. This thorough analysis was enabled by single-cell technology, and may have been overlooked with ensemble sequencing ²¹. Additionally, a single-cell analysis of patient-derived xenografts (PDXs) of GBM39 also found higher heterogeneity in resistant tumors than in responsive tumors ²². In line with these single-cell measurements, we previously discovered, measured, and statistically described heterogeneity in breast cancer xenografts by quantifying vascular endothelial growth factor plasma membrane receptor (VEGFR) concentrations at the single-cell level ²³. When we combined this quantitative analysis with computational modeling, we arrived at the prediction that tumors having "high" concentrations of plasma membrane VEGFR1 could be resistant to anti-VEGF drugs (angiogenesis inhibitors) ²⁴. Clinical work supports this prediction for colorectal cancer ²⁵, and application of this quantification and prediction should offer a new paradigm for biomarker discovery in cancer medicine.

To address the need for quantitative, single-cell analysis of GBM heterogeneity, we apply our optimized and provisionally patented VEGFR quantitative flow (qFlow) cytometry approach to GBM ^{23,24,26–30}. We describe expanded measurement to several RTKs critical to tumor development. To provide further context we, briefly, review the roles of RTKs in cancer and present connections between RTK heterogeneity and drug resistance. We then present our approach, qFlow cytometry, and report promising findings of a "proof of concept" PDX GBM study.

5.1.1 Roles of RTKs in cancer

RTKs are widely expressed transmembrane proteins ^{31,32}. Upon ligand binding, they are activated via canonical ^{33,34} and non-canonical ^{28,35–37} ligand-induced dimerization and tyrosine phosphorylation mechanisms. Importantly, unligated receptors can dimerize ^{33,38–44} and signal ^{33,45,46}, although ligand binding stabilizes the dimeric receptor structure. These receptor-initiated signaling events regulate cell survival, proliferation, differentiation, and motility ^{47,48}.

VEGFRs are upregulated in many cancers ^{49–51}. Signals through endothelial VEGFRs and the neuropilin (NRP) co-receptors ^{23,52–55} induce the sprouting angiogenic hallmarks of cell proliferation and cell migration ⁵⁶. These sprouting angiogenesis hallmarks also sustain tumor growth and enable tumor metastasis ^{57,58}. VEGF and other pro-angiogenic factors, may also regulate vascular growth and regression in tumors that co-opt pre-existing blood vessels ^{59–61}.

In addition to these canonical pathways, cross-family signaling may also affect tumor vascularization. In this paradigm, ligands from one growth factor family bind to and signal through receptor(s) of another family. For instance, we have shown VEGF-mediated

downregulation of PDGFRs ²⁸, and discovered that both VEGF–PDGFR binding and PDGF– VEGFR binding is high affinity ³⁷. Other cross-family studies have identified VEGF–PDGFR binding and signaling ^{62,63} and VEGFR–PDGFR dimerization in tumor associated pericytes ⁶⁴. Altogether, these canonical and cross-family RTK mechanisms suggest several possible receptor activation landscapes that can contribute to tumor growth and drug resistance.

5.1.2 GBM-associated cell heterogeneity: stem and endothelial

An accepted origin of tumor heterogeneity involves clonal evolution; an reiterative process of genetic mutation, clonal selection, and expansion, which drives the growth of single cancer cells into heterogeneous tumor masses ^{65–67}. In addition to cancer cells, other cell types within the tumor may also differentiate or transition as tumor develops. Some such cells include: tumor-associated fibroblasts, macrophages/monocytes, endothelial cells (ECs), and stem cells ¹⁹. Here, we describe glioblastoma stem cells (GSCs) and ECs, which we focus on in our pilot study.

GSCs are an important tumor cell component, because despite their small number (~0.5% - 10%) ⁶⁸, GSCs are more resistant to radiotherapy and chemotherapy than other cancer cells ^{69,70}. Furthermore, their resistance can amplify tumor heterogeneity, because they have self-renewing and tumor-initiating capabilities ⁷¹. GSCs are often identified by CD133 ⁷², which is associated with poor prognosis in a number of tumor types. There is controversy surrounding the usage of CD133 as a GSC marker ^{70,73,74}. Early studies showed a subpopulation of GBM cells expressing CD133 were able to form tumors ⁷⁵ and further studies showed subpopulations of CD133⁻ cells were also able to form tumors in vivo ⁷⁶. While these studies do not negate the possible role of CD133 in identifying GSCs, they do highlight the importance of heterogeneity and the need for additional markers. Therefore, establishing a "barcode" of RTK plasma membrane concentrations on GSCs may help to identify novel markers, aiding in the isolation and understanding of these stem cells.

ECs, the primary structural unit of the vasculature, are an important contributor to GBM development. Unlike normal vessels, tumor vasculature is leaky, tortuous, and dilated ^{77,78}. In addition to typical tumor vascular pathological features, brain tumor vasculature exhibits the loss of the important blood-brain-barrier feature of tight EC-EC junctions when tumor size grows beyond 1–2 mm in diameter ⁷⁹. The close interaction between tumors and tumor vessels, and the observation of extensive EC heterogeneity supports the need for profiling tumor-associated ECs.

5.1.3 A paradigm shift in single-cell technologies: from gene-centric to proteomics

Studies characterizing GBM heterogeneity primarily focus on genetic and transcriptomic profiling ^{80–86}, which does not always correlate with functional changes^{87–89}. Moreover, multiple studies show discordance between sequence data and protein expression in GBM, particularly with regards to epidermal growth factor receptor (EGFR) ⁹⁰ and PDGFR ⁹¹ gene vs. protein expression. Because proteins are the effectors of signaling towards functional response ^{27,53,92}, there is a need for increased protein-based, functional measurements.

qFlow cytometry offers a powerful tool for protein-based, single-cell measurements. It applies fluorescent calibration to traditional flow cytometry, converting signal to absolute protein concentrations ^{27,93,94}. Absolute protein quantification allows detection of variations in proteins across published studies, tissues, replicates, and instrument settings ^{95–99}. Moreover, qFlow cytometry advances systems biology, providing the quantitative data needed for computational studies ^{100,101}. For example, using qFlow cytometry coupled with systems biology, we predicted

122

that anti-VEGF efficacy depends on tumor endothelial VEGFR1 plasma membrane concentrations ²⁴. Furthermore, a receptor-internalization computational model recently predicted that small increases in plasma membrane RTK concentrations (< 1,000 receptors/cell) may double nuclear-based RTK signaling ¹⁰², which further implicates RTK concentrations as a determinant of signal transduction. These predictions were only possible with the accurate experimental data offered by qFlow cytometry.

5.2 RESULTS AND DISCUSSION

We performed a "proof of concept" qFlow cytometry study on a PDX, GBM39 (**Fig. 19**). GBM39 is known for EGFR^{vIII} and low invasiveness, *in vivo*^{6,103}. The xenograft was established with tumor tissue from patients undergoing surgical treatment at Mayo Clinic, Rochester, MN. Multiple studies characterize these PDX models and report maintenance of patient morphologic and molecular characteristics including EGFR amplification as well as tumor invasiveness ^{11,104}.

Following dissociation, PDX cells were stained with Sytox Blue (a live/dead cell stain), CD45 ¹⁰⁵, CD34 ^{106,107}, and CD133 ^{108–112} fluorophore-conjugated antibodies that target EC-like cells and GSCs, respectively (**Fig. 19**). This labeling scheme excludes both dead cells and hematopoietic cells and enables identification of human tumor EC-like cells (hCD34⁺), mouse tumor EC-like cells (mCD34⁺), and GSCs (hCD133⁺) from the live CD45⁻ pool (**Fig. 20A**). To obtain reliable data, we obtained fluorescence signals from 2–3 samples/RTK with 10,000 – 35,000 live single cells collected per sample. As expected, the bulk GBM39 PDX sample was primarily non-EC, non-GSC cells (62.46%). In addition, we found 6-fold higher mouse tumor EC-like cells than human tumor EC-like cells (**Fig. 20B**). This quantification aligned with prior studies of GBM xenograft showing ~7.1% EC population (CD45⁻CD31⁺CD34⁺). Consistent with

our quantification of GSCs, a primary human study of 37 patients reported a range of 0.5%–10% ⁶⁸, when identifying GSCs using the CD133 marker.

We labeled and screened 9 plasma membrane RTKs on these cells, which included two established GBM biomarkers, EGFR and insulin-like growth factor receptor (IGFR)¹⁰, and angiogenic signaling biomarkers: VEGFRs, PDGFRs, NRP1, and Tie2^{31,113–116}. Using qFlow cytometry and statistical models, we quantitatively characterized GBM39 PDX via four patented metrics (**Fig. 19**): cell composition, ensemble RTK concentration, cell-by-cell analysis with Gaussian mixture modeling, and heterogeneity analysis ³⁰.

Percentage of gated cell populations were exported using FlowJo software (TreeStar). Ensemble RTK concentrations and cell-by-cell analysis were performed as previously described ^{27,28}. We then applied Gaussian mixture modeling to identify log-normal sub-populations within each distribution, described by its mean, standard deviation, and density. We reduced the chance of overfitting the subpopulations by using Bayesian Information Criterion (BIC) ^{117,118}. A detailed description of heterogeneity quantification is provided in section 5.5.

5.2.1 Human tumor EC-like cells have high EGFR and IGFR on plasma membrane

EGFR and IGFR are expressed on tumor cells and contribute to tumor progression. Interestingly, the human tumor EC-like population had high plasma membrane EGFR and IGFR concentrations (~21,000/cell and ~20,000/cell, respectively) (**Fig. 20C**), consistent with qualitative findings of higher EGFR on breast carcinoma-derived ECs compared to normal ECs ¹¹⁹. Our results of high EGFR on human tumor EC-like cells from GBM39 is also consistent with results of clinical GBM samples ¹²⁰.

The mixture modeling revealed that 8% of human tumor EC-like subpopulations had a ~12-fold higher membrane localization of EGFRs than average. We found a similar pattern for IGFRs in human tumor EC-like subpopulations. Together, the ensemble-averaged data and the mixture modeling indicated significant plasma membrane localization of EGFR and IGFR on human tumor EC-like cells. High concentrations of EGFR and IGFR suggest an opportunity for targeted inhibition, which could be a mechanism for disrupting tumor vessels on GBMs with a similar profile.

5.2.2 Mouse tumor EC-like cells have similar plasma membrane VEGFR concentrations as healthy mouse ECs from skeletal muscle

VEGFRs are key regulators of tumor angiogenesis, so their quantification can offer insight into the tumor vasculature. Furthermore, as biomarkers of vasculature, these receptors have been proposed as diagnostic biomarkers of anti-angiogenic drug efficacy ^{121,122} with computational ²⁴ and clinical ²⁵ support to their use. We found that VEGFR1 and VEGFR2 had similar concentrations and ratios on mouse tumor EC-like cells (~3,100 VEGFR1/cell and ~1,000 VEGFR2/cell) as on healthy ECs obtained from mouse skeletal muscle ⁵² (**Fig. 20E**). This finding of a low VEGFR2:VEGFR1 ratio aligns with a previous study on breast cancer xenografts ²⁴; however, the receptor abundance we report here is much lower. These findings of EC-like cells from GBM39 having VEGFRs at levels similar to normal mouse skeletal muscle ECs suggests a need for further quantification of normal brain ECs VEGFR concentrations to establish tissue standards. Similarly, it suggests a need to examine other GBM specimens to identify whether this is a property of co-opted vessels or specific to this GBM strain. We analyzed the human tumor EC-like population (5.20% of the population, **Fig. 20B**), which should reflect the original tumor vessels from the patient (**Fig. 20C**). We found similar plasma membrane VEGFR1 and VEGFR2 ratios (~3,600 VEGFR1/cell & ~5,800 VEGFR2/cell) as previous reports *in vitro* ^{28,54}. However, these data show that not all tumors have the same concentrations or ratios of plasma membrane VEGFRs on their endothelium. Importantly, tumor EC-like cells display much greater heterogeneity than normal ECs with subpopulations that have high concentrations of VEGFRs. Indeed, cell-by-cell analysis and mixture modeling of human tumor EC-like cells reveals the existence of a high-VEGFR1 subpopulation (~10%) with ~41,000 VEGFR1/cell, while the highest VEGFR2 subpopulation is ~18,500 VEGFR2/cell, comprising ~35% of the total human tumor EC-like population (**Fig. 20D & 20G**). The difference in VEGFR2:VEGFR1 ratio and receptor concentrations between human and mouse tumor EC-like population shows a significant level of endothelial heterogeneity. Such data may enable correlations between these tumor vessel regulators and anti-angiogenic drug efficacy.

5.2.3 Plasma membrane PDGFRs localize on tumor EC-like cells

PDGFRs serve important roles in supporting vasculature in tumor microenvironments ¹²³. We observed lower levels of PDGFRs on human tumor EC-like cell membranes than on mouse (**Fig. 20C & 20E**). The cell-by-cell analysis and mixture modeling suggests that this ensemble average does not capture the subpopulations having high-PDGFR plasma membrane localization: 66% and 16% of mouse tumor EC-like cell membrane had ~23,400 PDGFR α and ~19,800 PDGFR β , respectively (**Fig. 20F**). This significant heterogeneity may be attributed to the use of the CD34 marker to designate EC-like cells, because it is also found on stem cells/precursors, mast cells, and neurons ^{23,124,125}. PDGFR α is also considered an important mesenchymal stem

cell marker ¹²⁶. So, the co-labeling of PDGFR α and CD34 suggests these cells may be mesenchymal stem cells ¹²⁷.

If these CD34⁺PDGFR⁺ cells are endothelial, then our data correlates with studies finding PDGFRs on tumor ECs ^{128–130}. PDGFR localization on ECs is controversial, because it is characteristic of mural cells and not of ECs ^{131–136}. However, they have been observed on monolayer microvascular ECs, *in vitro* ^{136,137} and on angiogenic ECs that formed sprout and tubes *in vitro* ¹³¹. If we subscribe to the canonical PDGFR localization understanding, then these tumor vessels induce "non-conventional" PDGFR localization patterns.

5.2.4 GSCs have little-to-no surface EGFR or IGFR

Multiple studies suggest that a higher degree of GSC "stemness" is associated with *EGFR* amplification ^{138,139}; however, we observed ~13-fold lower EGFRs on GSC plasma membranes compared to the bulk PDX cells (**Fig. 20C**). This trend was also seen with IGFR (**Fig. 20C**). The low membrane EGFR concentrations on GSCs is concerning, given reports that EGFR signaling is necessary for GSC proliferation and tumor-sphere formation ^{140,141}. Yet, this may explain the lower percentage of GSCs in the PDX sample (~0.9%) compared to the expected stem cell fraction (0.5%–10% ⁶⁸). A possible explanation is that serially transplanted tumors can lose their EGFR overexpression, even *in vivo* (Liffers et al. 2015). Clearly, further investigation of both gene expression and protein quantification on other GBM PDX GSCs is necessary to understand their contribution to heterogeneity and drug-resistance.

5.2.5 Quantification of cell-RTK heterogeneity

To quantify heterogeneity of each cell subpopulation, we used two parameters: number of mixture components and Quadratic Entropy (QE). To quantitatively assess the number of subpopulations within each cell population, we fit each cell-by-cell RTK distribution with mixture models consisting of 1 - 9 log-normal Gaussian sub-distributions (mixture components); we then applied BIC as the criterion to select the mixture model with the lowest BIC. The number of mixture components is determined by how many log-normal Gaussian sub-distributions are in the mixture model. The number of mixture components, thus, is a measurement of cell heterogeneity. Generally, 1-2 mixture components are considered low heterogeneity ²⁷, while more than 2 components is considered highly heterogeneous ^{23,24}.

Alternatively, QE requires equally spaced bins, here we chose 500 bins, from each cell-by-cell distribution (**Fig. 20D & 20F**). QE then sums the weighted differences of the means between two bins ^{142–144}. Thus, QE is a measurement of the increase in random variation in the cellular response. Because healthy ECs and human fibroblasts *in vitro* have shown QE within 0.2–0.7 ²⁸, we describe QE<0.7 as low heterogeneity and QE>0.7 as high heterogeneity. QE provides a quantitative measure of the diversity of cellular phenotypes in cancer tissue sections for diagnostic applications ¹⁴⁵ and drug discovery ¹⁴⁶. Interestingly, human tumor EC-like cells showed lower QE and number of mixture components when compared to mouse tumor EC-like cells (**Fig. 20G**). We suspect that the likely loss of human tumor-associated cells over time in a PDX model ¹⁴⁷ may be the reason why human tumor EC-like cells present a more homogenous state than the mouse tumor EC-like cells.

5.2.6 Clinical implications of GBM heterogeneity

We envision that RTK quantification can identify ideal receptor targets across the bulk tumor specimen and on specific cell populations in the tumor. First, the ideal receptor target would be highly available ^{5,15,148}: it would have high concentrations on a high percentage of bulk cells or specific cells. Next, the target RTK would exhibit low heterogeneity: it would have low QE in bulk cells or on the specific cell subpopulation ^{103,149}. An ideal receptor target would also be highly specific to the tumor, which would manifest as higher receptor concentrations in the tumor versus healthy tissue ¹⁵.

Based on these guidelines, we offer possible targets on GBM39. If the goal is targeting tumor vessels, then VEGFR2 and PDGFR α are highly targetable: >70% target cells have > 6,000 VEGFR2 or PDGFR α /cell plasma membrane with QE=0.20 or 0.32, respectively. Furthermore, they are likely targets, because they are more highly expressed in GBM specimens than health tissue ²⁸: ~5-fold higher VEGFR2 and ~4-fold higher PDGFR α . Therefore, targeting VEGFR2 and PDGFR α should preferentially target the tumor.

Our work suggests that targeting EGFR and IGFR on tumors like GBM39 may not be effective by itself. Although, they have high concentrations on ~70-90% EC-like and non-EC-like GBM cells, their high GBM heterogeneity (QE=~1.0) and high concentration on healthy tissue (2- $2,000 \times 10^3$ EGFR/fibroblast or epithelial cell; 2.5×10^4 IGFR/NIH 3T3 mouse fibroblasts ^{80,102,150} may lower their targeting specificity, resulting in lower drug efficacy ¹⁵¹. Better drug delivery to the tumor site will likely improve targeting specificity without disrupting healthy tissue. An alternative strategy is to develop dual-inhibitors targeting both EGFR/IGFR and VEGFR2 to increase their specificity for tumor EC-like cells. We believe our method can also identify cellular and molecular mechanisms underlying reduced response to drugs. For example, upregulation of alternative signaling pathways has been implicated in anti-VEGF drug resistance ^{152,153}. This mechanism of drug resistance is often accompanied by significant tumor heterogeneity ^{14,84,86}. Therefore, these alternative pathways may be overlooked in bulk studies if they are only present on small cell subpopulations. From this study, we suggest targeting RTKs that are localized on plasma membrane at high concentrations on small cell populations (<10%) for combination therapy. For example, VEGFR1 and Tie2 on tumor ECs may become "alternative" RTKs for anti-VEGF treatment, because we found ~10% human tumor EC-like cell subpopulations had 41,000 VEGFR1 and ~8% had 65,700 Tie2 on the plasma membrane. Identifying alternative RTK pathways that contribute to resistance can provide tumor-specific drug targets for combination therapy.

5.3 CONCLUSIONS

Our study of the GBM39 PDX model, arrived at 4 key findings and 2 recommendations: (1) tumor EC-like subpopulations have high concentrations of plasma membrane VEGFR1 and VEGFR2; (2) human versus mouse tumor EC-like cells have inverted VEGFR2:VEGFR1 ratios; (3) tumor EC-like subpopulations have high plasma membrane EGFR, IGFR, and PDGFR concentrations; and (4) GSCs compose a low percentage of cells in the tumor and have little-tono EGFRs and IGFRs on their plasma membranes.

Based on findings in this study and our RTK-targeting criteria, VEGFR2 or PDGFRα would be likely drug targets for GBM39. In addition, VEGFR1 and Tie2 are likely drug targets for combination therapy. The next step would be to test these targets in a GBM PDX model.

The results of this "proof of concept" study should be interpreted as such: it offers an approach for continued measurement of tumor samples, broadly, and GBM samples, specifically, with the GBM39 PDX sample as a first example. We present the novel method, qFlow cytometry, and show its application in characterizing GBM heterogeneity. Larger and well powered samples are warranted to expand the current preliminary results, and to discover ideal drug targets and mechanisms underlying drug resistance.

Future opportunities for expanding this research lies in establishing protein concentration ranges on additional samples and continued development of biomimetic tumor models. Firstly, additional measurements of protein concentration on normal ECs and other cells would provide the baselines needed to compare to tumor. In establishing EC baselines, isolation of a pure EC population may be a challenge. Previous qFlow studies have identified ECs using both the CD34 and CD31 markers ^{23,26,52}. However, it is important to note, that using multiple markers can bias cell collection: CD34 is a progenitor marker, so its use biases selection from more mature cells. Whereas, CD31 is a mature cell marker that is found on ECs, platelets, natural killer cells, monocytes, macrophages, and among other cells ¹⁵⁴, so its use can lead to sample impurity. Here, we chose to bias towards progenitor-like ECs; however, expanded studies may determine if protein concentrations correlate with marker presentation (e.g., identifying whether progenitorlike cells having higher or lower protein concentrations).

Another opportunity for advancement lies in our quantitative single-cell RTK mapping, moving towards multiplexed measurement of RTKs. Towards multiplexed quantification, Lee-Montiel et al. developed a quantum dot method for receptor labeling and calibration ^{94,155} that can be translated to qFlow cytometry. Another approach could be to adapt receptor quantification to

mass cytometry (CyTOF) ¹⁵⁶. Such advancements would provide multi-RTK, multi-cell insight into tumor heterogeneity.

In conclusion, cancer research is experiencing a paradigm shift from ensemble analysis to cellto-cell variability ^{157–159} because of the increasing evidence correlating drug resistance with tumor heterogeneity. The perspective and work that we present here offers sensitive methods for heterogeneity characterization in tumors that should enable improved treatment. We believe that continued quantification of single-cell receptor heterogeneity is a new frontier that will offer significant clinical impact.

5.4 FIGURES AND FIGURE LEGENDS



Figure 19. An overview of the workflow for characterizing tumor heterogeneity in GBM39 PDX samples. The GBM39 PDX is established with tumor tissue from patients at Mayo Clinic, Rochester, MN. Following dissociation, multi-channel flow cytometer is used to characterize PDX cells. Briefly, dead cells are excluded using a live/dead cell stain, and hematopoietic cells are excluded using the CD45 antigen, then the endothelial marker CD34 and CD133 can be used to identify EC-like cells and GSCs respectively from the CD45⁻ pool. Percentage of GSCs, EC-like cells and other PDX cells within all live cells can be exported from the flow cytometer. Cells are also stained with phycoerythrin (PE)-conjugated antibodies targeting one of the 9 plasma membrane RTKs. qFlow cytometry is performed as described previously, and ensemble averaged plasma membrane RTK concentrations and cell-by-cell RTK distributions can be obtained. We use two parameters to quantify RTK heterogeneity across EC-like and non EC-like cells: number of mixture components and Quadratic entropy of the cell-by-cell RTK distribution.



Figure 20. Characterization of plasma membrane RTK concentrations and tumor heterogeneity in GBM39 PDX sample. (A) Representative flow cytometry plots for gating GSCs (hCD45-hCD133+), human EC-like cells (hCD45-hCD34+), and mouse EC-like cells (mCD45-mCD34+) from live cell population. (B) Percentage of GSCs, human EC-like, mouse EC-like, and tumor & other PDX cells in the GBM39 PDX sample. (C) Ensemble-averaged concentrations and (D) cell-by-cell distributions of plasma membrane VEGFRs, Tie2, NRP1, PDGFRs, EGFR, and IGFR on human EC-like cells. (E) Ensemble-averaged concentrations and (F) cell-by-cell distributions of plasma membrane VEGFRs, Tie2, NRP1, PDGFRs, EGFR, and IGFR on human EC-like cells. (E) Ensemble-averaged concentrations and (F) cell-by-cell distributions of plasma membrane VEGFRs, Tie2, NRP1, and PDGFRs on mouse EC-like cells. (G) Heterogeneity analysis of RTKs in EC-like and non EC-like cell populations. Number of mixture components estimates how many cell subpopulations there are having different plasma membrane RTK concentrations. Quadratic entropy represents the diversity of RTK concentrations within EC-like and non EC-like populations.

5.5 REFERENCES

- 1. Yadav, V. N. *et al.* Molecular Ablation of Tumor Blood Vessels Inhibits Therapeutic Effects of Radiation and Bevacizumab. *Neuro. Oncol.* (2018). doi:10.1093/neuonc/noy055
- 2. Smith, C. L. *et al.* Migration Phenotype of Brain-Cancer Cells Predicts Patient Outcomes. *Cell Rep.* **15**, 2616–24 (2016).
- 3. Hawkins-Daarud, A., Rockne, R. C., Anderson, A. R. A. & Swanson, K. R. Modeling Tumor-Associated Edema in Gliomas during Anti-Angiogenic Therapy and Its Impact on Imageable Tumor. *Front. Oncol.* **3**, 66 (2013).
- 4. Massey, S. C. *et al.* Simulating PDGF-Driven Glioma Growth and Invasion in an Anatomically Accurate Brain Domain. *Bull. Math. Biol.* **80**, 1292–1309 (2018).
- 5. Cloughesy, T. F., Cavenee, W. K. & Mischel, P. S. Glioblastoma: From Molecular Pathology to Targeted Treatment. *Annu. Rev. Pathol. Mech. Dis.* **9**, 1–25 (2014).
- 6. Johnson, H. *et al.* Molecular characterization of EGFR and EGFRvIII signaling networks in human glioblastoma tumor xenografts. *Mol. Cell. Proteomics* **11**, 1724–40 (2012).
- Furnari, F. B., Cloughesy, T. F., Cavenee, W. K. & Mischel, P. S. Heterogeneity of epidermal growth factor receptor signalling networks in glioblastoma. *Nat. Rev. Cancer* 15, 302–10 (2015).
- Plate, K. H., Breier, G., Weich, H. A., Mennel, H. D. & Risau, W. Vascular endothelial growth factor and glioma angiogenesis: coordinate induction of VEGF receptors, distribution of VEGF protein and possible in vivo regulatory mechanisms. *Int. J. Cancer* 59, 520–9 (1994).
- Kuczynski, E. A., Patten, S. G. & Coomber, B. L. VEGFR2 Expression and TGF-β Signaling in Initial and Recurrent High-Grade Human Glioma. *Oncology* 81, 126–134 (2011).
- Sangar, V. *et al.* Quantitative proteomic analysis reveals effects of epidermal growth factor receptor (EGFR) on invasion-promoting proteins secreted by glioblastoma cells. *Mol. Cell. Proteomics* 13, 2618–31 (2014).
- 11. Giannini, C. *et al.* Patient tumor EGFR and PDGFRA gene amplifications retained in an invasive intracranial xenograft model of glioblastoma multiforme. *Neuro. Oncol.* **7**, 164–176 (2005).
- 12. Murat, A. et al. Stem Cell-Related "Self-Renewal" Signature and High Epidermal Growth

Factor Receptor Expression Associated With Resistance to Concomitant Chemoradiotherapy in Glioblastoma. *J. Clin. Oncol.* **26**, 3015–3024 (2008).

- 13. Popescu, A. M. *et al.* Targeting the VEGF and PDGF signaling pathway in glioblastoma treatment. *Int. J. Clin. Exp. Pathol.* **8**, 7825–37 (2015).
- 14. Lu, K. V & Bergers, G. Mechanisms of evasive resistance to anti-VEGF therapy in glioblastoma. *CNS Oncol.* **2**, 49–65 (2013).
- 15. Rich, J. N. & Bigner, D. D. Development of novel targeted therapies in the treatment of malignant glioma. *Nat. Rev. Drug Discov.* **3**, 430–446 (2004).
- 16. Candolfi, M. *et al.* Targeted toxins for glioblastoma multiforme: pre-clinical studies and clinical implementation. *Anticancer. Agents Med. Chem.* **11**, 729–38 (2011).
- 17. De Witt Hamer, P. C. Small molecule kinase inhibitors in glioblastoma: a systematic review of clinical studies. *Neuro. Oncol.* **12**, 304–16 (2010).
- Szopa, W., Burley, T. A., Kramer-Marek, G. & Kaspera, W. Diagnostic and Therapeutic Biomarkers in Glioblastoma: Current Status and Future Perspectives. *Biomed Res. Int.* 2017, 1–13 (2017).
- 19. Saunders, N. A. *et al.* Role of intratumoural heterogeneity in cancer drug resistance: molecular and clinical perspectives. *EMBO Mol. Med.* **4**, 675–84 (2012).
- 20. Qazi, M. A. *et al.* Intratumoral heterogeneity: pathways to treatment resistance and relapse in human glioblastoma. *Ann. Oncol.* **28**, 1448–1456 (2017).
- 21. Tirosh, I. *et al.* Dissecting the multicellular ecosystem of metastatic melanoma by single-cell RNA-seq. *Science* **352**, 189–96 (2016).
- 22. Shi, Q. *et al.* Single-cell proteomic chip for profiling intracellular signaling pathways in single tumor cells. *Proc. Natl. Acad. Sci. U. S. A.* **109**, 419–24 (2012).
- Imoukhuede, P. I. & Popel, A. S. Quantitative fluorescent profiling of VEGFRs reveals tumor cell and endothelial cell heterogeneity in breast cancer xenografts. *Cancer Med.* 3, 225–44 (2014).
- 24. Weddell, J. C. & Imoukhuede, P. I. Quantitative characterization of cellular membranereceptor heterogeneity through statistical and computational modeling. *PLoS One* **9**, e97271 (2014).
- 25. Weickhardt, A. J. *et al.* Vascular endothelial growth factor D expression is a potential biomarker of bevacizumab benefit in colorectal cancer. *Br. J. Cancer* **113**, 37–45 (2015).

- 26. Imoukhuede, P. I., Dokun, A. O., Annex, B. H. & Popel, A. S. Endothelial cell-by-cell profiling reveals the temporal dynamics of VEGFR1 and VEGFR2 membrane localization after murine hindlimb ischemia. *Am J Physiol Hear. Circ Physiol* **304**, H1085–H1093 (2013).
- 27. Chen, S. *et al.* in *Methods Mol Biol* (eds. Hurst Petrosko, S. & S. Day, E.) 117–138 (Springer New York, 2017).
- 28. Chen, S., Guo, X., Imarenezor, O. & Imoukhuede, P. I. Quantification of VEGFRs, NRP1, and PDGFRs on Endothelial Cells and Fibroblasts Reveals Serum, Intra-Family Ligand, and Cross-Family Ligand Regulation. *Cell. Mol. Bioeng.* **8**, 383–403 (2015).
- 29. Imoukhuede, P. I. & Popel, A. S. Quantification and cell-to-cell variation of vascular endothelial growth factor receptors. *Exp. Cell Res.* **317**, 955–965 (2011).
- 30. Imoukhuede, P. I. & Chen, S. Characterizing heterogeneity via single-cell receptor quantification. (2018).
- 31. Ferrara, N., Gerber, H.-P. & LeCouter, J. The biology of VEGF and its receptors. *Nat Med* **9**, 669–676 (2003).
- 32. Cadena, D. L. & Gill, G. N. Receptor tyrosine kinases. *FASEB J.* 6, 2332–7 (1992).
- 33. Sarabipour, S. & Hristova, K. Mechanism of FGF receptor dimerization and activation. *Nat. Commun.* **7**, 10262 (2016).
- 34. Mac Gabhann, F. & Popel, A. S. Dimerization of VEGF receptors and implications for signal transduction: A computational study. *Biophys Chem* **128**, 125–139 (2007).
- 35. Steinkamp, M. P. *et al.* erbB3 Is an Active Tyrosine Kinase Capable of Homo- and Heterointeractions. *Mol. Cell. Biol.* **34**, 965–977 (2014).
- Pennock, S., Kim, L. A. & Kazlauskas, A. Vascular Endothelial Cell Growth Factor A Acts via Platelet-Derived Growth Factor Receptor α To Promote Viability of Cells Enduring Hypoxia. *Mol. Cell. Biol.* 36, 2314–27 (2016).
- 37. Mamer, S. B. *et al.* Discovery of High-Affinity PDGF-VEGFR Interactions: Redefining RTK Dynamics. *Sci. Rep.* **7**, 16439 (2017).
- 38. King, C., Stoneman, M., Raicu, V. & Hristova, K. Fully quantified spectral imaging reveals in vivo membrane protein interactions. *Integr. Biol.* **8**, 216–229 (2016).
- 39. Sarabipour, S., Ballmer-Hofer, K. & Hristova, K. VEGFR-2 conformational switch in response to ligand binding. *Elife* **5**, e13876 (2016).

- Comps-Agrar, L., Dunshee, D. R., Eaton, D. L. & Sonoda, J. Unliganded Fibroblast Growth Factor Receptor 1 Forms Density-independent Dimers. *J. Biol. Chem.* 290, 24166–24177 (2015).
- 41. Chung, I. *et al.* Spatial control of EGF receptor activation by reversible dimerization on living cells. *Nature* **464**, 783–787 (2010).
- 42. Low-Nam, S. T. *et al.* ErbB1 dimerization is promoted by domain co-confinement and stabilized by ligand binding. *Nat Struct Mol Biol* **18**, 1244–1249 (2011).
- 43. Lin, C.-C. *et al.* Inhibition of Basal FGF Receptor Signaling by Dimeric Grb2. *Cell* **149**, 1514–1524 (2012).
- Ruch, C., Skiniotis, G., Steinmetz, M. O., Walz, T. & Ballmer-Hofer, K. Structure of a VEGF–VEGF receptor complex determined by electron microscopy. *Nat. Struct. Mol. Biol.* 14, 249–250 (2007).
- 45. Kazlauskas, A. PDGFs and their receptors. *Gene* **614**, 1–7 (2017).
- 46. Wu, F. T. H. *et al.* A systems biology perspective on sVEGFR1: its biological function, pathogenic role and therapeutic use. *J. Cell. Mol. Med.* **14**, 528–52 (2010).
- 47. Hubbard, S. R. & Miller, W. T. Receptor tyrosine kinases: mechanisms of activation and signaling. *Curr. Opin. Cell Biol.* **19**, 117–123 (2007).
- 48. Volinsky, N. & Kholodenko, B. N. Complexity of receptor tyrosine kinase signal processing. *Cold Spring Harb. Perspect. Biol.* **5**, a009043 (2013).
- 49. Ferrara, N. VEGF and the quest for tumour angiogenesis factors. *Nat Rev Cancer* **2**, 795–803 (2002).
- 50. Gabhann, F. Mac & Popel, A. S. Systems Biology of Vascular Endothelial Growth Factors. *Microcirculation* **15**, 715–738 (2008).
- 51. Kut, C., Mac Gabhann, F. & Popel, A. S. Where is VEGF in the body? A meta-analysis of VEGF distribution in cancer. *Br. J. Cancer* **97**, 978–85 (2007).
- 52. Imoukhuede, P. I. & Popel, A. S. Expression of VEGF receptors on endothelial cells in mouse skeletal muscle. *PLoS One* **7**, e44791 (2012).
- 53. Gelfand, M. V *et al.* Neuropilin-1 functions as a VEGFR2 co-receptor to guide developmental angiogenesis independent of ligand binding. *Elife* **3**, (2014).
- 54. Simons, M., Gordon, E. & Claesson-Welsh, L. Mechanisms and regulation of endothelial
VEGF receptor signalling. Nat. Rev. Mol. Cell Biol. 17, 611–625 (2016).

- 55. Hanahan, D. & Weinberg, R. A. Hallmarks of cancer: the next generation. *Cell* **144**, 646–74 (2011).
- 56. Shibuya, M. VEGF-VEGFR Signals in Health and Disease. *Biomol. Ther. (Seoul).* **22**, 1–9 (2014).
- 57. Jayson, G. C., Kerbel, R., Ellis, L. M. & Harris, A. L. Antiangiogenic therapy in oncology: current status and future directions. *Lancet* **388**, 518–529 (2016).
- Kuczynski, E. A. *et al.* Co-option of Liver Vessels and Not Sprouting Angiogenesis Drives Acquired Sorafenib Resistance in Hepatocellular Carcinoma. *J. Natl. Cancer Inst.* 108, (2016).
- 59. Holash, J. *et al.* Vessel cooption, regression, and growth in tumors mediated by angiopoietins and VEGF. *Science* **284**, 1994–8 (1999).
- Pennock, S., Kim, L. A. & Kazlauskas, A. Vascular Endothelial Cell Growth Factor A Acts via Platelet-Derived Growth Factor Receptor α To Promote Viability of Cells Enduring Hypoxia. *Mol. Cell. Biol.* 36, 2314–27 (2016).
- 61. Ball, S. G., Shuttleworth, C. A. & Kielty, C. M. Vascular endothelial growth factor can signal through platelet-derived growth factor receptors. *J. Cell Biol.* **177**, 489–500 (2007).
- 62. Greenberg, J. I. *et al.* A role for VEGF as a negative regulator of pericyte function and vessel maturation. *Nature* **456**, 809–13 (2008).
- 63. McGranahan, N. & Swanton, C. Clonal Heterogeneity and Tumor Evolution: Past, Present, and the Future. *Cell* **168**, 613–628 (2017).
- 64. Greaves, M. & Maley, C. C. Clonal evolution in cancer. *Nature* **481**, 306–313 (2012).
- 65. Greaves, M. Evolutionary determinants of cancer. *Cancer Discov.* 5, 806–20 (2015).
- 66. Pallini, R. *et al.* Expression of the stem cell marker CD133 in recurrent glioblastoma and its value for prognosis. *Cancer* **117**, 162–174 (2011).
- Schonberg, D. L., Lubelski, D., Miller, T. E. & Rich, J. N. Brain tumor stem cells: Molecular characteristics and their impact on therapy. *Mol. Aspects Med.* 39, 82–101 (2014).
- 68. Seymour, T., Nowak, A. & Kakulas, F. Targeting Aggressive Cancer Stem Cells in Glioblastoma. *Front. Oncol.* **5**, 159 (2015).

- 69. Lathia, J. D., Mack, S. C., Mulkearns-Hubert, E. E., Valentim, C. L. L. & Rich, J. N. Cancer stem cells in glioblastoma. *Genes Dev.* **29**, 1203–17 (2015).
- Mak, A. B. *et al.* CD133 Protein *N* -Glycosylation Processing Contributes to Cell Surface Recognition of the Primitive Cell Marker AC133 Epitope. *J. Biol. Chem.* 286, 41046– 41056 (2011).
- 71. Golebiewska, A. *et al.* Side population in human glioblastoma is non-tumorigenic and characterizes brain endothelial cells. *Brain* **136**, 1462–75 (2013).
- Bradshaw, A. *et al.* Cancer Stem Cell Hierarchy in Glioblastoma Multiforme. *Front. Surg.* 3, 21 (2016).
- 73. Singh, S. K. *et al.* Identification of human brain tumour initiating cells. *Nature* **432**, 396–401 (2004).
- 74. Beier, D. *et al.* CD133(+) and CD133(-) glioblastoma-derived cancer stem cells show differential growth characteristics and molecular profiles. *Cancer Res.* **67**, 4010–5 (2007).
- 75. Jain, R. K. Normalization of tumor vasculature: an emerging concept in antiangiogenic therapy. *Science* **307**, 58–62 (2005).
- 76. Aird, W. C. Molecular heterogeneity of tumor endothelium. *Cell Tissue Res.* **335**, 271–81 (2009).
- 77. Jain, R. K. et al. Angiogenesis in brain tumours. Nat. Rev. Neurosci. 8, 610–622 (2007).
- 78. Brennan, C. W. *et al.* The Somatic Genomic Landscape of Glioblastoma. *Cell* **155**, 462–477 (2013).
- 79. Verhaak, R. G. W. *et al.* Integrated genomic analysis identifies clinically relevant subtypes of glioblastoma characterized by abnormalities in PDGFRA, IDH1, EGFR, and NF1. *Cancer Cell* **17**, 98–110 (2010).
- 80. Patel, A. P. *et al.* Single-cell RNA-seq highlights intratumoral heterogeneity in primary glioblastoma. *Science* (80-.). **344**, 1396–1401 (2014).
- 81. Ellis, H. P. *et al.* Current Challenges in Glioblastoma: Intratumour Heterogeneity, Residual Disease, and Models to Predict Disease Recurrence. *Front. Oncol.* **5**, 251 (2015).
- 82. Snuderl, M. *et al.* Mosaic Amplification of Multiple Receptor Tyrosine Kinase Genes in Glioblastoma. *Cancer Cell* **20**, 810–817 (2011).
- 83. Dunn, G. P. *et al.* Emerging insights into the molecular and cellular basis of glioblastoma.

Genes Dev. 26, 756–784 (2012).

- 84. Szerlip, N. J. *et al.* Intratumoral heterogeneity of receptor tyrosine kinases EGFR and PDGFRA amplification in glioblastoma defines subpopulations with distinct growth factor response. *Proc. Natl. Acad. Sci. U. S. A.* **109**, 3041–6 (2012).
- 85. Simonson, A. B. & Schnitzer, J. E. Vascular proteomic mapping in vivo. *J. Thromb. Haemost.* **5**, 183–187 (2007).
- Feng, Y., Mitchison, T. J., Bender, A., Young, D. W. & Tallarico, J. A. Multi-parameter phenotypic profiling: using cellular effects to characterize small-molecule compounds. *Nat. Rev. Drug Discov.* 8, 567–578 (2009).
- 87. Taniguchi, Y. *et al.* Quantifying E. coli proteome and transcriptome with single-molecule sensitivity in single cells. *Science* **329**, 533–8 (2010).
- 88. Brennan, C. *et al.* Glioblastoma subclasses can be defined by activity among signal transduction pathways and associated genomic alterations. *PLoS One* **4**, e7752 (2009).
- 89. Hermanson, M. *et al.* Platelet-derived growth factor and its receptors in human glioma tissue: expression of messenger RNA and protein suggests the presence of autocrine and paracrine loops. *Cancer Res.* **52**, 3213–9 (1992).
- 90. Grecco, H. E., Schmick, M. & Bastiaens, P. I. H. Signaling from the living plasma membrane. *Cell* **144**, 897–909 (2011).
- 91. Lyer, S. et al. QuantiBRITE: a new standard for PE flourescence quantitation. In: White Paper. (Becton Dickinson Immunocytometry Systems, 1997).
- 92. Lee-Montiel, F. T. & Imoukhuede, P. I. Engineering quantum dot calibration standards for quantitative fluorescent profiling. *J. Mater. Chem. B* **1**, 6434 (2013).
- 93. Rocha-Martins, M., Njaine, B. & Silveira, M. S. Avoiding pitfalls of internal controls: validation of reference genes for analysis by qRT-PCR and Western blot throughout rat retinal development. *PLoS One* **7**, e43028 (2012).
- 94. Vigelsø, A. *et al.* GAPDH and β-actin protein decreases with aging, making Stain-Free technology a superior loading control in Western blotting of human skeletal muscle. *J. Appl. Physiol.* **118**, 386–94 (2015).
- 95. Baumgartner, R. *et al.* Identification and validation of platelet low biological variation proteins, superior to GAPDH, actin and tubulin, as tools in clinical proteomics. *J. Proteomics* **94**, 540–51 (2013).

- 96. Nguyen, R., Perfetto, S., Mahnke, Y. D., Chattopadhyay, P. & Roederer, M. Quantifying spillover spreading for comparing instrument performance and aiding in multicolor panel design. *Cytometry*. *A* **83**, 306–15 (2013).
- 97. Wheeless, L. L. *et al.* Measurement variability in DNA flow cytometry of replicate samples. *Cytometry* **10**, 731–738 (1989).
- 98. Chen, S. *et al.* Current State-of-the-Art and Future Directions in Systems Biology. *Progress and Communication in Sciences* **1**, 12–26 (2014).
- Weddell, J. C., Imoukhuede, P. I., Weddell, J. C. & Imoukhuede, P. I. in *Encyclopedia of Cardiovascular Research and Medicine* 659–676 (Elsevier, 2018). doi:10.1016/B978-0-12-809657-4.99548-6
- 100. Weddell, J. & Imoukhuede, P. I. Integrative meta-modeling identifies endocytic vesicles, late endosome and the nucleus as the cellular compartments primarily directing RTK signaling. *Integr. Biol.* (2017). doi:10.1039/C7IB00011A
- Wei, W. *et al.* Single-Cell Phosphoproteomics Resolves Adaptive Signaling Dynamics and Informs Targeted Combination Therapy in Glioblastoma. *Cancer Cell* 29, 563–573 (2016).
- 102. Sarkaria, J. N. *et al.* Identification of molecular characteristics correlated with glioblastoma sensitivity to EGFR kinase inhibition through use of an intracranial xenograft test panel. *Mol. Cancer Ther.* **6**, 1167–74 (2007).
- 103. Patenaude, A., Parker, J. & Karsan, A. Involvement of endothelial progenitor cells in tumor vascularization. *Microvasc. Res.* **79**, 217–223 (2010).
- 104. Moghadam, S. A., Abadi, A. M. & Mokhtari, S. Immunohistochemical analysis of CD34 expression in salivary gland tumors. *J. Oral Maxillofac. Pathol.* **19**, 30–3 (2015).
- Soares, A. B., Juliano, P. B., Araujo, V. C., Metze, K. & Altemani, A. Angiogenic switch during tumor progression of carcinoma ex-pleomorphic adenoma. *Virchows Arch.* 451, 65–71 (2007).
- 106. Naujokat, C. Monoclonal antibodies against human cancer stem cells. *Immunotherapy* **6**, 290–308 (2014).
- 107. Singh, S. K. *et al.* Identification of human brain tumour initiating cells. *Nature* **432**, 396–401 (2004).
- 108. Soeda, A. et al. The Evidence of Glioblastoma Heterogeneity. Sci. Rep. 5, 7979 (2015).

- Molina, E. S., Pillat, M. M., Moura-Neto, V., Lah, T. T. & Ulrich, H. Glioblastoma stemlike cells: approaches for isolation and characterization. *J. Cancer Stem Cell Res.* 1, 1 (2014).
- 110. Calabrese, C. *et al.* A Perivascular Niche for Brain Tumor Stem Cells. *Cancer Cell* **11**, 69–82 (2007).
- 111. Carmeliet, P. & Jain, R. K. Angiogenesis in cancer and other diseases. *Nature* **407**, 249–257 (2000).
- 112. Carmeliet, P. & Jain, R. K. Molecular mechanisms and clinical applications of angiogenesis. *Nature* **473**, 298–307 (2011).
- 113. Dudley, A. C. Tumor endothelial cells. *Cold Spring Harb. Perspect. Med.* **2**, a006536 (2012).
- 114. Ferrara, N. & Kerbel, R. S. Angiogenesis as a therapeutic target. *Nature* **438**, 967–74 (2005).
- Raftery, A. E. Bayesian Model Selection in Social Research. Social. Methodol. 25, 111 (1995).
- Huedo-Medina, T. B., Sánchez-Meca, J., Marín-Martínez, F. & Botella, J. Assessing heterogeneity in meta-analysis: Q statistic or I² index? *Psychol. Methods* 11, 193–206 (2006).
- 117. Amin, D. N., Hida, K., Bielenberg, D. R. & Klagsbrun, M. Tumor Endothelial Cells Express Epidermal Growth Factor Receptor (EGFR) but not ErbB3 and Are Responsive to EGF and to EGFR Kinase Inhibitors. *Cancer Res.* 66, 2173–2180 (2006).
- 118. Soda, Y. *et al.* Transdifferentiation of glioblastoma cells into vascular endothelial cells. *Proc. Natl. Acad. Sci. U. S. A.* **108,** 4274–80 (2011).
- 119. Lambrechts, D., Lenz, H.-J., de Haas, S., Carmeliet, P. & Scherer, S. J. Markers of response for the antiangiogenic agent bevacizumab. *J. Clin. Oncol.* **31**, 1219–30 (2013).
- 120. Wehland, M., Bauer, J., Magnusson, N. E., Infanger, M. & Grimm, D. Biomarkers for anti-angiogenic therapy in cancer. *Int. J. Mol. Sci.* **14**, 9338–64 (2013).
- 121. Andrae, J., Gallini, R. & Betsholtz, C. Role of platelet-derived growth factors in physiology and medicine. *Genes Dev.* **22**, 1276–312 (2008).
- 122. AbuSamra, D. B. *et al.* Not just a marker: CD34 on human hematopoietic stem/progenitor cells dominates vascular selectin binding along with CD44. *Blood Adv.* **1**, 2799–2816

(2017).

- 123. Nielsen, J. S. & McNagny, K. M. Novel functions of the CD34 family. *J. Cell Sci.* **121**, 3683–3692 (2008).
- Farahani, R. M. & Xaymardan, M. Platelet-Derived Growth Factor Receptor Alpha as a Marker of Mesenchymal Stem Cells in Development and Stem Cell Biology. *Stem Cells Int.* 2015, 362753 (2015).
- 125. Aguirre, A., Planell, J. A. & Engel, E. Dynamics of bone marrow-derived endothelial progenitor cell/mesenchymal stem cell interaction in co-culture and its implications in angiogenesis. Biochemical and Biophysical Research Communications **400**, (2010).
- 126. Werner, S., Hofschneider, P. H., Heldin, C. H., Ostman, A. & Roth, W. K. Cultured Kaposi's sarcoma-derived cells express functional PDGF A-type and B-type receptors. *Exp. Cell Res.* 187, 98–103 (1990).
- 127. Hermansson, M. *et al.* Endothelial cell hyperplasia in human glioblastoma: coexpression of mRNA for platelet-derived growth factor (PDGF) B chain and PDGF receptor suggests autocrine growth stimulation. *Proc. Natl. Acad. Sci. U. S. A.* **85**, 7748–52 (1988).
- Plate, K. H., Breier, G., Farrell, C. L. & Risau, W. Platelet-derived growth factor receptorbeta is induced during tumor development and upregulated during tumor progression in endothelial cells in human gliomas. *Lab. Invest.* 67, 529–34 (1992).
- Battegay, E. J., Rupp, J., Iruela-Arispe, L., Sage, E. H. & Pech, M. PDGF-BB modulates endothelial proliferation and angiogenesis in vitro via PDGF beta-receptors. *J. Cell Biol.* 125, 917–28 (1994).
- Raines, E. W., Bowen-Pope, D. F. & Ross, R. in *Peptide Growth Factors and Their Receptors I* 173–262 (Springer New York, 1991). doi:10.1007/978-1-4612-3210-0_5
- Kazlauskas, A. & DiCorleto, P. E. Cultured endothelial cells do not respond to a plateletderived growth-factor-like protein in an autocrine manner. *Biochim. Biophys. Acta* 846, 405–12 (1985).
- 132. Bowen-Pope, D. & Ross, R. Platelet-derived growth factor. II. Specific binding to cultured cells. *J. Biol. Chem.* **257**, 5161–5171 (1982).
- 133. Heldin, C. H., Westermark, B. & Wasteson, a. Specific receptors for platelet-derived growth factor on cells derived from connective tissue and glia. *Proc. Natl. Acad. Sci. U. S.* A. 78, 3664–8 (1981).

- Marx, M., Perlmutter, R. A. & Madri, J. A. Modulation of platelet-derived growth factor receptor expression in microvascular endothelial cells during in vitro angiogenesis. *J. Clin. Invest.* 93, 131–9 (1994).
- 135. Bar, R. S. *et al.* The effects of platelet-derived growth factor in cultured microvessel endothelial cells. *Endocrinology* **124**, 1841–8 (1989).
- 136. Liffers, K., Lamszus, K. & Schulte, A. EGFR Amplification and Glioblastoma Stem-Like Cells. *Stem Cells Int.* **2015**, 427518 (2015).
- 137. Mazzoleni, S. *et al.* Epidermal growth factor receptor expression identifies functionally and molecularly distinct tumor-initiating cells in human glioblastoma multiforme and is required for gliomagenesis. *Cancer Res.* **70**, 7500–13 (2010).
- 138. Griffero, F. *et al.* Different response of human glioma tumor-initiating cells to epidermal growth factor receptor kinase inhibitors. *J. Biol. Chem.* **284**, 7138–48 (2009).
- 139. Soeda, A. *et al.* Epidermal Growth Factor Plays a Crucial Role in Mitogenic Regulation of Human Brain Tumor Stem Cells. *J. Biol. Chem.* **283**, 10958–10966 (2008).
- Rao, C. R. Diversity and dissimilarity coefficients: A unified approach. *Theor. Popul. Biol.* 21, 24–43 (1982).
- 141. Pavoine, S. & Dolédec, S. The apportionment of quadratic entropy: a useful alternative for partitioning diversity in ecological data. *Environ. Ecol. Stat.* **12**, 125–138 (2005).
- 142. Botta-Dukát, Z. Rao's quadratic entropy as a measure of functional diversity based on multiple traits. *J. Veg. Sci.* **16**, 533–540 (2005).
- 143. Potts, S. J. *et al.* Evaluating tumor heterogeneity in immunohistochemistry-stained breast cancer tissue. *Lab. Invest.* **92**, 1342–57 (2012).
- 144. Gough, A. H. *et al.* Identifying and quantifying heterogeneity in high content analysis: application of heterogeneity indices to drug discovery. *PLoS One* **9**, e102678 (2014).
- Chao, C. *et al.* Patient-derived Xenografts from Colorectal Carcinoma: A Temporal and Hierarchical Study of Murine Stromal Cell Replacement. *Anticancer Res.* 37, 3405–3412 (2017).
- 146. Cloughesy, T. F., Cavenee, W. K. & Mischel, P. S. Glioblastoma: From Molecular Pathology to Targeted Treatment. *Annu. Rev. Pathol. Mech. Dis.* **9**, 1–25 (2014).
- 147. Weis, S. M. & Cheresh, D. A. Tumor angiogenesis: molecular pathways and therapeutic targets. *Nat Med* **17**, 1359–1370 (2011).

- 148. Jain, R. K. *et al.* Biomarkers of response and resistance to antiangiogenic therapy. *Nat. Rev. Clin. Oncol.* **6**, 327–38 (2009).
- 149. Heath, J. R., Ribas, A. & Mischel, P. S. Single-cell analysis tools for drug discovery and development. *Nat. Rev. Drug Discov.* **15**, 204–16 (2016).
- 150. Sorkin, A. & Duex, J. E. in *Current Protocols in Cell Biology* Chapter 15, Unit 15.14 (John Wiley & Sons, Inc., 2010).
- Wheeler, D. L., Dunn, E. F. & Harari, P. M. Understanding resistance to EGFR inhibitors[mdash]impact on future treatment strategies. *Nat Rev Clin Oncol* 7, 493–507 (2010).
- 152. Bergers, G. & Hanahan, D. Modes of resistance to anti-angiogenic therapy. *Nat. Rev. Cancer* **8**, 592–603 (2008).
- Shojaei, F. & Ferrara, N. Role of the microenvironment in tumor growth and in refractoriness/resistance to anti-angiogenic therapies. *Drug Resist. Updat.* 11, 219–30 (2008).
- 154. Liu, L. & Shi, G.-P. CD31: beyond a marker for endothelial cells. *Cardiovasc. Res.* **94,** 3–5 (2012).
- 155. Lee-Montiel, F. T., Li, P. & Imoukhuede, P. I. Quantum dot multiplexing for the profiling of cellular receptors. *Nanoscale* **7**, 18504–14 (2015).
- 156. Spitzer, M. H. & Nolan, G. P. Mass Cytometry: Single Cells, Many Features. *Cell* **165**, 780–791 (2016).
- 157. Hoppe, P. S., Coutu, D. L. & Schroeder, T. Single-cell technologies sharpen up mammalian stem cell research. *Nat. Cell Biol.* **16**, 919–927 (2014).
- 158. Niepel, M., Spencer, S. L. & Sorger, P. K. Non-genetic cell-to-cell variability and the consequences for pharmacology. *Curr. Opin. Chem. Biol.* **13**, 556–61 (2009).
- 159. Dar, R. D. & Weiss, R. Perspective: Engineering noise in biological systems towards predictive stochastic design. *APL Bioeng.* **2**, 020901 (2018).

CHAPTER 6 MULTIPLEXING ANGIOGENIC RECEPTOR QUANTIFICATION VIA QUANTUM DOTS¹

Clinical and biomedical research seeks single-cell quantification to better understand their roles in a complex, multi-cell environment. Recently, quantification of vascular endothelial growth factor receptors (VEGFRs) provided important insights into endothelial cells characteristics and response in tumor microenvironments. However, existing technologies for quantifying plasma membrane receptor tyrosine kinases (RTKs) lack multiplexing capabilities, limiting detailed characterization. Here, we use the unique spectral properties of quantum dots (Qdots) to optimize and dually quantify VEGFR1 and VEGFR2 on human umbilical vein endothelial cells (HUVECs). To enable this quantification, we reduce nonspecific binding between Qdotconjugated antibodies and cells via buffer optimization. Second, we identify optimal labeling conditions by examining Qdot-conjugated antibody binding to five receptors: VEGFRs (VEGFR1 and VEGFR2), their co-receptor Neuropilin1 (NRP1), and platelet-derived growth factor receptor (PDGFR α and PDGFR β). We establish that 800 – 20,000 is the dynamic range where accurate Qdot-enabled quantification can be achieved. Through these optimizations, we demonstrate measurement of 1,100 VEGFR1 and 6,900 VEGFR2 per HUVEC. We induce ~90% upregulation of VEGFR1 and ~30% downregulation of VEGFR2 concentration via 24 h VEGF-A165 treatment. We observe no change in VEGFR1 or VEGFR2 concentration with 24 h VEGF-B167treatment. We further apply the Qdots to analyze HUVEC heterogeneity and observe that 24 h VEGF-A165 treatment induces ~15% decrease in VEGFR2 heterogeneity, but little to no change in VEGFR1 heterogeneity 24 h VEGF-B167 induced little to no changes in either VEGFR1 or VEGFR2-dependent heterogeneity. Overall, we demonstrate experimental and analysis strategies for quantifying two or more RTKs at single-level using Qdots, which will provide new insights into biological systems.

¹ This chapter has been submitted for publication.

6.1 INTRODUCTION

Tyrosine kinase receptors (RTKs) are transmembrane proteins that initiate signaling events that regulate cell survival, proliferation, differentiation, and motility. Here we examine two receptor tyrosine kinase (RTK) families, vascular endothelial growth factor receptors (VEGFRs) and platelet-derived growth factor receptors (PDGFRs), which are both critical to angiogenesis, and are upregulated in many cancers ^{1–3}. Signals through endothelial VEGFRs and the neuropilin (NRP) co-receptors ^{4–8} induce the sprouting angiogenic hallmarks of cell proliferation and cell migration ⁹. PDGFR signaling regulates vascular stability ^{10,11}, stimulates wound-healing ^{12–14}, and induces vascular growth and reperfusion ¹⁵. We and others have also discovered that cross-family signaling between VEGFR and PDGFR exists ^{16–19}, which may affect tumor vascularization ²⁰. Therefore, the coordinated analysis of VEGF and PDGF signaling would advance our knowledge for RTK signaling mechanisms, while uncovering novel approaches for controlling cell survival, proliferation, differentiation, motility, and angiogenesis.

Quantitative flow (qFlow) cytometry offers a powerful tool for analyzing RTKs and other plasma membrane proteins on a single-cell level. It is an advancement upon traditional flow cytometry which converts signal to absolute protein concentrations via fluorescent calibration standards ^{21,22}. Absolute protein quantification is advantageous, because it detects protein variations across published studies, tissues, replicates, and instrument settings ^{23–28}. Moreover, qFlow cytometry advances systems biology, providing the quantitative data needed for computational studies ^{29–31}. For instance, a computational model that included ligand-receptor binding and receptor-internalization predicted that small increases (< 1,000 receptors/cell) in plasma membrane RTK concentration may double nuclear-based RTK signaling ³², which further implicates RTK concentration as a determinant of signal transduction. Furthermore, via a

systems biology paradigm, we predicted that anti-VEGF efficacy can depend on tumor endothelial VEGFR1 plasma membrane concentrations ³³. This prediction was further supported by a clinical post-hoc analysis of anti-VEGF efficacy in colorectal cancer ³⁴. This prediction was only possible with the accurate experimental data offered by qFlow cytometry.

A complete understanding of biological system response to environmental stimuli requires examining multiple signals at once; however few technologies provide absolute quantification³⁵. In fact, current qFlow cytometry regularly provides only one absolute quantitative readout at a time due to the wide emission spectra of organic fluorescent dyes, such as phycoerythrin (PE) ^{21,22}. In contrast, the narrow, largely symmetric emission range of Qdots³⁶ allows multiple Qdots to be monitored simultaneously ^{37–39}. Our lab recently optimized Qdot-antibody labeling for imaging VEGFRs on endothelial cells ³⁸ and engineered Qdot calibration standards for receptor quantification ⁴⁰. Here, we advance towards multiplexed absolute receptor quantification by combining these calibration standards and Qdot-antibody conjugates (**Fig. 21**). The RTK concentrations obtained using our method can be further analyzed to characterize and quantify the cell-by-cell heterogeneity within a biological system, and how it responds to environmental changes.

To achieve multiplexed absolute receptor quantification, we investigate and optimize Qdot labeling and analysis via two advancements. First, nonspecific binding is a hurdle for applying Qdot-conjugated antibodies ^{41,42}. Conventional blocking buffers, like PBS buffers containing BSA ^{7,21,43}, serum ⁴⁴, may not effectively minimize nonspecific binding without modification of Qdot materials ^{45,46}. So, we increase binding specificity by identifying an ideal blocking buffer. Second, researchers have reported that receptor densities affect antibody binding efficiency ^{47,48}.

However, no study has quantitatively characterized the effect of receptor density on binding between Qdot-antibody and receptors. So, we identify the dynamic range for Qdot receptor quantification. Ultimately, we present multiplexed VEGFR1 & VEGFR2 quantification and heterogeneity analysis, as few technologies provide us absolute quantification.

6.2 MATERIALS AND METHODS

Cell culture. The human umbilical vein endothelial cells (HUVECs) and human adult dermal fibroblasts (HDFs) were obtained from individual donors (Lonza, Allendale, NJ). HUVECs were cultured in Endothelial Cell Basal Medium (EBM)-2 supplemented by the Endothelial Cell Growth Medium (EGM)-2 SingleQuot Kit (Lonza). HDFs were cultured in Fibroblasts Growth Medium (FGM)-2 (Lonza) for the first passage; for passage 2 – 12, HDFs were cultured in DMEM/high-glucose medium supplemented with 5% (v/v) FBS and 1% (v/v) penicillin and streptomycin. The media was filtered using Nalgene[™] Rapid-Flow[™] Sterile Disposable Bottle Top Filters containing a polyethersulfone (PES) Membrane (Nalge Nunc International Corp., Rochester, NY). The pore size of the filter is 0.20 μm. The media was stored at 4 °C and warmed to 37 °C upon usage. All cultures were incubated at 37°C in a humidified 5% CO₂ incubator and then harvested to collect independent samples upon confluency. HUVECs were used up to passage 6 as described previously ⁴⁹ and HDFs were used up to passage 12.

Growth factor application. Near-confluent HUVECs were cultured in EGM-2 media supplemented with 25 ng/mL VEGF-A₁₆₅ and 25 ng/mL VEGF-B₁₆₇ (Shenandoah Biotechnology, Warmack, PA) prior to cell labeling ⁷.

Qdot calibration standards. The Qdot calibration standards were established as described previously ⁴⁰. Briefly, biotin-functionalized polystyrene beads (Spherotech, Lake Forest, IL)

were labeled with a dilution series of Innovator's Tool Kit (ITK)-streptavidin Qdots (Invitrogen, Carlsbad, CA) at 500, 5 000, 20 000, 60 000, and 100 000 Qdots per bead.

Qdots–antibody conjugation. We conjugated four different Qdots CdSe/ZnS nanocrystals with 525, 605, 655 and 705 nm emission (Invitrogen, Carlsbad, CA) to monoclonal human antibodies using the copper free click chemistry Qdot–antibody conjugation kits (catalog numbers: S10449, S10450, S10469, S10453, S10454). The copper-free click conjugation consists of three steps: antibody carbohydrate domain modification, azide attachment to the antibody, and conjugation with the DIBO-modified label. It relies on copper-free click chemistry to covalently link the label containing the dibenzocyclooxtyne (DIBO) moiety with the azide-modified antibody without reducing the protein. The molar ratio of antibody fragments to the Qdots at mixing is \sim 3: 1. Conjugates with bigger Qdots (Qdot605 and Qdot705) are concentrated by ultrafiltration.

Cell labeling. HUVECs and HDFs of 85 – 90% confluency were harvested from flasks and dissociated as described ^{16,21}. Dissociated cells were resuspended using BlockaidTM blocking solution (Thermo Fisher Scientific) for 30 min on ice prior to staining. Then we added 25 μ L aliquots of cell suspension containing 1 x 10⁵ cells to 5 ml polystyrene round-bottom tubes (BD Biosciences, NJ). Cells were labeled with either Qdot-conjugated antibodies or phycoerythrin (PE)-conjugated monoclonal antibodies. PE-conjugated antibodies were added at previously established concentrations to ensure saturated labeling ^{16,21}. Qdot-conjugated antibodies were titrated at various concentrations from 0.5 nM to 200 nM, and added to the cell suspension. Samples were protected from light and incubated for 40 minutes on ice in stain buffer (PBS containing 0.2% FBS and 0.05% sodium azide) to prevent receptor internalization. After two

washes with 2 mL of stain buffer, samples were resuspended in 300 μ L stain buffer and kept on ice until flow cytometric measurements.

Quantitative flow cytometry. Flow cytometry was performed on a LSR Fortessa (BD) Flow cytometer; BD FACSDIVA software was used for data acquisition, and FlowJo (TreeStar) software was used for data analysis. Upon analysis, 5 µg/mL Sytox Blue (for PE, Qdot655, and Qdot705) or Sytox Red (for Qdot525 and Qdot 605) live/dead cell stain (Invitrogen) was added to all samples. Tubes were vortexed immediately prior to placement in the flow cytometer. 8,000 - 10,000 live cells were collected from each tube. For each experiment we collected 2-4 tubes of Qdot-labeled cell samples for each receptor under each condition, i.e. control and treated. To subtract cell auto-fluorescence and background noise, 1-2 tubes of unlabeled cell samples were collected as well. Sytox blue was excited with a solid-state laser (407 nm) and its emission was collected using a 450/50 band-pass filter. Qdot 525, 605, 655, and Sytox Blue were excited with a 403-nm violet laser; Qdot 705 was excited with a 488-nm blue laser; Sytox Red was excited with a 640-nm red laser. Fluorescence of Qdot 525, 605, 655, 705, Sytox Blue, and Sytox Red were obtained with band filters at 525/30 nm, 610/20 nm, 670/30 nm, 695/40 nm, 450/50 nm, and 670/30 nm, respectively. Qdot calibration beads, along with the QuantiBRITE[™] PE calibration beads (Becton Dickinson), were analyzed by flow cytometry under the same setting as cell samples.

Statistical analysis: ensemble averages. For samples incubated with PE-conjugated antibodies, cell surface receptor concentration was quantified with PE calibration standards as previously described ^{7,16,21}. Similarly, a calibration curve that correlates Qdot fluorescence intensity with the number of Qdots was determined. Following flow cytometry, single beads were gated using

FlowJo (TreeStar) to ensure accurate fluorescence measurement (Fig. S9A). Histograms of fluorescence intensity of each Qdot655-conjugated beads population were plotted and geometric means of each histogram was measured (Fig. S9B). Qdot calibration curve was determined by fitting the geometric mean of fluorescence histograms of five bead populations that were conjugated with 500, 5 000, 20 000, 60 000, and 100 000 Qdots per bead respectively to a linear regression (Fig. S9C): y = mx+b, where $x = log_{10}$ (Number of Qdots per bead), $y = log_{10}$ (Qdot fluorescent intensity geometric mean). A calibration curve was generated for every type of Qdot used in each experiment. Number of Qdots per cell for each labeled and unlabeled sample was calculated using the equations below:

Number of
$$Qdots/cell_{labeled} = 10^{\frac{1}{m}(\log_{10}Qdot_{labeled} geometric mean-b)}$$

Number of $Qdots/cell_{unlabeled} = 10^{\frac{1}{m}(\log_{10}Qdot_{unlabeled} geometric mean-b)}$

where m and b were obtained from the Qdot calibration curve. Finally, ensemble receptor concentration was calculated by subtracting averaged number of Qdots/cell of all unlabeled samples from averaged number of Qdots/cell of all labeled samples.

Saturation study and K_D. Binding of the conjugated antibody to the receptor follows the law of mass action, therefore, equilibrium binding characteristics including the dissociation constant and maximum number of binding sites can be determined from saturation binding studies ^{48,50,51}. This method is often used to assess binding characteristics of radiolabeling ligands, or to determine whether a given drug acts as a competitive antagonist to a receptor of interest ⁵⁰. Here, we adapted he method to quantitatively characterize Qdot-conjugated antibody binding to

receptors. Briefly, ensemble-averaged number of bound antibody-receptor pairs and the corresponding antibody concentrations were fitted using OriginLab software to the equation

$$y = \frac{B_{max} \cdot x}{x + K_D}$$

where x is the concentration of Qdot or PE-conjugated antibody, B_{max} is the maximal density of binding sites for the conjugated antibody, and K_D is the equilibrium dissociation constant, which is a measure of the binding affinity (ratio of unbinding, off, to binding, on, rates) between conjugated antibody and the targeted receptor.

Statistical analysis: cell-by-cell analysis. A 2D histogram of RTK concentration within a cell population was calculated using cell-by-cell Qdot fluorescence intensity, similar to the established PE-based qFlow cytometry ²¹. Briefly, we derived a signal to noise ratio by dividing the weighted integral of the labeled cell signal with the unlabeled cell signal, and deriving the histogram of RTK concentration using this signal to noise ratio:

$$H_{RTK} = H_{signal} \left(1 - \frac{\sum Qdot_{unlabeled} / N_{unlabeled}}{\sum Qdot_{labeled} / N_{labeled}} \right)$$

where H_{signal} is the histogram of Qdot fluorescence from labeled cells, H_{RTK} is the histogram of RTK concentration, $Qdot_{labeled}$ and $Qdot_{unlabeled}$ are the fluorescence signal from labeled cells and unlabeled cells respectively, and N is the number of cells in a dataset (may contain multiple samples). The cell-by-cell analysis was performed using R (<u>www.r-project.org</u>), and the 2D histogram was plotted using OriginPro.

Heterogeneity analysis. To quantitatively characterize cell heterogeneity at receptor-level, we employed quadratic entropy (QE). QE provides a quantitative measure of the diversity of cellular phenotypes in cancer tissue sections for diagnostic applications ⁵² and drug discovery ^{53,54}. Each log-scaled cell-by-cell distribution was divided into 500 equally spaced bins. QE then sums the weighted differences of the means between two bins ^{55–57}.

Cell staining for TIRF imaging. 4.4×10^5 HUVECs and mouse 3T3 fibroblasts were seeded overnight on 14 mm No. 0 glass microwells coated manufacturer coated with poly-d-lysine attached to 35 mm diameter plastic dishes (Mattek, P35GC-0-14-C). Live cells were pre-fixed by adding 500 µL of 4% formaldehyde (Fisher; Lot: 122604) in phosphate buffered saline (PBS) to each dish for 2 min at room temperature (RT). The pre-fixation solution was replaced with 1 mL of 4% formaldehyde for 20 min at RT. Cells were washed with 1 mL PBS. Free aldehyde groups were quenched, to reduce binding of antibody by incubating at RT with 100 mM glycine (Fisher; Lot #131855) in PBS. Cells were washed twice with wash buffer containing PBS plus 0.1% bovine serum albumin (BSA), corresponding to 500 mg BSA + 500 mL PBS. A 1:25 antibody dilution in wash buffer was used and added to cells and incubated overnight at 4°C. Cells were washed twice with wash buffer, and DAPI was added to stain nuclei.

TIRF Microscopy. TIRF imaging was performed on an inverted microscope (Nikon, Eclipse Ti) configured for evanescent wave excitation at the Beckman Institute (University of Illinois at Urbana-Champaign). Briefly, the beam was generated by a 20 mW, 405 nm laser (Power Technology Inc.) was focused using a Nikon 60x 1.4 NA oil objective. TIR was introduced via a single mode fiber to produce a better spatial mode, and then guided through a fiber collimator and a Chroma dichroic mirror to finally focus at the back focal plane of the objective lens. In the

detection path, a Chroma Notch filter was used to eliminate laser stray light and emission was filtered via a YFP filter cube. Images were captured with an Evolve 512 Delta EMCCD camera (Photometrics), which was equipped single molecule imaging. A custom software program was developed in Labview and C++ for microscope control and image acquisition.

6.3 RESULTS AND DISCUSSION

6.3.1 Minimize non-specific binding by optimizing staining buffer

Ensuring antibody specificity is the first step to accurately quantify receptor density and characterize novel receptor-targeting biosensors. If the antibody binds to targeted receptors specifically, the number of antibody binding sites should be saturable, since a finite number of receptors is present on each cell plasma membrane ⁵⁸. To assess saturability, HDFs at a fixed concentration (4×10^6 cells/mL) were labeled with increasing concentrations of Qdot-conjugated anti-PDGFR α . As shown in Figure 22, binding sites on stain buffer-blocked HDFs for Qdot-conjugated PDGFR antibody did not saturate (as indicated by a steeper slope), whereas binding sites on HDFs incubated with Blockaid buffer (Thermo Fisher) saturated (as indicated by a plateau). At saturating concentrations of Qdot-conjugated PDGFR α antibody (~40 nM), we measured ~7,800 total binding sites per stain buffer-blocked HDF and ~4,000 specific binding sties per Blockaid-blocked HDF. Therefore, we reduced ~49% non-specific binding by using Blockaid buffer. These results showed that optimizing staining buffer has a significant impact on increasing saturability of Qdot-conjugated antibody.

We further assessed nonspecific binding by applying Qdot-conjugated human antibodies on cells of a different specie; therefore, the number of binding sites detected will be due to nonspecific binding between Qdot-antibodies and cells. We observed that nonspecific binding is a linear function of conjugated-antibody concentration when human PDGFR α antibody conjugated to Qdot655 (Qdot655-anti-hPDGFR α) is applied to mouse 3T3 fibroblasts (**Fig. 22**). We measured ~250 non-specific binding sites per mouse 3T3 cell at concentrations lower than 40 nM (where antibody plateaued on HDFs). We assessed VEGFR1, VEGFR2, and PDGFR β using the same method, and observed less than ~800 nonspecific binding sites per plasma membrane (**Fig. S10**). By comparison, mouse antibodies conjugated with PE measured ~3,200 mouse VEGFR1, ~21,500 mouse PDGFR α , and ~32,800 mouse PDGFR β , confirming that the low receptor counts measured via Qdot-antibody conjugates was not due to low concentrations of receptors present but high specificity of the antibodies.

Nonspecific binding poses an challenge to accurate receptor quantification on cell plasma membranes ³⁷. We showed that number of nonspecific binding sites increased as Qdot-antibody concentration increased, as nonspecific binding is usually linear with the labeling concentration ⁵⁸. More quantitatively, Healey et al. suggest that nonspecific binding for radioligand should be less than 50% of the total binding to be considered "not too high" ⁵⁹. Here, we reported ~2-25% of the total binding sites on HDFs for nonspecific binding sites between Qdot-conjugated human antibody and mouse 3T3 cells, ensuring specific binding between Qdot-conjugated antibodies and targeted receptors. The detected low non-specific binding along with our comparison between conventional stain buffer and optimized buffer BlockaidTM, suggests that optimizing buffer can increase antibody specificity and minimize nonspecific binding in Qdot-antibody staining.

6.3.2 Determining optimal labeling concentration and K_D from saturation binding studies Importantly, we verified antibody saturation to ensure accurate receptor quantification. We identified the saturating concentration (optimal staining concentration) at the plateau of the saturation curves of other Qdot-conjugated antibodies: VEGFR1=30 nM (**Fig. 23A**), VEGFR2=40 nM (**Fig. 23B**), NRP1=40 nM (**Fig. 23C**), PDGFR α =40 nM (**Fig. 23D**), and PDGFR β =200 nM (**Fig. 23E**). These results established the optimal staining concentrations for Qdot-conjugated antibodies. Our optimal staining concentrations for Qdot-conjugated antibodies aligned with previously established PE-conjugated antibodies (20 – 40 nM) ^{7,16,21} expect for PDGFR β . This similarity is expected, because same human monoclonal antibody clones were used in PE-conjugates and Qdot-conjugates. The higher Qdot-conjugate concentration required than PE-antibody to saturate PDGFR β may be due to the lower binding efficiency of larger Qdots, i.e. Qdot705. We will discuss the effect of steric hindrance and other implications when using larger Qdots in later sections.

We derived K_D of Qdot-conjugated antibodies from saturation binding curves (**Fig. 23A-E**): Qdot525-anti-VEGFR1=13.8 nM, Qdot605-anti-VEGFR2= 3.2 nM, Qdot655-anti-NRP1=9.1 nM, Qdot655-anti-PDGFR α = 5.0 nM, Qdot705-anti-PDGFR β = 64 nM. Previous study showed that monoclonal IgG antibody-receptor binding affinity can range from several pM to several nM ⁴⁸, which aligns with what we observed except for Qdot705-anti-PDGFR β .

 K_D is affected by several factors including the intrinsic binding affinity of the monoclonal IgG antibody to the targeted receptor ^{48,60}, fluorophore conjugation ⁶¹, antibody conjugate size and shape ^{60,62,63}, cell-surface receptor density ^{48,64}, and the valency of the conjugates or "degree of labeling", the mean number of fluorophores per antibody ^{42,61,64}. Some of these factors affect

each other, for example, if receptor density is low, the valency of the Qdot-antibody conjugates would matter less than if the receptor density is high. Another example is that if the size of Qdotantibody conjugates is too large, steric hindrance may prevent a conjugate from binding to multiple receptors even if the antibody: Qdot ratio is higher than 1.

6.3.3 Single-Qdot labeling provides accurate quantification of VEGFRs and PDGFR α Using the optimized buffer, and Qdot-antibody saturating concentrations (**Fig. 23A-E**), we quantified VEGFR1, VEGFR2, and NRP1 on HUVEC surface, and PDGFRs on HDF surface, as these receptors were previously located on the two cell types ^{7,16}. For VEGFR1, VEGFR2, and PDGFR α , Qdot-conjugated antibodies yielded similar quantification compared to PE-conjugated antibodies (p>0.05, **Fig. 23F**): 1,520±120 VEGFR1 per HUVEC, 3,030±110 VEGFR2 per HUVEC, and 4,440±190 PDGFR α per HDF. We observed ~2-fold lower NRP1 concentrations quantified using Qdot-conjugated antibodies than PE-conjugated antibodies, and ~14-fold lower for PDGFR β (p<0.01, **Fig. 23F**). The quantification disparity for NRP1 and PDGFR β may be due to Qdot multivalency, as our previous reports found a ~4:1 IgG antibodies to Qdot ratio per each Qdot565-antibody conjugate ³⁸. These results show that Qdot-antibody used in this study may be able to accurately measure receptor concentration within a dynamic range.

6.3.4 Impact of receptor density on Qdot-antibody quantification.

To determine the dynamic range for accurate Qdot-conjugated antibody measurement, we applied Qdot-conjugated antibodies on HUVECs having various plasma membrane NRP1 concentrations. We chose NRP1, because it is highly present on HUVECs (~50,000 NRP1/HUVEC ^{7,16}), and its concentration can be manipulated via serine protease exposure ^{7,16}. By incubating HUVECs with PBS buffer containing 0-80% TrypLE at 37 °C for 5 minutes, we

measured 5,900 – 50,900 NRP1 per HUVEC using PE-conjugated antibody. Qdot605conjugated NRP1 antibody can only measure ~60% NRP1 comparing to PE-conjugated antibody (p < 0.05, **Fig. 24**) on HUVECs having >20,000 plasma membrane NRP1/cell. Conversely, Qdot-anti-NRP1 bound to 85-100% plasma membrane NRP1 on HUVECs having fewer than 20,000 plasma membrane NRP1 per cell (p > 0.05, **Fig. 24**). Altogether, Qdot-conjugated antibodies can ensure accurate receptor quantification of plasma membranes having 20,000 receptors or lower.

The correlation between receptor concentration and Qdot-antibody labeling suggests that receptor clustering may also cause steric hindrance, and therefore, prevent Qdot-conjugated antibodies from binding to receptor targets. Clustering is indeed a common phenomenon among highly expressed membrane proteins ⁶⁵. For example, epidermal growth factor receptors (EGFRs) form clusters of 2-3 receptors on BAF/3 or COS7 cells, which express 50,000 EGFRs/cell ⁶⁶. In another study, A431 cells, a cancer cell line that express abnormally high levels of EGFR (2×10⁶ EGFRs/cell), form clusters of 10-15 receptors ⁶⁷. Similarly, we observed inhomogeneous NRP1 distribution on HUVEC (50,900 NRP1s/cell), indicating receptor clustering (Fig. S11A). In comparison, we observed low levels of autofluorescence of nonlabeled HUVECs (Fig. S11B) and low non-specific binding of conjugated human NRP1 antibodies on 3T3 mouse fibroblasts (Fig. 23C). These results confirmed that the small fluorescence puncta observed on stained HUVECs were not from autofluorescence or nonspecific binding. Therefore, when applying Qdot-conjugated antibodies, researchers should use similar methods as we have outlined, to ensure that their antibodies are specific and determine the measuring range for receptor density with their Qdot-conjugated antibodies.

In addition to receptor clustering, receptor concentration itself can affect antibody binding, and therefore may affect measurement accuracy. A study measured IgG antibody apparent binding affinity on tumor cells expressing varying levels of EGFR, and showed a correlation between antibody apparent affinity and receptor concentration ⁴⁸. Therefore, when developing antibody-based nanosensors, it is important to quantitatively determine the measuring limit to ensure accurate quantification. This study and our results also suggest that antibodies of different apparent binding affinities can be tested to achieve the optimal pairing between antibody and receptor concentration.

6.3.5 Impact of Qdot size on Qdot-antibody quantification

To investigate whether smaller Qdots can exceed the measuring limit in receptor concentration, we conjugated Qdots of different sizes to human PDGFR β antibody and applied these conjugates along with PE-conjugated PDGFR β antibody on HDFs (**Fig. S12A**). The emission maxima of Qdots are dependent on their size; the emission peak for large Qdots, like Qdot705, is in the red end of the spectra and smaller Qdots, like Qdot525, in the blue region ³⁶. Binding affinity of antibody conjugates decreases as Qdot size increases due to the reduced steric hindrance; in turn, higher binding efficacy of smaller Qdots leads to higher number of bound cell-surface receptors. Indeed, we detected ~4-fold higher bound membrane-PDGFR β using smaller Qdots, Qdot525 or Qdot605-conjugated antibody, than larger Qdot, Qdot705 (**Fig. S12B**). However, neither of the smaller Qdots we tested, Qdot525 or Qdot605 exceeded the measuring limit (20,000 receptors per cell), while PE-conjugated antibody measured ~78,800 PDGFR β per cell.

6.3.6 Multiplexed VEGFR quantification reveals receptor surface regulation by VEGF-A₁₆₅ but not VEGF-B₁₆₇

In order to validate the performance of our Qdot-antibody conjugates, we recapitulated the VEGFR regulation induced by 24 h VEGF-A₁₆₅-treatment⁷. We observed that 20-24 h VEGF-A₁₆₅-treatment induced an increase of ~990 VEGFR1s and a downregulation of ~2,300 VEGFR2s per HUVEC plasma membrane via Qdot525-anti-VEGFR1 and Qdot605-anti-VEGFR2 co-staining (p < 0.05, **Fig. 25A**). In contrast, long-term, 20 – 24 h VEGF-B₁₆₇ treatment did not induce significant changes in plasma membrane VEGFR1 and VEGFR2 concentrations using either PE-based or Qdot-based quantification (p >0.05, **Fig. 25B**). Together, these results demonstrate Qdot-conjugated antibodies can measure at least two receptors presented within the detection limit.

6.3.7 Multiplexed cell-by-cell analysis reveals changes in bivariate receptor distribution by VEGF-A₁₆₅ but not VEGF-B₁₆₇

In addition to regulating VEGFR1 and VEGFR2 concentrations, VEGF-A₁₆₅ induced changes in cell heterogeneity. We observed a shift of cell frequency distribution on a two-dimensional surface mapped by VEGFR1 and VEGFR2 plasma membrane concentrations, when HUVECs were treated with VEGF-A₁₆₅ but not VEGF-B₁₆₇ (**Fig. 26A**). To quantitatively understand these changes in cell heterogeneity at receptor-level, we employed quadratic entropy (QE). QE provides a quantitative measure of the diversity of cellular phenotypes in cancer tissue sections for diagnostic applications ⁵² and drug discovery ^{53,54}. QE requires equally spaced bins, here we chose 500 bins, from each log-scaled cell-by-cell distribution. QE then sums the weighted differences of the means between two bins ^{55–57}. Thus, QE is a measurement of the increase in

random variation in the cellular response. Prior to VEGF treatment, the QE of VEGFR1 and VEGFR2 was 0.06 and 0.07, respectively; QE of the dual-receptor distribution was 0.14 (**Fig. 26B**). VEGF-A₁₆₅ induced little to no change in VEGFR1 heterogeneity and ~15% decrease in QE of VEGFR2. The dual-receptor distribution of HUVECs showed ~8% decrease in QE when treated with VEGF-A₁₆₅. Here, we observe ~3-11-fold decrease in receptor QE on healthy ECs and human fibroblasts using Qdot-antibody conjugate compared to PE-based quantification. Previously, we have shown that healthy ECs and human fibroblasts in vitro have QE within 0.2– 0.7¹⁶. This discrepancy in QE may be due to that Qdot-antibody has a narrower measurable receptor density range than PE-conjugated antibody. Therefore, it is important to establish a baseline for multiplexed heterogeneity using standard cell lines.

Not surprisingly, VEGF-B₁₆₇-treated HUVECs show little to no changes in either VEGFR1 or VEGFR2-dependent heterogeneity. Changes in the dual-receptor heterogeneity highly correlates with VEGFR2 heterogeneity, despite of the changes of receptor density in both receptors. Together, this analysis revealed, for the first time, changes in endothelial heterogeneity defined by dual-receptor distribution of VEGFR1 and VEGFR2, upon VEGF activation.

Our observation of VEGF-A₁₆₅-induced downregulation of cell heterogeneity in VEGFR2 concentration aligns with previous findings ⁷, however, we did not observe a significant upregulation of cell heterogeneity in VEGFR1 concentration. This is likely due to the heterogeneity analysis done in this study, which is based on log-scaled receptor distribution, whereas a previous study was done on linear-scaled distributions.

6.4 CONCLUSIONS

In summary, we have established a receptor quantification method for multiplexing more than one receptor, using Qdots. In particular, we optimized and established the buffer to minimize non-specific antibody binding; we identified the dynamic range of our conjugates to be 800 - 20,000 receptors per cell; we confirmed significant changes in VEGFR1 & VEGFR2 concentration and heterogeneity when cells were treated with VEGF-A₁₆₅, and found no significant change induced by VEGF-B₁₆₇; and we validated the method by comparing our results to previously established PE-based qFlow cytometry.

The Qdot-based qFlow cytometry has several advantages and limitations among cytometry-based proteomic technologies. The wide usage of flow cytometry in both clinical and laboratory settings permits easier and cheaper access, than more advanced technologies, i.e. mass cytometry (CyTOF) 68 . In addition, the commercial availability of Qdot-antibody conjugation kits allows for easy development of protein-specific nanosensors that requires little to no training. The Qdot-based qFlow cytometry could potentially be expanded to 3 - 5 RTKs, depending on their plasma membrane concentrations; whereas CyTOF can provide measurement of over 40 parameters at single-cell level 68 . Despite these features for multiplexed measurements, both technologies are limited by their reliance on antibodies.

Here, we identified a 20,000 receptors per cell measuring limit for the commercial Qdots, so above this single-receptor concentration, PE-based qFlow cytometry is preferred over Qdots. Qdots should still enable heterogeneity measurements, as has previously been reported ^{69–71}, and multiplexed quantification may still be achieved by using a calibration standard for each Qdot. Given our previous reports of a 4:1 IgG antibodies to Qdot ratio per each Qdot-antibody

conjugate ³⁸, advancements towards higher-ranged Qdot-based multiplexed receptor quantification may still be achieved via emerging monovalent Qdot-antibody conjugates ⁷². Similarly, the development of smaller Qdot-antibody probes ^{73,74} has the potential to measure high-density receptors by overcoming steric hindrance and/or multivalency.

qFlow cytometry has enabled computational modeling ^{29,31}; multiplexed qFlow cytometry enabled by Qdots should improve the accuracy of such models, because model parameters such as RTK concentrations of each cell or cell population will be more accurate. For example, our previous model predicted tumor resistance with high VEGFR1 plasma membrane concentrations to anti-VEGF drugs ⁶; with multiplexed qFlow cytometry, models will be able to incorporate concentrations of other RTKs that may contribute to drug resistance for a more accurate prediction. Other researchers have shown that in quantitative models of RTK signaling, small changes in cell-specific parameters including receptor concentrations and rate constant for receptor activation can drastically influence modeling outcome, thus significantly affect the accuracy of model prediction ^{75,76}. Therefore, the cell-specific, quantitative receptor concentrations obtained via multiplexed qFlow cytometry will advance computational models for receptor signal transduction.

In order to apply this technology to clinical tissue samples, it is important to establish RTK concentration on cells under normal and diseased conditions in preclinical models. qFlow cytometry has been applied towards quantifying key angiogenic receptors (VEGFRs, PDGFRs, Tie receptors) on several preclinical models ^{4–7,16,54}, and multiplexed qFlow cytometry should be able to expand these quantitative data. Ideal models for using Qdot-based qFlow cytometry requires RTK concentrations to be lower than 20,000 receptors/cell. For VEGFR1 and VEGFR2,

human ECs *in vitro*⁷, mouse ECs from nonischemic limb and ischemic limb ^{4,5}, tumor cells and tumor ECs from breast cancer xenografts ⁶, and human EC-like cells in glioblastoma 39 xenografts ⁵⁴ are ideal models. Human EC-like cells in glioblastoma 39 xenografts ⁵⁴ showed PDGFRs and NRP1 concentrations within the ideal measuring range of Qdot-based qFlow cytometry. For RTKs having concentrations higher than 20,000 receptors per cell in these preclinical models, PE-conjugated antibodies are still applicable to be used alone or combined with Qdots. The application of quantitative Qdot probes to these preclinical models will provide us valuable insights into RTK profile in clinical models.

Other preclinical applications of our multiplexed RTK quantification method can range from biomaterials to tissue engineering. For example, stem cells have become a popular target for researchers due to its wide applications including cancer therapy ^{77,78} and regenerative biomaterials ^{79,80}; however, there is a critical need for better identify and isolate stem cells from tissues ^{81,82}. Our multiplexed qFlow cytometry can help establish a "barcode" of RTK plasma membrane concentrations on stem cells may help to identify novel markers, aiding in the isolation and understanding of these stem cells. Multiplexed RTK quantification can improve other tissue engineering applications: cell RTK concentrations can be used to assess the functionality of biomaterials such as such as 3D hydrogel matrices ⁸³, carbon nanotubes ⁸⁴, to better direct tissue engineering.



Figure 21. An overview of the workflow for characterizing plasma membrane VEGFR1 and VEGFR2 concentrations and heterogeneity on HUVECs.



Figure 22. Saturation binding studies show difference between specific versus nonspecific cell labeling. Saturation curves of Qdot655-anti-PDGFR α on PDGFR α -expressing human dermal fibroblasts pre-incubated with Blockaid buffer vs. stain buffer (PBS supplemented with 0.2% BSA and 0.05% sodium azide). Blockaid buffer reduced nonspecific Qdot-cell labeling, demonstrated by a saturated curve. Nonspecific binding was characterized by applying Qdot655-anti-PDGFR α on mouse 3T3 fibroblasts, resulting in a linear-like curve.



Figure 23. Quantification of VEGFRs, NRP1, and PDGFRs concentration on HUVECs and HDFs and Qdot-antibody binding affinity. (A)-(E) K_D , the equilibrium dissociation constant for Qdot-antibody binding to cell surface receptors, and B_{max} , maximal density of binding sites for the conjugated antibody were determined by fitting the saturation binding data to the Lineweaver-Burk equation (see Supporting Information, Methods and Materials). Optimal Qdot-antibody concentrations (> 2-fold of K_D) were indicated by arrows. (F) Single staining of Qdot-antibodies provides accurate quantification for plasma membrane VEGFRs on HUVECs and PDGFR α on HDFs when compared with previously established phycoerythrin (PE)-antibodies. Significance tests were conducted using ANOVA where *** indicates p<0.001.



Figure 24. Receptor concentration affects accurate quantification of Qdot-conjugated antibodies. HUVECs were pretreated with increasing concentrations of TrypLE, an enzymatic cell dissociation buffer that cleaves NRP1 antibody epitope. Quantified NRP1 concentrations via Qdot-antibody was compared with previously established phycoerythrin (PE)-antibody. Significance tests were conducted using ANOVA where * indicates p<0.05 and *** indicates p<0.001.



Figure 25. Dual-staining of Qdot-conjugated VEGFR1 and VEGFR2 antibodies on HUVECs yields similar receptor concentrations as phycoerythrin (PE)-antibodies. (A) Qdot-based qFlow cytometry accurately quantify changes in VEGFR1 & VEGFR2 membrane plasma concentrations on VEGF-A₁₆₅treated HUVECs. (B) 2.6 nM VEGF-B₁₆₇ did not cause significant changes in VEGFR1 or VEGFR2 levels. Significance tests were conducted using ANOVA where * indicates p<0.05 and *** indicates p<0.001; NS: Not significant.



Figure 26. Characterizing receptor heterogeneity on single HUVECs treated by VEGF-A₁₆₅ and VEGF-B₁₆₇. (A) 3D cell-by-cell analysis shows increase in VEGFR1 & VEGFR2 heterogeneity on HUVECs treated with VEGF-A₁₆₅ and no significant change induced by VEGF-B₁₆₇ treatment. (B) Quantification of cell heterogeneity using quadratic entropy. Significance tests were conducted using ANOVA where * indicates p<0.05 and *** indicates p<0.001.

Supplemental Figures



Supplemental Figure 9. Qdot calibration curve. (A) Gated single beads conjugated with Qdot655 on a forward scatter vs. side scatter plot. (B) Histograms of fluorescence intensity from five groups of beads conjugated with 500, 5000, 20 000, 60 000, and 100 000 Qdot655s respectively. (C) Linear regression of log₁₀(geometric mean of fluorescence of Qdot655-conjugated beads) plotted against log₁₀(number of Qdot655 per bead).



Supplemental Figure 10. Characterization of nonspecific binding of Qdot-conjugated antibodies. Phycoerythrin (PE)-conjugated human antibody on mouse 3T3 fibroblasts showed little to no binding cites and high specificity of the antibody conjugates, while Qdot-conjugate human antibody showed maximum ~800 non-specific binding sites per 3T3 cell (indicated by red dashed line). The ensemble average of each receptor were mean of receptor concentrations measured at 10 nM, 25 nM, and 50 nM of Qdot-antibody conjugates.


Supplemental Figure 11. Inhomogeneous NRP1 distribution on HUVEC plasma membranes indicates receptor clustering. (A) Total internal reflection fluorescence (TIRF) imaging of fixed HUVECs stained with PE-conjugate antibodies showed NRP1 clustering, indicated by inhomogeneous PE distribution on cell plasma membrane. (B) TIRF imaging of non-labeled HUVECs showed little to no autofluorescence. (C) TIRF imaging of 3T3 mouse fibroblasts stained with PE-conjugated human NRP1 antibody showed low level of fluorescence, indicating high specificity of conjugated NRP1 antibody.



Supplemental Figure 12. Impact of Qdot size on cell-surface receptor quantification. (A) Size comparison between Qdots and PE used to conjugate with anti-PDGFR β . (B) Saturation binding curves of PDGFR β antibody conjugated with Qdots of varying sizes resulted in different binding affinities and receptor quantification.

6.6 REFERENCES

- 1. Ferrara, N. VEGF and the quest for tumour angiogenesis factors. *Nat Rev Cancer* **2**, 795–803 (2002).
- 2. Stefanini, M. O., Wu, F. T. H., Mac Gabhann, F. & Popel, A. S. A compartment model of VEGF distribution in blood, healthy and diseased tissues. *BMC Syst. Biol.* **2**, 77 (2008).
- 3. Kut, C., Mac Gabhann, F. & Popel, A. S. Where is VEGF in the body? A meta-analysis of VEGF distribution in cancer. *Br. J. Cancer* **97**, 978–85 (2007).
- 4. Imoukhuede, P. I. & Popel, A. S. Expression of VEGF receptors on endothelial cells in mouse skeletal muscle. *PLoS One* **7**, e44791 (2012).
- 5. Imoukhuede, P. I., Dokun, A. O., Annex, B. H. & Popel, A. S. Endothelial cell-by-cell profiling reveals the temporal dynamics of VEGFR1 and VEGFR2 membrane localization after murine hindlimb ischemia. *Am J Physiol Hear. Circ Physiol* **304**, H1085–H1093 (2013).
- 6. Imoukhuede, P. I. & Popel, A. S. Quantitative fluorescent profiling of VEGFRs reveals tumor cell and endothelial cell heterogeneity in breast cancer xenografts. *Cancer Med.* **3**, 225–44 (2014).
- 7. Imoukhuede, P. I. & Popel, A. S. Quantification and cell-to-cell variation of vascular endothelial growth factor receptors. *Exp. Cell Res.* **317**, 955–965 (2011).
- 8. Gelfand, M. V *et al.* Neuropilin-1 functions as a VEGFR2 co-receptor to guide developmental angiogenesis independent of ligand binding. *Elife* **3**, (2014).
- 9. Simons, M., Gordon, E. & Claesson-Welsh, L. Mechanisms and regulation of endothelial VEGF receptor signalling. *Nat. Rev. Mol. Cell Biol.* **17**, 611–625 (2016).
- 10. Carmeliet, P. Mechanisms of angiogenesis and arteriogenesis. *Nat. Med.* **6**, 389–95 (2000).
- 11. Zhang, J. *et al.* Differential roles of PDGFR-alpha and PDGFR-beta in angiogenesis and vessel stability. *FASEB J.* **23**, 153–63 (2009).
- 12. Greenhalgh, D. G., Sprugel, K. H., Murray, M. J. & Ross, R. PDGF and FGF stimulate wound healing in the genetically diabetic mouse. *Am. J. Pathol.* **136**, 1235–46 (1990).
- Lynch, S. E., Nixon, J. C., Colvin, R. B. & Antoniades, H. N. Role of platelet-derived growth factor in wound healing: synergistic effects with other growth factors. *Proc. Natl. Acad. Sci. U. S. A.* 84, 7696–700 (1987).

- 14. Pierce, G. F., Mustoe, T. A., Altrock, B. W., Deuel, T. F. & Thomason, A. Role of platelet-derived growth factor in wound healing. *J. Cell. Biochem.* **45**, 319–65 (1991).
- 15. Brown, L. F. *et al.* Expression of vascular permeability factor (vascular endothelial growth factor) and its receptors in breast cancer. *Hum. Pathol.* **26**, 86–91 (1995).
- 16. Chen, S., Guo, X., Imarenezor, O. & Imoukhuede, P. I. Quantification of VEGFRs, NRP1, and PDGFRs on Endothelial Cells and Fibroblasts Reveals Serum, Intra-Family Ligand, and Cross-Family Ligand Regulation. *Cell. Mol. Bioeng.* **8**, 383–403 (2015).
- Pennock, S., Kim, L. A. & Kazlauskas, A. Vascular Endothelial Cell Growth Factor A Acts via Platelet-Derived Growth Factor Receptor α To Promote Viability of Cells Enduring Hypoxia. *Mol. Cell. Biol.* 36, 2314–27 (2016).
- 18. Ball, S. G., Shuttleworth, C. A. & Kielty, C. M. Vascular endothelial growth factor can signal through platelet-derived growth factor receptors. *J. Cell Biol.* **177**, 489–500 (2007).
- 19. Mamer, S. B. *et al.* Discovery of High-Affinity PDGF-VEGFR Interactions: Redefining RTK Dynamics. *Sci. Rep.* **7**, 16439 (2017).
- 20. Greenberg, J. I. *et al.* A role for VEGF as a negative regulator of pericyte function and vessel maturation. *Nature* **456**, 809–13 (2008).
- 21. Chen, S. *et al.* in *Methods Mol Biol* (eds. Hurst Petrosko, S. & S. Day, E.) 117–138 (Springer New York, 2017).
- 22. Lyer, S. et al. QuantiBRITE: a new standard for PE flourescence quantitation. In: White Paper. (Becton Dickinson Immunocytometry Systems, 1997).
- 23. Rocha-Martins, M., Njaine, B. & Silveira, M. S. Avoiding pitfalls of internal controls: validation of reference genes for analysis by qRT-PCR and Western blot throughout rat retinal development. *PLoS One* **7**, e43028 (2012).
- Vigelsø, A. *et al.* GAPDH and β-actin protein decreases with aging, making Stain-Free technology a superior loading control in Western blotting of human skeletal muscle. *J. Appl. Physiol.* 118, 386–94 (2015).
- 25. Baumgartner, R. *et al.* Identification and validation of platelet low biological variation proteins, superior to GAPDH, actin and tubulin, as tools in clinical proteomics. *J. Proteomics* **94**, 540–51 (2013).
- 26. Keyt, B. A. *et al.* Identification of vascular endothelial growth factor determinants for binding KDR and FLT-1 receptors. Generation of receptor-selective VEGF variants by

site-directed mutagenesis. J. Biol. Chem. 271, 5638–46 (1996).

- 27. Nguyen, R., Perfetto, S., Mahnke, Y. D., Chattopadhyay, P. & Roederer, M. Quantifying spillover spreading for comparing instrument performance and aiding in multicolor panel design. *Cytometry*. A **83**, 306–15 (2013).
- 28. Wheeless, L. L. *et al.* Measurement variability in DNA flow cytometry of replicate samples. *Cytometry* **10**, 731–738 (1989).
- 29. Chen, S. *et al.* Current State-of-the-Art and Future Directions in Systems Biology. *Progress and Communication in Sciences* **1**, 12–26 (2014).
- 30. Weddell, J. C., Chen, S. & Imoukhuede, P. I. VEGFR1 promotes cell migration and proliferation through PLCγ and PI3K pathways. *npj Syst. Biol. Appl.* **4**, 1 (2018).
- Weddell, J. C., Imoukhuede, P. I., Weddell, J. C. & Imoukhuede, P. I. in *Encyclopedia of Cardiovascular Research and Medicine* 659–676 (Elsevier, 2018). doi:10.1016/B978-0-12-809657-4.99548-6
- 32. Weddell, J. & Imoukhuede, P. I. Integrative meta-modeling identifies endocytic vesicles, late endosome and the nucleus as the cellular compartments primarily directing RTK signaling. *Integr. Biol.* (2017). doi:10.1039/C7IB00011A
- 33. Weddell, J. C. & Imoukhuede, P. I. Quantitative characterization of cellular membranereceptor heterogeneity through statistical and computational modeling. *PLoS One* **9**, e97271 (2014).
- 34. Weickhardt, A. J. *et al.* Vascular endothelial growth factor D expression is a potential biomarker of bevacizumab benefit in colorectal cancer. *Br. J. Cancer* **113**, 37–45 (2015).
- 35. Schulenberg, B. & Patton, W. F. in *Handbook of Proteomic Methods* 107–115 (Humana Press, 2003). doi:10.1007/978-1-59259-414-6_8
- 36. Bruchez, M., Moronne, M., Gin, P., Weiss, S. & Alivisatos, A. P. Semiconductor nanocrystals as fluorescent biological labels. *Science (80-.).* **281,** 2013–2016 (1998).
- Chattopadhyay, P. K. *et al.* Quantum dot semiconductor nanocrystals for immunophenotyping by polychromatic flow cytometry. *Nat. Med.* 12, 972–7 (2006).
- 38. Lee-Montiel, F. T., Li, P. & Imoukhuede, P. I. Quantum dot multiplexing for the profiling of cellular receptors. *Nanoscale* **7**, 18504–14 (2015).
- 39. Kairdolf, B. A. *et al.* Semiconductor quantum dots for bioimaging and biodiagnostic applications. *Annu. Rev. Anal. Chem. (Palo Alto. Calif).* **6**, 143–62 (2013).

- 40. Lee-Montiel, F. T. & Imoukhuede, P. I. Engineering quantum dot calibration standards for quantitative fluorescent profiling. *J. Mater. Chem. B* **1**, 6434 (2013).
- 41. Diagaradjane, P. *et al.* Imaging Epidermal Growth Factor Receptor Expression In vivo: Pharmacokinetic and Biodistribution Characterization of a Bioconjugated Quantum Dot Nanoprobe. *Clin. Cancer Res.* **14**, 731–741 (2008).
- 42. Pathak, S., Davidson, M. C. & Silva, G. A. Characterization of the functional binding properties of antibody conjugated quantum dots. *Nano Lett.* **7**, 1839–45 (2007).
- 43. Roxworthy, B. J. *et al.* Plasmonic optical trapping in biologically relevant media. *PLoS One* **9**, e93929 (2014).
- 44. Buchwalow, I., Samoilova, V., Boecker, W. & Tiemann, M. Non-specific binding of antibodies in immunohistochemistry: fallacies and facts. *Scientific Reports* **1**, (2011).
- 45. Kairdolf, B. A., Mancini, M. C., Smith, A. M. & Nie, S. Minimizing nonspecific cellular binding of quantum dots with hydroxyl-derivatized surface coatings. *Anal. Chem.* **80**, 3029–34 (2008).
- 46. Zhang, B., Wang, X., Liu, F., Cheng, Y. & Shi, D. Effective reduction of nonspecific binding by surface engineering of quantum dots with bovine serum albumin for cell-targeted imaging. *Langmuir* **28**, 16605–13 (2012).
- 47. Caré, B. R. *et al.* Impact of receptor clustering on ligand binding. *BMC Syst. Biol.* **5**, 48 (2011).
- 48. Zhou, Y. *et al.* Impact of intrinsic affinity on functional binding and biological activity of EGFR antibodies. *Mol. Cancer Ther.* **11**, 1467–76 (2012).
- 49. Willett, C. G. et al. Direct evidence that the VEGF-specific antibody bevacizumab has antivascular effects in human rectal cancer. Nature medicine **10**, (2004).
- 50. Hein, P. *et al.* in *Practical Methods in Cardiovascular Research* 723–783 (Springer-Verlag, 2005). doi:10.1007/3-540-26574-0_37
- 51. Hulme, E. C. & Trevethick, M. A. Ligand binding assays at equilibrium: validation and interpretation. *Br. J. Pharmacol.* **161**, 1219–37 (2010).
- 52. Potts, S. J. *et al.* Evaluating tumor heterogeneity in immunohistochemistry-stained breast cancer tissue. *Lab. Invest.* **92**, 1342–57 (2012).
- 53. Gough, A. H. *et al.* Identifying and quantifying heterogeneity in high content analysis: application of heterogeneity indices to drug discovery. *PLoS One* **9**, e102678 (2014).

- Chen, S., Le, T., Harley, B. A. C. & Imoukhuede, P. I. Characterizing Glioblastoma Heterogeneity via Single-Cell Receptor Quantification. *Front. Bioeng. Biotechnol.* 6, 92 (2018).
- 55. Rao, C. R. Diversity and dissimilarity coefficients: A unified approach. *Theor. Popul. Biol.* **21**, 24–43 (1982).
- 56. Pavoine, S. & Dolédec, S. The apportionment of quadratic entropy: a useful alternative for partitioning diversity in ecological data. *Environ. Ecol. Stat.* **12**, 125–138 (2005).
- 57. Botta-Dukát, Z. Rao's quadratic entropy as a measure of functional diversity based on multiple traits. *J. Veg. Sci.* **16**, 533–540 (2005).
- 58. Limbird, L. E. *Cell surface receptors : a short course on theory & amp; methods.* (Springer Science+Business Media, 2005).
- Healey, G. F. Fitting models to biological data using linear and nonlinear regression Motulsky H, Christopoulos A (2004)ISBN 0195171802; 352 pages; £18.50 Oxford University Press; *Pharm. Stat.* 4, 147–148 (2005).
- 60. Fujimori, K., Covell, D. G., Fletcher, J. E. & Weinstein, J. N. Modeling analysis of the global and microscopic distribution of immunoglobulin G, F(ab')2, and Fab in tumors. *Cancer Res.* **49**, 5656–63 (1989).
- 61. Gnes Szabó, A. ´*et al.* The Effect of Fluorophore Conjugation on Antibody Affinity and the Photophysical Properties of Dyes. *Biophysj* **114**, 688–700 (2018).
- 62. Han, H.-S. *et al.* Quantum dot/antibody conjugates for in vivo cytometric imaging in mice. *Proc. Natl. Acad. Sci. U. S. A.* **112**, 1350–5 (2015).
- Francis, J. E., Mason, D. & Lévy, R. Evaluation of quantum dot conjugated antibodies for immunofluorescent labelling of cellular targets. *Beilstein J. Nanotechnol.* 8, 1238–1249 (2017).
- Rhoden, J. J., Dyas, G. L. & Wroblewski, V. J. A Modeling and Experimental Investigation of the Effects of Antigen Density, Binding Affinity, and Antigen Expression Ratio on Bispecific Antibody Binding to Cell Surface Targets. J. Biol. Chem. 291, 11337– 47 (2016).
- 65. Grecco, H. E., Schmick, M. & Bastiaens, P. I. H. Signaling from the living plasma membrane. *Cell* **144**, 897–909 (2011).
- 66. Clayton, A. H. A. et al. Ligand-induced dimer-tetramer transition during the activation of

the cell surface epidermal growth factor receptor-A multidimensional microscopy analysis. *J. Biol. Chem.* **280**, 30392–9 (2005).

- Zidovetzki, R., Yarden, Y., Schlessinger, J. & Jovin, T. M. Rotational diffusion of epidermal growth factor complexed to cell surface receptors reflects rapid microaggregation and endocytosis of occupied receptors. *Proc. Natl. Acad. Sci. U. S. A.* 78, 6981–5 (1981).
- 68. Spitzer, M. H. & Nolan, G. P. Mass Cytometry: Single Cells, Many Features. *Cell* **165**, 780–791 (2016).
- 69. Xing, Y. *et al.* Bioconjugated quantum dots for multiplexed and quantitative immunohistochemistry. *Nat. Protoc.* **2**, 1152–1165 (2007).
- 70. Liu, J. *et al.* Molecular Mapping of Tumor Heterogeneity on Clinical Tissue Specimens with Multiplexed Quantum Dots. *ACS Nano* **4**, 2755–2765 (2010).
- 71. Zhang, R. *et al.* Probing the Heterogeneity of Protein Kinase Activation in Cells by Superresolution Microscopy. *ACS Nano* **11**, 249–257 (2017).
- 72. Howarth, M. *et al.* Monovalent, reduced-size quantum dots for imaging receptors on living cells. *Nat. Methods* **5**, 397–399 (2008).
- 73. Yang, L. *et al.* Single Chain Epidermal Growth Factor Receptor Antibody Conjugated Nanoparticles for in vivo Tumor Targeting and Imaging. *Small* **5**, 235–243 (2009).
- 74. Cai, E. *et al.* Stable small quantum dots for synaptic receptor tracking on live neurons. *Angew. Chem. Int. Ed. Engl.* **53**, 12484–8 (2014).
- 75. Linderman, J. J. Modeling of G-protein-coupled receptor signaling pathways. *J. Biol. Chem.* **284**, 5427–31 (2009).
- Kinzer-Ursem, T. L. & Linderman, J. J. Both Ligand- and Cell-Specific Parameters Control Ligand Agonism in a Kinetic Model of G Protein–Coupled Receptor Signaling. *PLoS Comput. Biol.* 3, e6 (2007).
- 77. Bao, S. *et al.* Glioma stem cells promote radioresistance by preferential activation of the DNA damage response. *Nature* **444**, 756–760 (2006).
- 78. Nadkarni, A. *et al.* ATM inhibitor KU-55933 increases the TMZ responsiveness of only inherently TMZ sensitive GBM cells. *J. Neurooncol.* **110**, 349–357 (2012).
- 79. Nissar, A. A., Martowirogo, A. & Gilbert, P. M. Targeting the stem cell niche with regenerative biomaterials. *Curr. Opin. Solid State Mater. Sci.* **20**, 180–192 (2016).

- 80. Choi, J. S. & Harley, B. A. C. Marrow-inspired matrix cues rapidly affect early fate decisions of hematopoietic stem and progenitor cells. *Sci. Adv.* **3**, e1600455 (2017).
- 81. Singh, S. K. *et al.* Identification of human brain tumour initiating cells. *Nature* **432**, 396–401 (2004).
- 82. Beier, D. *et al.* CD133(+) and CD133(-) glioblastoma-derived cancer stem cells show differential growth characteristics and molecular profiles. *Cancer Res.* **67**, 4010–5 (2007).
- Zustiak, S. P. & Leach, J. B. Hydrolytically degradable poly(ethylene glycol) hydrogel scaffolds with tunable degradation and mechanical properties. *Biomacromolecules* 11, 1348–57 (2010).
- 84. Imaninezhad, M. *et al.* Cell Attachment and Spreading on Carbon Nanotubes Is Facilitated by Integrin Binding. *Front. Bioeng. Biotechnol.* **6**, 129 (2018).

APPENDIX A: CURRENT STATE-OF-THE-ART AND FUTURE DIRECTIONS IN SYSTEMS BIOLOGY

This Chapter has been published in *Progress in Communication in Sciences* (2014), Volume 1, Issue 1, pp12-26. We conducted an extensive review on current experimental approaches and computational approaches used in the field of systems biology, with an emphasis on its application in studying angiogenesis and vessel formation. We also included a section of how systems biology can advance personalized medicine, using examples of several diseases that present to be challenging to treat.

Current State-of-The-Art and Future Directions in Systems Biology

Abstract—Systems Biology offers the promise of decoding genetic information, optimizing pharmaceutical design, and aiding in the development of precision medicine. These advances require the bimodal approach of deriving information from experimental data and integrating such information via computational modeling. However, choosing an appropriate experimental assay and computational model is paramount to the accuracy and relevancy of the output. Here, we delve into the fundamental concept of several commonly used modeling approaches, their advantages and limitations, as well as potential applications. We review and compare experimental assays used in systems biology, based on the throughput, simplicity and possibility for quantification. In addition, we review current experimental models used in conjunction with assays to provide parameters and/or validation for computational modeling. Lastly, we present applications of systems biology.

Keywords—computational modeling, high-throughput, deterministic, stochastic, agent-based, qFlow cytometry.

A1. INTRODUCTION

SYSTEMS biology has positively impacted several translational and clinical research areas: diagnostics, drug discovery and personalized medicine [1]-[3]. Our bodies consist of many integrated biological and chemical components that communicate on multiple scales: from genomes to molecules to cells that make up the organs. Some fields of biology focus on probing and studying components of a system one at a time, and one scale at a time. However, such a linear approach is not enough when it comes to solving systemic problems, such as cancer. Systems biology, on the other hand, aims to understand biological functions by integrating experimental data across different scales with predictive computational modeling. This integrative approach is driven by three major fields of research and technology: 1) Physiologically-relevant experimental models that allow us to define biological systems; 2) High-throughput technologies that probe biological systems at molecular-level with single-cell resolution; 3) Multi-scale predictive modeling that can integrate such biological data to predict system mechanism and responses.

Experimental models offer a window to empirically define and probe a biological system, and test response from a given stimulation. Without experimental models, no definitive statements

regarding the structure or function of biological systems could be made. However, experimental models are often difficult to choose from, due to the large variety and heterogeneity among those models. In addition, each model has their advantages and limitations depending on the experimental parameters one would like to probe. To alleviate confusion and provide guidance on which experimental model to use, we lay out the strengths and weakness of some commonly used experimental models, and the relevancy between those models and the physiological systems that they represent.

The complexity of the biological system and heterogeneity at different scales has posed great challenges in systems biology and hindered its application in biomarker discovery and drug development (**Figure 27**). In order to expand the approach of systems biology in those areas, it is crucial to develop technologies for genomic, transcriptomic and multiplex proteomic analyses at single-cell resolution [4][5]. In the past two decades, a variety of high-throughput technologies such as cDNA microarrays have greatly benefited the advancement of systems biology [6][7]. These technologies also generate "big data" or -omics data, providing insight into novel therapeutic targets or critical nodes. However, one challenge that remains is how to interpret and integrate these massive datasets in order to provide meaningful insights. Computational modeling is a useful tool to integrate big data and possibly overcome this challenge.

Computational modeling allows big datasets generated from experimental models and highthroughput assays to be analyzed in a physiological context in shorter times with lower material costs. Computational models allow probing of a biological system, using experimentally derived knowledge of the system, to provide new insights into the system function and predict system responses to various stimuli [8]. However, the biological system must first be experimentally defined to a certain extent before computational models can be developed, otherwise the computational models will fail to be physiological relevant. As knowledge and data of biological systems continues to grow, computational models will be critical for organizing, interpreting, and utilizing these data to improve decision making in pharmaceutical development.

Systems biology, the iteration between experimental models that represent a system, highthroughput assays that map parameters within the system, and computational models that predict system function and response, will accelerate discovery in several fields of biomedical research (**Figure 28**). As such, this review will help contextualize this field by first presenting several types of computational models: describing metrics for selection of appropriate modeling paradigms. We will then outline four types of commonly used experimental models in systems biology and discuss quantitative high-throughput techniques, with a focus on angiogenesis assays and quantitative proteomics. Furthermore, we present selected examples of recent research contributions that applied systems biology to better understand and treat diseases such as breast cancer and Alzheimer's. We conclude by proposing two possible opportunities for incorporating systems biology approaches towards cancer treatment.



Figure 27 Experimental approaches to systems biology in the pharmaceutical industry.

A2. COMPUTATIONAL MODEL SELECTION

You et al described succinctly the major driving force behind the creation of computational models: "To be confident how the car works, we should be able to put the [individual] parts back together and demonstrate that the car works" [9]. One goal of modeling is to probe empirical data and derive the mechanisms and critical nodes of interaction, i.e., the 'parts', that comprise the system, the 'car'. A model of the system can be defined using data derived from literature, experiments, -omic databases, or clinical studies. Models are iteratively tested and improved by comparing model predictions with results, measured empirically. Experimentally validated models

can provide predicted responses of systems, and provide insight into mechanisms and pathways of interacting system components. The ideal computational model should be robust, computationally efficient, and faithful to empirical data. Not all models are created equally. It is important when building a model to choose the most efficient type of model for the given system. For example, one model type may better represent phenomena at a specific scale than another type. In this section, we will review some of the different modeling approaches that are commonly used in systems biology studies, focusing on their strengths and weaknesses for modeling a specific system.



Figure 28 The continuous cycle that systems biology studies generally follow. Experimental studies derive the first information about the system components, which are utilized to develop a computational model. After performing model validation, models give additional insight into the system, which are then confirmed empirically. Additional experimental studies allow refinement of the model, and the cycle starts anew.

a. Kinetic Equation -based Modeling

Kinetic equations, possibly the most common form of representing biochemical interactions, can be derived from stoichiometric and empirical data. Kinetic equations are implemented through the use of ordinary differential equations (ODEs) and they can give information about the fluctuations in concentrations as a function of time (

Table). Kinetic equations require explicit definition of every state, interaction, and rate constants, and are thusly described as "deterministic".

Table 4 ODE Modeling:

Reaction Equation	ODE		
$A + B \xrightarrow[k_r]{k_r} AB$	$\frac{d[AB]}{dt} = k_{f}[A][B] - k_{r}[AB]$		
$A + X \cdot B \xleftarrow{k_f}{\underset{k_r}{\longleftarrow}} AB_X$	$\frac{d[AB_x]}{dt} = k_f[A][B]^x - k_r[AB_x]$		
$Y \cdot A + X \cdot B \xrightarrow[k_{i}]{k_{i}} A_{Y}B_{X}$	$\frac{d[A_{Y}B_{X}]}{dt} = k_{f}[A]^{Y}[B]^{X} - k_{r}[A_{Y}B_{X}]$		

The interaction of two species with a forward rate constant k_f, and reverse rate constant k_r.

Kinetic equation based modeling is useful as it allows hypothesized interactions to be explored and validated. If the hypothesized model is unable to match empirical results, the model parameters and interaction networks can easily be redefined iteratively, through the use of parameter estimation, until the empirical results are reproduced. Several methods of estimating parameters [10][11] and measuring parameter uncertainty [12][13] have been described. One of the main drawbacks associated with kinetic based modeling is the amount of information that is necessary: reaction definitions, reaction rates, and initial protein concentrations [14]. A second drawback is when simulating several orders of magnitude of reactions, the computational strain becomes quite large. One way to reduce this computational intensity is to make the interaction network Boolean, meaning every state exhibits one of two cases- on or off, allowing great simplification of the system [9]. While the Boolean model is very robust for gene networks, cellular interactions or compartmental changes are not very amenable to the system. Thus, systems with more complex interaction networks are usually modeled with ODEs. Additionally, ODEs can only be accurately applied to a continuum. Thus in situations where reactions occur stochasticity, such as at low concentrations, kinetic based approaches can be highly inaccurate. Altogether, kinetic equation based modeling can provide straightforward insight into the hypothesized network.

b. Stochastic Modeling

Stochastic modeling is the modeling of random, "non-deterministic," processes. It can be applied to a variety of topics including metabolite diffusion and receptor coupling. One stochastic technique with widespread use for systems of chemical reactions is the Gillespie algorithm. It captures stochastic processes and predicts the next state of the system [15]. This allows for better prediction of highly random interactions compared to deterministic modeling, but it is not without challenges. The randomness introduced into the system may not necessarily correlate with inherent system stochasticity. In addition, the added randomness makes it possible for the Gillespie algorithm to arrive at different simulation results, a phenomenon that does not occur in deterministic modeling. It is best used for few reactions and few species; otherwise, the calculation time necessary for a purely stochastic model can be significant. The Gillespie algorithm without kinetic equations can also be implemented, giving a homogeneously distributed system that contains only basic elementary reactions. Such conditions may not well represent intercellular signaling, and thus it is a very rare exception [9].

Another stochastic approach involves combining differential equations with to create a combinational model. One commonly used combinational model is that of Langevin. Each Langevin equation is comprised of two terms: (1) the differential equation, derived via deterministic modeling, and (2) a noise term. Introduction of both noise and dynamics provide accuracy to the model, without introducing significant computational time.

Stochastic modeling has also been explored to better reflect the randomness of small scale binding-unbinding behavior. Modeling techniques such as Monte Carlo methods allow for the creation of randomized data inputs through random sampling and/or probability distributions. This allows for the creation of "trials" in computational modeling for interrogating model robustness. However, it is debatable whether the increased computing power and number of trials necessary for a Monte Carlo simulation results in meaningful accuracy improvement. A study by Mac Gabhann et al compared a Mcell Monte Carlo simulation to a Gillespie model and to a purely deterministic model to determine if there were differences among these approaches [16]. Here, they examined the binding of the vascular endothelial growth factor (VEGF)-A variant, VEGF165, to its receptors, VEGFR1 and VEGFR2 on the cellular scale. They determined that there was not an appreciable difference in output amongst the three models, when examining a sample size representative of a cell surface. This suggests that for applications on the cellular scale, the more convenient deterministic models are sufficient. This study did have some notable limitations: it did not account for receptor clustering, receptor internalization, or ligand secretion. Furthermore, simulations over small surface areas revealed differences between stochastic and deterministic models, indicating that further studies might help identify the deterministic to stochastic relevancy threshold [16].

Stochastic systems can also be modeled using Bayesian methods to represent probabilistic noise [17]. Capturing probabilistic noise is advantageous as physiological processes are not purely deterministic in nature, and every process has some degree of stochasticity. One Bayesian method, Markov chain Monte Carlo simulation, is used to estimate initial parameters, discover motifs, or predict transcription factor binding sites [17]. Markov chain Monte Carlo simulations are able to include biological noise into their simulations, and have achieved 95% physiologically accurate results [18]. Markov chain Monte Carlo simulations have been applied to calculate mRNA transcript concentration in a series of spotted cDNA wells. Bayesian methods have also been applied to discriminate physical interactions across thousands of genes in cancer tumor models, such as gliomas. These statistical network models study the topology of cellular systems to find key genetic interactions regulating cancer development [19]. One major disadvantage of Bayesian methods is that it causes large computational loads, resulting from the large amount of calculations and processes; as such, a complete Bayesian analysis of models containing high physiological complexity is infeasible with current technology.

In stochastic models, it is important to note that noise introduced externally is typically assumed to be additive. This means that noise is not accounted for directly in the differential parameters, but as a constant, input separately. Additionally, fluctuations due to noise only affect the measurements, as the reality of the dynamics being affected by noise would require the differential equations factoring in that noise, which may make calculations more complex. This would involve the creation of a differential term that varied the noise on a temporal scale, which could complicate the model.

c. Agent-based Modeling

Agent-based modeling involves the creation of a series of rules that constrain the model. Instead of a series of deterministic equations such as the kinetic ODE model, agent based modeling is much more open-ended as the rules can be implemented to incorporate such aspects as orientation, proximity, velocity and even time. One example of this model type is the simulation of angiogenic sprouting. With this model, researchers were able to visualize the vessel filopodia as they grew, and were able to verify the presence of delta-notch mediated tip and stalk cell selection [20]. Recently, agent based modeling has been used to examine leukocyte rolling, adhesion, and

extravasation in microvascular networks [21]; to understand angiogenesis by surveying the rules for cell behavior compared against experimental results [22]; and to examine breast cancer progression [23]. Agent based modeling is very useful as the implementation of rules introduces more noise than a kinetic ODE model. The open-endedness of the model comes at a cost however, as creating the rules for reactions are not trivial, and the equations can become computationally complex when modeling how each agent within the model responds to the rules. Doing five to ten equations for a single agent (molecule/gene/protein/cell/etc.) may be computationally inexpensive, but scale-up to hundreds or thousands of such agents would result in higher computational strain (**Figure 29**).



Figure 29 An example of the interaction between a blue particle and a red one and the increasing complexity of the model with experiment duplication, several time steps, and several particles (Figure is adapted from Janes et al 2005 [24]).

d. Selection of Model and Parameter

In order to create models with high physiological relevance, it is important to implement an effective model type, as incorrect model assignment can result in non-trivial deviations from empirical results. For example, if a kinetic ODE based model is utilized for a dilute, low volume system where stochastic movement is integral, the model will not be able to capture the random fluctuations in particle quantities. The random fluctuations are an essential feature of the system, and as such, its lack of integration is a non-trivial simplification in the model. Forcing the kinetic model to fit this stochastic process would be much more difficult than implementing it as a stochastic model. Relatedly, using a kinetic ODE based model, or even a Langevin stochastic

model to simplify a complex interaction may result in lost information; whereas describing complexity via agent based model rules may better capture the system.

Deciding what type of model best represents the system of interest can also be subjective. One important aspect to note when comparing models is that a model or hypothesis cannot be proven - -- merely rejected. Meaning, even if a model is suggested and fits the empirical data, there is no proof that it is correct or that a "more accurate model" does not exist. Therefore, the success of a model should be judged not simply by whether it best fits empirical data, but also by whether the outcome makes sense biologically or physiologically. Additionally, models that can predict system outcomes that canbe further validated experimentally are often desired [25]. Models that help experimental design, predicting more effective experiments has been well examined [26][27].

A3. EXPERIMENTAL APPROACHES

Experimental research offers new insight into fundamental biological processes. It can serve several purposes including providing insight into signals, providing parameters for computational modeling, identifying potential biomarkers for clinical applications (early-stage detection, progress, and predicted outcome), and providing potential targets for drug development (**Figure 28**). The complexity of cellular systems often necessitates high-throughput approaches, while the data requirements of computational models require quantification. Additionally, the development of quantitative and high-throughput technology can significantly improve the efficiency and accuracy of experimental measurements and allow us compare data between facilities, days.

a. Experimental Assays

Systems biology has catalyzed the development of quantitative and high-throughput biological tools. In turn, the development of these new technologies has revolutionized the way we practice biology. The majority of the current experimental tools used in systems biology can be classified into genomic technologies and high-throughput proteomics (**Table 5**). Genomic technologies are used to determine the sequence or abundance of individual gene to an entire genome at either DNA level or the transcriptional level. Whereas, post-translational modifications, protein abundance, and protein-protein interactions can be identified and quantified using high-throughput proteomics. We have detailed examples of these experimental tools in Table 5, detailing their respective throughput, simplicity, quantitative nature.

b. Genomic Technologies

There are three primary genomic technologies that are used in systems biology [28]-[31]: 1) DNA/RNA sequencing, 2) microarray, and 3) quantitative polymerase chain reaction (qPCR) (Table 5). Since the invention of the first automated DNA sequencer in 1986, the throughput of DNA sequencing has increased more than 2000-fold [30][32]. The most powerful tool to decipher the complexity of genomes, next-generation sequencing (NGS), have been evolving over the past decade, leading to improvements such as longer read, higher throughput, and lower cost [29][33][34]. However, the relatively higher error rate and higher cost compared to other DNA platforms remains the limitation for NGS to be used clinically. Microarrays are widely used in genomic research due to its lower cost compared to NGS routines. Microarrays can be used to identify single-nucleotide polymorphisms (SNPs) [35][36] as well as measure expression levels of thousands of genes inexpensively [37]. Normalization for microarray measurement is considered to be challenging due to the variations in hybridizations; therefore, RNA sequencing is sometimes recommended in order to achieve more precise output [29][38][39]. qPCR is the gold standard for clinical gene detection due to its high sensitivity and specificity. With primers designed for targets of interest, qPCR can quickly and robustly detect specific targets, making it preferable for pointof-care applications [29][40][41].

Table 5 Examples of experimental techniques used in systems biology research.Within each assay subset, we evaluate and rank throughput, simplicity, and how quantitative theiroutput is.

Genomic Technologies	<u>Throughput</u>	Simplicity	Quantitative Output
DNA/RNA Sequencing	High	Complex	Moderate
Microarray	High	Moderate	Low
qPCR	Moderate	Simple	High
High-throughput Proteomics			
Protein structure	<u>Throughput</u>	<u>Simplicity</u>	Resolving power
NMR	High	Simple	Low
Crystallography	Moderate	Moderate	Moderate
Mass spectrometry	High	Complex	High
Protein concentrations	<u>Throughput</u>	<u>Simplicity</u>	Quantitative Output
Mass spectrometry	High	Complex	High
qFlow cytometry	Moderate	Simple	High
Quantitative ELISA	Moderate	Simple	High
Protein interaction	<u>Throughput</u>	<u>Simplicity</u>	Quantitative Output
Yeast 2-hybrid	Moderate	Complex	Low
MS-based affinity purification	High	Moderate	Moderate
Quantitative SPR	Low	Simple	High

c. High-throughput Proteomics

It is clear that proteins are the effectors of several critical cell behaviors. Biological systems cannot be decoded simply by studying the genes or RNAs due to the poor correlation between gene/RNA expression and protein expression [42]-[45]. Therefore, proteomics research applied to systems biology can enlighten on protein function, delineating how proteins regulate cell behavior on several levels. Towards this goal, proteomic technologies have been developed to characterize protein structure, protein-protein interactions, protein concentrations, and localization.

Here we focus on technologies used in systems biology that examine: protein structure, protein concentrations, and protein-protein interactions (**Table 5**).

1) Protein structure

There are three primary technologies used to identify protein structure in systems biology [42][46][47]: 1) Nuclear Magnetic Resonance (NMR), 2) crystallography and 3) mass spectrometry (MS). The advantage of NMR over the other two technologies listed here is that it allows us to identify the secondary structure, which means it can solve the 3D structure of a protein. However, the resolving power of NMR is lower compared to other technologies, and the molecular mass of protein complex is limited[48]. Crystallography allows high-resolution identification of protein structure, but requires the most prior knowledge of protein function of all the techniques outlined here. Relatively large amounts of protein need to be available for purification to form crystalline structures and the weight of the protein of interest needs to be approximately known.

Mass spectrometry is a means of peptide and protein identification by ionization and mass analysis. Biomarker identification by mass spectrometry allows for high resolution. However, mass spectrometry technology is limited by scalability as the large sample size requirement and pre-processing limit single cell analyses. Additionally, the distinction of molecules by mass is complicated by extensive proteomes in which several molecules are similar in mass. One example of advances in resolving protein complexes is given by Ho et al., where they successfully applied mass spectrometry to systematically identify protein complexes in *Saccharomyces cerevisiae* [49].

2) Protein concentrations

There are three primary technologies that are used to quantify protein concentrations in systems biology [50]-[53]: 1) Mass spectroscopy, 2) quantitative flow (qFlow) cytometry, 3) quantitative

ELISA, and 4) Immunohistochemistry. Here we start with several MS-based proteomic assays used in systems biology.

Protein phosphorylation, a post-translational modification that is critical to intracellular signaling, can be identified along with the sites of phosphorylation through the use of mass spectroscopy assays. While useful in assessing the amount and nature of phosphorylation, mass spectroscopy techniques cannot easily trace the act of phosphorylation itself, leaving the kinetics unknown. However, a powerful benefit of this technique is that these assays can be made in physiologically relevant environment and cellular conditions. These techniques are usually aided through the use of stable isotope labeling of amino acids in culture, such as with isotope tagged amine reactive agents or isotope-coded affinity tags [44], allowing for quantification of many different phosphorylation sites under different biological conditions [54]. A similar technique, selected reaction monitoring (SRM), is a form of double mass spectroscopy where the sample is ionized, fractionated, reionized, and then refractionated. Using heavy isotopes to quantitatively label certain ions allows for very accurate quantitation of preselected proteins or protein motifs. High-throughput techniques can increase the applicability of selected reaction monitoring [55] to new proteins. When SRM assay mixtures are available, they provide specific information about the concentrations of several proteins of interest simultaneously [56].

Multidimensional protein identification technology (MudPIT) is a mass spectroscopy technique that relies on data mining to identify and sort the protein fragments generated. Proteins are denatured and liquid chromatography is used to separate them by size, then tandem mass spectroscopy fragments the proteins and measures their distribution. Using computational techniques, the original protein mixture can be reconstructed [57]. MudPIT samples require several days of preparation, and the spectroscopy itself can take many hours. However, MudPIT allows quantitative analysis of thousands of proteins across a wide dynamic range.

Metabolic foot printing is a novel method that uses mass spectrometry in conjunction to measuring the metabolites that are excreted or not consumed by the cells from the media [58]. Instead of tracing what the cells have consumed through labelling, this technique finds the consumption through the analysis of cellular remains and excretion. This method is done through the stimulation of "overflow metabolism" which up-regulates the amount of excreted metabolites from the cells. This technique is very high-throughput, at the scale of 250 experiments in triplicate

in three days. Additionally, this system has been proven to be very useful in detecting the metabolic patterns in single-gene knock-out strains in functional genome analyses [58]. These assays give mass/charge data which contain vital information about the metabolites remaining in the system. However, a purely direct injection system trades off some knowledge of the identity of the metabolites for speed. A method to remedy this tradeoff is to use time of flight mass spectrometry plus gas chromatography to give much more information about metabolite identity, while minimizing the run time to roughly 13-20 minutes per run [58].

Quantitative enzyme-linked immunosorbent assays (ELISAs) use enzyme-linked antibodies to tag proteins of interest. Proteins are detected by adding a substrate that reacts with the enzyme to produce a measureable signal. ELISAs offer protein quantification at the cellular scale, but also detect protein levels within fluid or dissociated tissue samples. Radiolabeling seeks to overcome the disadvantages to fluorescent tags by tagging proteins with radioactive isotopes. The emitted radiation is detected and combined with information about the isotope half-life and decay kinetics, which allows protein quantification at small scales. Despite these advances in receptor profiling, an inexpensive, high-throughput, and highly-sensitive experimental tool for in vivo measurements of protein profiles is still missing.

Quantitative flow (qFlow) cytometry allows measurement of several different fluorophores across hundreds of cells per second. Recently, qFlow cytometry has been used to profile angiogenic receptors on endothelial cells in vitro [59], and on endothelial cells from normal tissue [60], ischemic tissue [61], and breast cancer xenografts ex vivo [62]. Without compromising specificity, up to five different dyes can be used if spectral unmixing techniques and the near-infrared spectrum are used [63]. Recently, quantum dot-based nanosensors which exhibit narrow emission spectrums have been developed to target multiple molecules simultaneously [64]. Quantum dot-conjugated antibodies can be combined with qFlow cytometry to quantify membrane receptors in a multiplexed manner [65], [66]. Modern fluorophores and compatible antibodies can be used to stain for nearly any cellular protein, allowing intercellular diversity to be captured. In comparison to immunohistochemistry, qFlow cytometry images a greater number of cells but may sacrifice some subcellular resolution.

3) Protein interaction

Protein interaction assays seek to identify how proteins bind, and the function of protein binding.

Here we introduce three quantitative methods used in systems biology to determine protein-protein interaction [67][68]: 1) Yeast two-hybrid assay, 2) MS-based affinity purification, and 3) surface plasma resonance (SPR).

Yeast two hybrid screening (Y2H) uses protein interactions to create a transcription factor for a reporter product, which tests for binary protein interactions [69]. The screening is simple and effective, but does not necessarily reflect in vivo conditions. MS-based affinity purification also probes the interaction of different proteins, though it is more technically complex than Y2H. In return for that complexity, the composition of macromolecular complexes can be tracked in more physiologically relevant conditions [70]. Surface plasmon resonance (SPR) quantifies the binding affinity of two types of molecules by detecting changes in the local refractive index upon protein-protein interaction on a biosensor. It has important applications in drug discovery and basic research because on-and-off kinetics can be measured [71].

A4. EXPERIMENTAL MODELS

Although large-scale gene, protein, and metabolite measurements (-omics) provide accurate identification of molecules and potential molecular interactions, it remains challenging to understand biological mechanisms with this information by itself. Therefore, experimental models that allow incorporation of biological complexity with knowledge of cellular and higher level system responses are beneficial. In all, an ideal experimental model for systems biology studies is reproducible, technically straightforward, quantitative, and has physiological meaning. The selection of appropriate experimental models for probing a biological system can be a challenging task. Here we describe some commonly used experimental models and discuss their strengths and limitations, mostly focusing on angiogenesis assays.

In vitro models of angiogenesis (e.g. cell cultures on or in Matrigel, transwell, or scratch assays) typically serve to quantify cell migration, endothelial cell proliferation and tubule formation, or cell responses to pro- and anti-angiogenic factors. In vitro studies allow for precise control of cellular environments, cell isolation, and augmentation, providing a high level of reproducibility. However, in vitro cell lines may not accurately recapitulate the physiology present in vivo due to the lack of the complexity in cell environment. Environmental factors that cells have surrounding them such as vasculature, tensile stresses, stromal cells, and connective tissues contribute greatly to the overall cell response to stimuli [72]. Thus, monolayer endothelial cells cultured in static

media may display different pathology to that observed in vivo. A recent study showed that proliferation rates in micro-channel assays continuously decreased, reaching 5% of the rate of cells cultured in flasks after 48 hours, maintaining this rate for 5 days [73]. This discrepancy may be due to different surface antigen levels or growth factors expression from proliferating cells in culture compared to normally quiescent cells in adult blood vessels [74]. In addition, differences have also been present between human umbilical vein endothelial cells (HUVECs) and human dermal microvascular endothelial cells (MECs) [75]. Imoukhuede et al quantified angiogenic receptor levels on HUVECs and MECs using high-throughput quantitative flow cytometry, revealing previously heterogeneities within each cell line [59]. Recently, experimental techniques of co-culturing multiple cell lines have been developed to more accurately capture the physiological environment. Bryan et al proposed a protocol for co-culturing endothelial cells and pericytes, revealing important interactions between these two cell types in angiogenesis [76]. Despite these recent advances, intrinsic heterogeneity across cell types and within cell lines needs to be better understood, in addition to the standardization of experimental models to provide more accurate measurements.

Beyond in vitro assays, there are a plethora of in vivo assays which allow the study of interactions between cells and the supporting environment. It is important to note that the cells and tissue that support in vivo environments are inherently heterogeneous, and can be observed even in the membrane proteins found on these cells [60]. As such, the characterization of such heterogeneities in vivo systems offers more insight into the biological system than in vitro assays. In the study of vasculature, it is critical to capture these interactions including those between endothelial cells and their supporting smooth muscle cells, fibroblasts, pericytes, basement membrane, and extracellular matrix. One widely performed in vivo angiogenesis study, which captures the vascular structure, is the chick chorioallantoic membrane (CAM) assay [77]. New formation of vessels can be observed and semi-quantified on the chorioallantoic membrane of chick embryos using image processing tools such as ImageJ.

Two limitations of many in vivo models is their inherent complexity, which fails to separate the mechanisms of each system component, and their difficulty in scaling up to high-throughput measurement. To overcome these limitations, multi-culture protocols have been developed for screening pro-angiogenic and anti-angiogenic compounds. For instance, Arnaoutova and Kleinman developed a high-throughput angiogenesis assay of endothelial cell tube formation that

can be created in 3-6 hours [78], whereas CAM assays usually takes 15 days. Another approach is metabolic foot printing analyses, which primarily use yeast. Yeast experiments have the advantages of safety, ease of use, absence of ethical issues, large specificity of knock-out mutants are obtainable, and 42% of its genetic information has human homologues, so the data derived from this method is very similar to human data. This method allows for potential probing of parameter values without the use of complex computational analysis [58].

Beyond high-throughput in vitro and in vivo, there lies ex vivo models, which provide a convenient, higher throughput option than in vivo testing. The choroid sprouting assay involves removing choroid tissue from live test subjects, culturing on Matrigel, and observing subsequent angiogenesis [79]. Next, image analysis can be used to determine the extent of angiogenesis by observing vessel growth and formation, and much of the lab work can be automated. This allows for high-throughput testing of pharmacological compounds in a mostly life-like environment. This assay can be expensive, but has so far shown promise in accurately reflecting the effects of pharmacological compounds on a system. However, ex vivo samples can be difficult to maintain and thus may have lower survival rate compared to in vitro assays [80]. Additionally, certain cell phenotypes may be altered by the tissue extraction and cell isolation processes.

Lastly, in situ modeling involves the culturing of large tissue in order to study smaller cells within the tissue. For instance, liver cells can be cultured and studied within a large portion of the liver. The cells in the liver represents the native environment and thus will experience little environmental alternation. Additionally, this has the strengths of in vitro culturing; testing and controlling parameters is relatively easy. However, the removal of such a significant portion of tissue is invasive and cannot be performed on biopsies. Additionally, while the cell to cell environment is conserved, larger system to system interactions are lost, and thus potential signaling between systems may be sacrificed. Fluorescence in situ hybridization (FISH), one of the best-known in situ techniques, uses a labeled complementary DNA or RNA strand to probe and visualize a DNA or RNA sequence of interest within tissue sections. Although FISH is very useful for detecting and locating viral nucleic acids and distinguish infected cells, it is limited in providing quantitative data for sensitive computational modeling. However, recent advancement in microfluidic devices has made it possible for in situ high-throughput –omics characterization [81]. In the future, minimized microfluidic platforms that are integrated with sensitive microarrays, or "Lab on a chip", can be used for quantitative monitoring of gene expression or molecular

interaction in vivo or in situ. This will provide systems biology with not only spatial, but also temporal information for advancing quantitative modeling.

While in vivo human models would be the ideal test bed when probing for system interactions or responses, cross-talk from other components may add unnecessary complexity to the study [72]. Thus, isolated systems such as in vitro modeling, ex vivo modeling or even in situ modeling can be preferential to in vivo modeling for probing individual interactions or mechanisms.

A5. APPLICATIONS OF BIMODAL SYSTEMS BIOLOGY: EXPERIMENT + COMPUTATION

Creating comprehensive computational models to identify and test new therapies is the goal of many systems biology researchers. Even though no models are completely accurate they still provide insight where knowledge gaps exist and can therefore direct further experimentation. For example, one model determined that direction and rate of endothelial cell migration are decoupled, thereby directing further modeling and experimentation [82]. More recent models have focused on the molecular interactions that drive endothelial cell migration, specifically the VEGF family of signaling molecules and their tyrosine kinase receptors with the goal of inhibiting these molecules to prevent tumor angiogenesis. In one example, a hypothesis that the selective binding of PIGF to VEGFR1 would increasesVEGFR2 signaling by displacing VEGF from VEGFR1, thus making VEGF more available for VEGFR2 was tested and described using a deterministic model [16]. This model demonstrates two primary bases for computational modeling; it elucidated signaling mechanisms as well as tested design assumptions by performing sensitivity analysis on assumed parameters.

All models make assumptions to derive their results. Most ODEs and other equation based modeling use parameter or concentration assumptions. However, inaccurate parameters can cause lead to inaccurate or physiologically irrelevant predictions [83]. One way to make these models more accurate would be to seed the models with distribution data rather than discrete values. Usually, a singular value is imputed for a certain parameter in the model. If a distribution of values was inputted into the model, the model would able to resolve the entire range of possible parameter values. The resulting distribution of solutions could be used to better understand the system. To reduce this dataset, a sensitivity analysis could be done to interrogate the sensitive parameters that

would significantly affect model results, and then applying the distribution approach to these parameters to gauge response.

The future direction of deterministic modeling in systems biology includes the move from single-scale, spatially isolated systems into larger, more comprehensive, multi-scale models (**Figure 30**). For example, a multi-scale model would incorporate stochasticity into ligand-receptor interactions, whole-cell responses from that binding action, and properties of the tissues made up of such cells. While elaborate, complex models of specific subsystems are an important focus of many modeling endeavors. Incorporation of multiple scales allows for integration of data at other scales, which will improve model robustness.



Figure 30 Modeling potential at different scales. Scale increases up the list. Each successively larger scale would be built using the smaller scale information below it.

There are significant computational challenges that accompany the development of multi-scale models. These challenges arise primarily from the fact that models at different scales use different data and modeling techniques. For example, signal transduction pathways are commonly modeled using ODEs, whereas ion channel opening is commonly modeled with stochastic techniques [84]. Agent-based techniques have been used for modeling the cellular level, such as in modeling endothelial cell migration [21]. These modeling techniques are carefully chosen as the best approach to representing the dynamics, spatial scale, and temporal scale of a particular system. When systems at multiple scales are combined, a more robust set of information can be captured, but integrating these different techniques is a significant computational challenge.

One recent technique for multi-scale modeling is the rule-based approach [85]. Rule-based modeling allows the integration of processes at different scales, as well as providing an iterative approach to model development. For example, Chen and colleagues used rule-based modeling to

simplify the EGFR system from 400 interacting proteins to 21 proteins [86]. Rule-based modeling reduces ODE models to simplified rules based on the necessary inputs and outputs. Rule-based methods have been increasingly used in systems biology and are available with OLGIO, StochSim, and BioNetGen software.

Some techniques have been developed to alleviate the challenges of integrating model techniques at multiple scales. For example, Covert et al used flux-balance analysis and ODEs to model large-scale metabolic networks of carbohydrate uptake in *E. coli* [87]. The specific regulatory flux-balance analysis model was verified to show predictive power in different environmental conditions, while ODE modeling of transcriptional regulation from catabolites have been able to simulate observation in a wide range of experimental conditions [88]. The results of this integrated paradigm provide a framework to combining different modeling techniques and shows information gain that would not be possible when limited to a single modeling approach (**Figure 31**). However, the complexity of many systems would not allow for this approach. This approach is also computationally expensive: every iteration of model development involves recalculating the protein activity, gene expression, protein expression, flux distribution, and solving for new concentrations.

Regulatory Flux Balance Analysis Model (rFBA) • predictive of outcome from gene perturbation • not predictive of internal concentration of metabolites • ignores cAMP (not a required for growth-

optimal solution)

Integrated Flux Balance Analysis

 predictive of outcome from gene perturbation
predictive of internal concentration of metabolites
encompasses action of cAMP
predicts shift of metabolic flux from one

pathway to another

Ordinary Differential Equation Model

 predictive of internal concentration of metabolites
not predictive of outcome from gene perturbation
cannot predict shift of metabolic flux from one pathway to another

Figure 31 Illustration of the advantages of a combinatory model from different scales and modeling techniques based on information provided by Covert [87].

The integrated flux balance analysis approach provides a good example of the information gained from incorporating different model types. However, further approaches must be developed to integrate other techniques (i.e. agent-based models (ABM) and partial differential equations) and different scales. Another approach integrates an agent-based model of cell-level processes with a constrained mixture model (accounting for energy and mass balances) to model tissue-level

processes (**Figure 32**) [89]. The agent-based model was based on data obtained in vitro while the constrained mixture model was based on tissue data. Both models were individually validated for the respective scales. However, disagreement between the two scales needed to be alleviated to create an integrated model. Specifically, the outputs from models, collagen and smooth muscle cell masses, had to be converged through the use of a heuristic genetic algorithm. While computational tools such as this genetic heuristic algorithm provide potential for systems biology, further algorithms and computational tools relevant to biological systems must be developed and refined for future models.



Figure 32 Illustration of the process and results of integration of different models from different scales based on information provided by Hayenga [89].

Towards these multi-scale modeling goals, a problem that must be overcome is that many different models exist to model distinct processes, while a unified whole-body model that incorporates the models of several researchers fails to exist. Reactome.org offers a good movement towards incorporating several cell processes into a qualitative framework. However, the challenges in unifying models is due to two major deficiencies: first, there is a lack of model uniformity; second, there is a lack of transparency in the dissemination of models. The lack of uniformity is partially due to a lack of knowledge about the implementation and design of different model types, and so as the field develops and more educated models are designed, greater uniformity will enable greater sharing and compatibility across models. Systems Biology Markup Language (SBML) is a modeling language that is gaining traction and may help overcome this limitation. However, transparency is a challenge that must be tackled side-by-side with uniformity. The presence of curated databases that accept well-notated, standardized models will enable greater sharing and implementation of current models.

A6. APPLICATION OF SYSTEMS BIOLOGY IN PERSONALIZED MEDICINE

A grand challenge in interfacing engineering with the life sciences, is understanding the complexity and heterogeneity in disease [90]. Previous decades have seen the collection of "big data" [91] in the genetic [92] and proteomic fronts [93]. Mapping complex interactions between genetics and proteomics requires the application of systems biology principles. By modeling the physiological pathways and processes the cause of complex diseases can be identified and efficient treatments can be developed.

The progression of systems biology applications to disease can follow a standard process as described in **Figure 33**. First, the genetic variation contributing to the disease can be identified using high-throughput gene sequencing across large patient populations. Next, pathways regulating this genetic variation can be computationally modeled to understand the proteomic role in the disease. The cell and tissue behavior can also be computationally modeled, as necessary. As computational models advance, new insights are discovered into the disease mechanisms, leading to more efficient drug development and personalized medicine. Here we describe three diseases that can benefit from this systemic approach: Metabolic Syndrome, Alzheimer's Disease, and Cancer. We then present cancer in greater detail, highlighting modeling of ErB signaling, and anti-angiogenic approaches in cancer.



Figure 33 Schematic of systems biology approach to disease treatment.

a. Metabolic Syndrome

Metabolic Syndrome (MetSyn), which contributes to diabetes and cardiovascular diseases, has been of great interest to systems biology research [94]. Risk factors for MetSyn include obesity, high triglyceride levels, low high density lipoprotein (HDL) or high low density lipoprotein (LDL) levels, high blood pressure, and high blood glucose levels [94]. The chance of inheriting MetSyn is as high as 70%, but only a small fraction of syndrome occurrence is explained by known genetic variations [94]. Thus, scientists are still applying high-throughput genetic sequencing to try and identify specific genes regulating MetSyn. This genetic sequencing relies on single nucleotide polymorphisms (SNPs), which are locations in the genome where a single nucleotide varies between members of the population. By comparing disease-associated SNPs to eSNPs (SNPs at experimental trait loci) using causality analysis, we can determine the source of damaging mutations. Using microarrays, SNPs present in diseased subjects are compared with eSNP databases to specifically identify candidate genes. This approach has previously yielded results, such as allowing researchers to identify that the SORT1 and neighboring genes are responsible for elevated LDL expression leading to cardiovascular disease. This was determined by analyzing correlation between three candidate genes (SORT1, PSRC1, and CELSR2) and an SNP known to correlate to LDL elevation. This insight was then validated experimentally in mice where SORT1 was blocked [95]. The complexity of MetSyn makes treatment progress difficult: hundreds of genes and proteins are involved but their identity and relationships remain undefined. As these proteins and relationships are defined, computer models can be created to map the interactome behind MetSyn and identify effective treatments.

b. Alzheimer's Disease

Alzheimer's disease (AD) provides an example of a disease in the next stage of systems analysis. AD is a neurogenerative disorder common in aging patients characterized by loss of memory, judgment, and communications skills. The neurological causes are commonly understood to be Amyloid Plaques (a buildup of protein fragments called AB) and neurofibrillary tangles (strands of a protein called 'tau') [96]. Currently, models of the molecular mechanisms are being designed to probe further into the functionality of these proteins. In one such model, the synapse is treated as a complex machine made up of thousands of proteins and lipids on both sides of the synapse. Their interaction is tuned by modifying their relative geometry to create more or less transmission of action potential across the synapse. This tuning can increase or decrease the "weight" that the outgoing signals have on the incoming signal. This model revealed that plaque buildup inhibits synaptic tuning and causes the memory loss and other cognitive dysfunction seen in AD [97]. By further probing protein interactions and validating experimentally, the exact mechanism of disruption that leads to AD can be elucidated and treated. To this end, efforts are being made to computationally model neural systems. One such model uses mass-action to understand the interaction of calcium with NDMA receptors and calmodulin-dependent protein kinase II in regulating synaptic strength [98]. This type of synaptic modeling can be applied further to understand the precise causes of AD symptoms. The same principles could be applied to other CNS disorders such as PTSD, Bipolar Disorder, ALS, and others to improve understanding and treatment.

c. Cancer

Another prominent application of systems biology has been in cancer research. Genetic analysis allowed identification of numerous oncogenes and tumor-suppressor genes such as p53, ErbB receptors, and RAS [99]. The systems that these genes regulate were modeled computationally. For example, the ErbB signaling pathway was modeled using mass-action kinetics. This model investigated the effects of ErbB overexpression, and determined that high ErbB expression leads to sustained signaling. This response was verified through testing in MCF-7 cells to complete the feedback loop characteristic of systems biology [100].

One example of a proposed cancer model uses glioma cell imaging data to generate a model. Initial parameters for the glioma cells in brain tumors can be found from patient specific data such as location, structure, and vasculature through the use of contrast-enhanced MRI. More parameters such as cell density and microvasculature could be obtained from histopathology, gene arrays, tissue cultures, and proteomic profiling. This proposed model would allow for the creation of a temporally evolving and relevant model that could allow for further insight in the mechanisms of brain cancer, which is a particularly lethal cancer variant [101].

An eventual goal of systems biology is to produce personalized treatment plans that are based on an intricate understanding of cancer function. [102]. One way in which the advancement of personalized medicine will be accomplished is through a more sophisticated understanding of tumor make-up derived from lineage tracing. Tumors can grow from one cell or from a number of mutated cells, with most mature tumors being derived from one cell. Tracing epigenetic variations in the tumor by taking advantage of silencing one X chromosome in females allowed verification of this monoclonality [103]. With the knowledge that most tumor cells derive from a single source, computational models mapping the tumor genotype could provide personalized understanding of tumor phenotype and patient reaction to various treatment options.

Another application of computational modeling is in the integration of smaller scale models into a larger system. Many system models are individual cells or units inside an organ. Integrating these models into a larger "organ" or "organ system" model could be done by making these "cells" into small compartments from which several can be tied together into one larger unit. This can help with modeling larger scale interactions of diseases, as several diseases do not target one specific area. Modeling the initial tumor, as well as the organs at which metastasis is most to occur could give insight into tumor growth and metastasis prevention.

d. Applications of Modeling Targeted Regimes in Cancer Treatment

VEGF and its receptors are regularly over-expressed in a wide variety of human cancers, including breast cancer. VEGF overexpression is an early step in breast cancer progression, and frequently occurs before tumor invasion [104]. Furthermore, some breast cancers that overexpress VEGF show resistance to chemotherapy and hormonal therapy. Therefore, targeting VEGF and its signaling axis becomes a potential approach of inhibiting breast cancer progression and metastasis.

As Rebecca et al put succinctly in their 2014 paper, "Despite excitement about the development of targeted therapy strategies of cancer, few cures have been achieved." [105] Clinical studies have shown that tumors may develop resistance to drugs that inhibit VEGF signaling. This is believed to be because endothelial cells, cancer-associated fibroblasts, and pericytes within individual tumor display strong heterogeneity. Thus, therapies among different patients, different tumor types, and different cancer stages exhibit contrasting responses [106]. Another explanation is that tumors may develop an alternative signaling pathway, such as PDGF signaling, to recruit pericytes and/or tumor-associated fibroblasts to support angiogenesis after VEGF inhibition [107].

A good case-study for the challenge of anti-angiogenic drug resistance is bevacizumab. Bevacizumab is the first FDA-approved drug to inhibit VEGF by binding to all the isoforms of the parent VEGF-A molecule. Bevacizumab has activity in multiple tumor types, and clinical trials have indicated benefits of Bevacizumab when combined with chemotherapy for the treatment of non-small-cell lung cancer [108] and metastatic colorectal cancer [109]. However, phase III breast cancer trials showed the addition of Bevacizumab failed to prolong time to progression or overall survival due to unexplained heterogeneous responses, in addition to severe side effects and development of drug resistance [110].

More thorough studies of the balance of angiogenic receptor levels and crosstalk between angiogenic signaling axes under pathological and physiological conditions are required to understand the development of drug resistance and develop more efficient therapeutics. To this end, the balance between VEGFR1 and VEGFR2 on HUVECs and their response to VEGFA treatment in vitro has been profiled [59]. More recently, heterogeneity in MDA-MB-231 breast tumors was discovered by quantifying receptor levels of VEGFR1 and VEGFR2 on tumor endothelial cells in mouse xenografts [62]. Erber et al. showed resistance of tumor blood vessels to VEGFR2 targeting, by the tyrosine kinase inhibitor SU5416, is conferred by recruitment of pericytes, which provide endothelial cell survival signals through PDGF and Ang-Tie signaling [111].

The concept of phenotypic resistance to VEGFR2 blockade is a well-discussed idea in the field of angiogenesis. Cassanovas et al. in 2005 [112] show that resistance to VEGFR2 blockade was associated with induction of proangiogenic factors other than VEGF, including members of FGF family, in a pancreatic islet carcinogenesis mouse model. To reduce the drug resistance due to alternative signaling, dual-targeting drugs have been studied. For example, Erber et al showed SU6668, which targets both VEGFR2 and platelet-derived growth factor receptor (PDGFR)- β caused 40% regression of tumor blood vessels in a C6 tumor mice model [111]. Other inhibitor cocktails include TAK-593 targeting VEGFR2 and PDGFR β [113], sorafenib [114], a single-chain antibody targeting VEGF-A and PDGFR β [115] and combination of AZD6244 (targeting MEK) and corenolanib (targeting PDGFR) [116].

Systems biology has brought valuable insights to understand the multi-step process of angiogenesis [117]. The complexity of signaling pathways involved makes it an excellent candidate for systems biology; and the combination of experimental profiling and computational modeling could offer new hypothesis. As Logsdon et al stated in their review article in 2014, "A model investigating the efficacy of targeting the VEGF co-receptor neuropilin predicted that inhibiting NRP-VEGFR coupling is a more effective strategy than blocking NRP1 expression or preventing VEGF-NRP binding" [117][118]. Another whole-body model of VEGF signaling investigated the level of free VEGF in the tumor affected by tumor microenvironment and drug characteristics such as the clearance rate and binding affinity [119]. The model further predicted that targeting VEGF₁₂₁ can reduce free VEGF in the tumor and yield effective reduction in VEGF signaling [120].

These findings indicate that the balance of VEGFR, PDGFR, and other surface receptors could provide insight into the behavior of cancer cells. With this in mind, it would be useful to profile the surface concentrations of VEGFR and PDGFR in multiple cancers and correlate these
concentration profiles to cancer behavior, specifically response to anti-angiogenic therapies. This data could elucidate a series of classifications with distinct treatment profiles. Towards these goals, Imoukhuede and Popel have optimized qFlow cytometry, using it to characterize receptor concentrations on individual cells in a sample [59]. In concert with such new experimental techniques, computational models that involve multiple signaling pathways are being developed [121]. These models will provide insight into the significance of the receptor concentration profiles determined by the flow cytometry. By applying this to determine concentrations of VEGF and PDGF receptors on a variety of cancer samples, the necessary database could be produced and mined for insight.

e. Multi-Targeted Approaches to Anti-Angiogenesis

Endothelial cells in tumor vasculature exhibit a large amount of heterogeneity in expression as well as in vasculature structure. This heterogeneity is an important aspect to consider in treatment regimes, as differences in vascularity profiles can be the difference between an anti-angiogenic drug succeeding or failing in a patient [122]. There are many hypotheses for what causes these different expressions and modalities, one of which is that the extracellular matrix (ECM) contributes to the heterogeneity through molecular expression as well as physical parameters such as stiffness [123]. It is even believed that certain types of collagen in the extracellular matrix, type I and type IV, can induce chemo-resistance through interactions with certain integrin [124]. Cancer ECM are also much more stiffened than typical ECM, which results in a potentially drastic difference in the chemical environment of the cancer endothelial cells as stiffness correlates to a difference in growth factors and modifiers such as Yes-associated protein [125]. Further studies have showed that small-molecule inhibitors of collagen synthesis prevent angiogenesis and tumor growth [126]. As a result, a large amount of research has gone into the development of the "matrisome" a comprehensive library of proteins that complement the extracellular matrix [127]. This extensive library is the first step in developing a mechanism in differentiating tumor extracellular matrix from healthy matrix, which in turn can provide vital information on how endothelial cells are affected by these changes. These effects could be the exact criterion on which to discriminate cancerous endothelial cells from healthy endothelial cells.

Drugs targeted to the extracellular matrix can transport more easily, as it is not encapsulated by a plasma membrane. Extracellular matrix targets have been pursued in many applications already.

One such medicine, Lysyl oxide, has been tested in murine models to inhibit tumor growth. Lysyl oxide is up-regulated in tumor ECM, and is an enzyme that interacts with the ECM, which makes it a viable target. Using nanoparticles rather than antibodies conjugated to the lysyl oxide resulted in definitive decreases in tumor sizes in the mouse models [128]. There are more drugs currently in clinical trials that target extracellular matrix binding. Cilengitide (EMD 121974) targets $\alpha\nu\beta3$ and $\alpha\nu\beta5$ integrins and inhibits them. It is currently in phase one and two clinical trials. Intetumumab (CNTO 95) is a monoclonal antibody that targets the $\alpha\nu$ integrin. It has shown some promise as it slightly increases survivability in cancer treatments. It is currently in clinical trials [129].

These examples suggest that the ECM is a viable target. Considering the past research suggesting correlations between cancer ECM and tumor angiogenesis, a robust model of ECM proteins, structure, and angiogenic signals could potentially lead to drug targets for antiangiogenesis. For example, a factor that is commonly targeted for angiogenesis is VEGF. Tumor ECM can alter the uptake of VEGF through additional modification. Basement membrane matrix protein 9 (MMP9) is necessary for transport of angiogenic growth factor VEGFR, which means an increase in the MMP activity will increase the uptake of VEGF [130]. Tumors typically do increase MMP activity to facilitate growth and establish more vascularity. This upregulation in glycolytic activity increases the amount of lactic acid produced, which changes the local pH of the tumor to be more acidic. The local acidity further effects the endothelial cells inside the tumor as pH of the environment definitely affects the expression of the cells [131]. These changes in expression can be used to differential the healthy endothelial cells from the tumor endothelial cells.

A comprehensive model of ECM proteins, tumor endothelial cell growth pathways, and angiogenic factors may be a potential new arena for cancer drug target discovery. Moreover, combining not only kinetic reactions but also the effects of physical structure within the ECM and tumor vasculature growth —both which differ from that of normal cells– may result in a multi-scale model with robust predictability. These modeling techniques would allow for the probing of physical ECM effects on endothelial cells and could be an engine to develop new treatments and diagnostics for cancer. Since the amount of data pertaining to cancer and ECM interactions are so large, some models strive to simplify the overall model. This results in drastic decreases in computational processing time, while remaining faithful to the empirical data [132]. By elucidating

the mechanisms by which the ECM affects the expression of tumorous cells, drugs that restore normal cell function or probes that target irregular function could be developed.

A7. CONCLUDING REMARKS

Systems biology has developed substantially with recent advances in both computational modeling and sophistication of experimentation. As experimental models have begun to reach the limits of realism allowed by biology, the focus of experimentation has shifted to the advancement of high-throughput, quantitative technologies that allow us to screen organisms at multi-scales. The data obtained with such technologies in turn allows complex in silico testing and provides feedback to improve existing experimental models and predict stimuli responses. This iteration between experimentation and modeling has been applied to the treatment of diseases such as Metabolic Syndrome, Alzheimer's disease, and cancer. As the field moves forward, better understanding of the mechanisms behind these complex diseases will yield new treatment strategies and precision medicine.

A8. REFERENCES

- [1] R.-S. Wang, B. A. Maron, and J. Loscalzo, "Systems medicine: evolution of systems biology from bench to bedside.," *Wiley Interdiscip. Rev. Syst. Biol. Med.*, vol. 7, no. 4, pp. 141–61.
- [2] E. C. Butcher, E. L. Berg, and E. J. Kunkel, "Systems biology in drug discovery.," Nat. Biotechnol., vol. 22, no. 10, pp. 1253–9, Oct. 2004.
- [3] F. Mac Gabhann, B. H. Annex, and A. S. Popel, "Gene therapy from the perspective of systems biology.," *Curr. Opin. Mol. Ther.*, vol. 12, no. 5, pp. 570–7, Oct. 2010.
- [4] J. R. Heath, A. Ribas, and P. S. Mischel, "Single-cell analysis tools for drug discovery and development," *Nat. Rev. Drug Discov.*, vol. 15, no. 3, pp. 204–216, Dec. 2015.
- [5] M. Breker and M. Schuldiner, "The emergence of proteome-wide technologies: systematic analysis of proteins comes of age," *Nat. Rev. Mol. Cell Biol.*, vol. 15, no. 7, pp. 453–464, Jun. 2014.
- [6] R. Macarron, M. N. Banks, D. Bojanic, D. J. Burns, D. A. Cirovic, T. Garyantes, D. V. S. Green, R. P. Hertzberg, W. P. Janzen, J. W. Paslay, U. Schopfer, and G. S. Sittampalam, "Impact of high-throughput screening in biomedical research," *Nat Rev Drug Discov*, vol. 10, no. 3, pp. 188–195, Mar. 2011.
- [7] M. G. Acker and D. S. Auld, "Considerations for the design and reporting of enzyme assays in high-throughput screening applications," *Perspect. Sci.*, vol. 1, no. 1–6, pp. 56–73, May 2014.
- [8] G. W. Brodland, "How computational models can help unlock biological systems," *Semin. Cell Dev. Biol.*, vol. 47–48, pp. 62–73, Dec. 2015.
- [9] L. You, "Toward computational systems biology," *Cell Biochem Biophys*, vol. 40, no. 2, pp. 167–184, 2004.
- [10] C.-L. Ko, E. Voit, and F.-S. Wang, "Estimating parameters for generalized mass action models with connectivity information," *BMC Bioinformatics*, vol. 10, no. 1, p. 140, 2009.
- [11] I. C. Chou and E. O. Voit, "Recent developments in parameter estimation and structure identification of biochemical and genomic systems," *Math. Biosci.*, vol. 219, no. 2, pp. 57–83, 2009.
- [12] K. Beven, "A manifesto for the equifinality thesis," J. Hydrol., vol. 320, no. 1–2, pp. 18–

36, 2006.

- [13] C. Hutton, Z. Kapelan, L. Vamvakeridou-Lyroudia, and D. Savić, "Dealing with Uncertainty in Water Distribution System Models: A Framework for Real-Time Modeling and Data Assimilation," *J. Water Resour. Plan. Manag.*, vol. 140, no. 2, pp. 169–183, 2012.
- [14] N. Jamshidi and B. Ø. Palsson, "Mass Action Stoichiometric Simulation Models: Incorporating Kinetics and Regulation into Stoichiometric Models," *Biophys. J.*, vol. 98, no. 2, pp. 175–185, 2010.
- [15] D. Gillespie, "Exact stochastic simulation of coupled chemical reactions," *J. Phys. Chem.*, vol. 93555, no. 1, pp. 2340–2361, 1977.
- [16] F. Mac Gabhann and A. S. Popel, "Model of competitive binding of vascular endothelial growth factor and placental growth factor to VEGF receptors on endothelial cells.," *Am. J. Physiol. Heart Circ. Physiol.*, vol. 286, no. 1, pp. H153-64, Jan. 2004.
- [17] D. J. Wilkinson, "Bayesian methods in bioinformatics and computational systems biology.," *Brief. Bioinform.*, vol. 8, no. 2, pp. 109–16, Mar. 2007.
- [18] A. Frigessi, M. a van de Wiel, M. Holden, D. H. Svendsrud, I. K. Glad, and H. Lyng, "Genome-wide estimation of transcript concentrations from spotted cDNA microarray data.," *Nucleic Acids Res.*, vol. 33, no. 17, p. e143, Jan. 2005.
- [19] L. B. Edelman, J. a Eddy, and N. D. Price, "In silico models of cancer.," Wiley Interdiscip. Rev. Syst. Biol. Med., vol. 2, no. 4, pp. 438–59, 2010.
- [20] L. Jakobsson, C. A. Franco, K. Bentley, R. T. Collins, B. Ponsioen, I. M. Aspalter, I. Rosewell, M. Busse, G. Thurston, A. Medvinsky, S. Schulte-Merker, and H. Gerhardt, "Endothelial cells dynamically compete for the tip cell position during angiogenic sprouting," *Nat Cell Biol*, vol. 12, no. 10, pp. 943–953, 2010.
- [21] A. Bailey, B. Thorne, and S. Peirce, "Multi-cell Agent-based Simulation of the Microvasculature to Study the Dynamics of Circulating Inflammatory Cell Trafficking," Ann. Biomed. Eng., vol. 35, no. 6, pp. 916–936, 2007.
- [22] B. L. Long, R. Rekhi, A. Abrego, J. Jung, and A. A. Qutub, "Cells as state machines: Cell behavior patterns arise during capillary formation as a function of BDNF and VEGF," *J. Theor. Biol.*, vol. 326, no. 0, pp. 43–57, 2013.
- [23] K.-A. Norton, M. Wininger, G. Bhanot, S. Ganesan, N. Barnard, and T. Shinbrot, "A 2D

mechanistic model of breast ductal carcinoma in situ (DCIS) morphology and progression," *J. Theor. Biol.*, vol. 263, no. 4, pp. 393–406, 2010.

- [24] K. A. Janes and D. A. Lauffenburger, "A biological approach to computational models of proteomic networks," *Curr. Opin. Chem. Biol.*, vol. 10, no. 1, pp. 73–80, 2006.
- [25] G. Cedersund and J. Roll, "Systems biology: model based evaluation and comparison of potential explanations for given biological data.," *FEBS J.*, vol. 276, no. 4, pp. 903–22, Feb. 2009.
- [26] A. Chakrabarty, G. T. Buzzard, and A. E. Rundell, "Model-based design of experiments for cellular processes," *Wiley Interdiscip. Rev. Syst. Biol. Med.*, vol. 5, no. 2, pp. 181–203, 2013.
- [27] E. Walter and L. Pronzato, "Qualitative and quantitative experiment design for phenomenological models—A survey," *Automatica*, vol. 26, no. 2, pp. 195–213, 1990.
- [28] A. Aderem, "Systems Biology: Its Practice and Challenges," *Cell*, vol. 121, no. 4. pp. 511–513, 2005.
- [29] S. Goodwin, J. D. McPherson, and W. R. McCombie, "Coming of age: ten years of next-generation sequencing technologies," *Nat. Rev. Genet.*, vol. 17, no. 6, pp. 333–351, May 2016.
- [30] L. Hood, "Systems biology: integrating technology, biology, and computation," *Mech. Ageing Dev.*, vol. 124, no. 1, pp. 9–16, 2003.
- [31] T. Ideker, T. Galitski, and L. Hood, "A N EW A PPROACH TO D ECODING L IFE : Systems Biology," *Annu. Rev. Genomics Hum. Genet.*, vol. 2, no. 1, pp. 343–372, Sep. 2001.
- [32] L. M. Smith, J. Z. Sanders, R. J. Kaiser, P. Hughes, C. Dodd, C. R. Connell, C. Heiner, S. B. H. Kent, and L. E. Hood, "Fluorescence detection in automated DNA sequence analysis," *Nature*, vol. 321, no. 6071, pp. 674–679, Jun. 1986.
- [33] M. Kircher and J. Kelso, "High-throughput DNA sequencing concepts and limitations," *BioEssays*, vol. 32, no. 6, pp. 524–536, May 2010.
- [34] E. R. Mardis, "Next-Generation Sequencing Platforms," *Annu. Rev. Anal. Chem.*, vol. 6, no. 1, pp. 287–303, Jun. 2013.
- [35] J. Perkel, "SNP genotyping: six technologies that keyed a revolution," Nat. Methods, Publ. online 01 May 2008; / doi10.1038/nmeth0508-447, vol. 5, no. 5, p. 447, 2008.

- [36] A.-C. Syvänen, "Toward genome-wide SNP genotyping," *Nat. Genet.*, vol. 37, no. 6s, pp. S5–S10, Jun. 2005.
- [37] M. Schena, D. Shalont, R. Heller, A. Chai, P. Brown, and R. W. Davis, "Parallel human genome analysis: Microarray-based expression monitoring of 1000 genes," vol. 93, no. October, pp. 10614–10619, 1996.
- [38] S. Zhao, W.-P. Fung-Leung, A. Bittner, K. Ngo, X. Liu, G. Kerr, H. Ruskin, M. Crane, P. Doolan, G. Passador-Gurgel, W. Hsieh, P. Hunt, N. Deighton, G. Gibson, A. Mortazavi, B. Williams, K. McCue, L. Schaeffer, B. Wold, U. Nagalakshmi, K. Waern, M. Snyder, S. Montgomery, M. Sammeth, M. Gutierrez-Arcelus, R. Lach, C. Ingle, M. Beyer, M. Mallmann, J. Xue, A. Staratschek-Jox, D. Vorholt, K. Mutz, A. Heilkenbrinker, M. Lönne, J. Walter, F. Stahl, M. Garber, M. Grabherr, M. Guttman, C. Trapnell, D. Bottomly, N. Walter, J. Hunter, P. Darakjian, S. Kawane, W. Zhang, J. Ferguson, S. Ng, K. Hui, G. Goh, A. Sîrbu, G. Kerr, M. Crane, H. Ruskin, J. Marioni, C. Mason, S. Mane, M. Stephens, Y. Gilad, X. Fu, N. Fu, S. Guo, Z. Yan, Y. Xu, Z. Wang, M. Gerstein, M. Snyder, J. Bloom, Z. Khan, L. Kruglyak, M. Singh, A. Caudy, P. Hurd, C. Nelson, J. Bradford, Y. Hey, T. Yates, Y. Li, S. Pepper, J. Malone, B. Oliver, B. Bolstad, R. Irizarry, M. Astrand, T. Speed, R. Irizarry, B. Hobbs, F. Collin, Y. Beazer-Barclay, K. Antonellis, J. Hu, H. Ge, M. Newman, K. Liu, Y. Benjamini, Y. Hochberg, A. Jetten, R. Røge, J. Thorsen, C. Tørring, A. Ozbay, B. Møller, J. Carstens, I. Nookaew, M. Papini, N. Pornputtapong, G. Scalcinati, L. Fagerberg, M. Stalteri, A. Harrison, X. Cui, A. Loraine, H. Liu, B. Zeeberg, G. Qu, A. Koru, A. Ferrucci, R. Nurtdinov, M. Vasiliev, A. Ershova, I. Lossev, A. Karyagina, K. Hansen, S. Brenner, S. Dudoit, L. McIntyre, K. Lopiano, A. Morse, V. Amin, A. Oberg, H. Le, M. Schulz, B. McCauley, V. Hinman, Z. Bar-Joseph, D. Risso, K. Schwartz, G. Sherlock, S. Dudoit, W. Zheng, L. Chung, H. Zhao, A. Roberts, C. Trapnell, J. Donaghey, J. Rinn, L. Pachter, M. Baker, M. Schatz, B. Langmead, S. Salzberg, and L. Stein, "Comparison of RNA-Seq and Microarray in Transcriptome Profiling of Activated T Cells," PLoS One, vol. 9, no. 1, p. e78644, Jan. 2014.
- [39] Z. Wang, M. Gerstein, and M. Snyder, "RNA-Seq: a revolutionary tool for transcriptomics," *Nat. Rev. Genet.*, vol. 10, no. 1, pp. 57–63, Jan. 2009.
- [40] A. Ståhlberg, P. Aman, L. Strömbom, N. Zoric, A. Diez, O. Nilsson, M. Kubista, and B. Ridell, "Comparison of reverse transcription quantitative real-time PCR, flow cytometry, and immunohistochemistry for detection of monoclonality in lymphomas.," *ISRN Oncol.*, vol.

2014, p. 796210, Jan. 2014.

- [41] D. J. Eastburn, A. Sciambi, and A. R. Abate, "Ultrahigh-throughput Mammalian singlecell reverse-transcriptase polymerase chain reaction in microfluidic drops.," *Anal. Chem.*, vol. 85, no. 16, pp. 8016–8021, Aug. 2013.
- [42] Andrea D. Weston and Leroy Hood, "Systems Biology, Proteomics, and the Future of Health Care: Toward Predictive, Preventative, and Personalized Medicine," *Journal of Proteome Research*, vol. 3, no. 2, pp. 179-196, Mar. 2004.
- [43] T. J. Griffin, S. P. Gygi, T. Ideker, B. Rist, J. Eng, L. Hood, and R. Aebersold, "Complementary profiling of gene expression at the transcriptome and proteome levels in Saccharomyces cerevisiae.," *Mol. Cell. Proteomics*, vol. 1, no. 4, pp. 323–33, Apr. 2002.
- [44] S. P. Gygi, B. Rist, S. A. Gerber, F. Turecek, M. H. Gelb, and R. Aebersold, "Quantitative analysis of complex protein mixtures using isotope-codedaffinity tags," *Nat. Biotechnol.*, vol. 17, no. 10, pp. 994–999, Oct. 1999.
- [45] N. S. Baliga, M. Pan, Y. A. Goo, E. C. Yi, D. R. Goodlett, K. Dimitrov, P. Shannon, R. Aebersold, W. V. Ng, and L. Hood, "Coordinate regulation of energy transduction modules in Halobacterium sp. analyzed by a global systems approach.," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 99, no. 23, pp. 14913–8, Nov. 2002.
- [46] E. Brunk, N. Mih, J. Monk, Z. Zhang, E. J. O'Brien, S. E. Bliven, K. Chen, R. L. Chang,
 P. E. Bourne, and B. O. Palsson, "Systems biology of the structural proteome," *BMC Syst. Biol.*, vol. 10, no. 1, p. 26, Dec. 2016.
- [47] W. B. Dunn, D. I. Broadhurst, H. J. Atherton, R. Goodacre, and J. L. Griffin, "Systems level studies of mammalian metabolomes: the roles of mass spectrometry and nuclear magnetic resonance spectroscopy," *Chem. Soc. Rev.*, vol. 40, no. 1, pp. 387–426, 2011.
- [48] A. Ambrus and L. Fésüs, "Comparison of NMR and X-ray crystallography.".
- [49] Y. Ho, A. Gruhler, A. Heilbut, G. D. Bader, L. Moore, S.-L. Adams, A. Millar, P. Taylor, K. Bennett, K. Boutilier, L. Yang, C. Wolting, I. Donaldson, S. Schandorff, J. Shewnarane, M. Vo, J. Taggart, M. Goudreault, B. Muskat, C. Alfarano, D. Dewar, Z. Lin, K. Michalickova, A. R. Willems, H. Sassi, P. A. Nielsen, K. J. Rasmussen, J. R. Andersen, L. E. Johansen, L. H. Hansen, H. Jespersen, A. Podtelejnikov, E. Nielsen, J. Crawford, V. Poulsen, B. D. Sørensen, J. Matthiesen, R. C. Hendrickson, F. Gleeson, T. Pawson, M. F. Moran, D.

Durocher, M. Mann, C. W. V. Hogue, D. Figeys, and M. Tyers, "Systematic identification of protein complexes in Saccharomyces cerevisiae by mass spectrometry," *Nature*, vol. 415, no. 6868, pp. 180–183, Jan. 2002.

- [50] A. Bensimon, A. J. R. Heck, and R. Aebersold, "Mass spectrometry-based proteomics and network biology.," *Annu. Rev. Biochem.*, vol. 81, pp. 379–405, 2012.
- [51] S. Chen, J. Weddel, P. Gupta, G. Conard, J. Parkin, and P. I. Imoukhuede, "QFlow Cytometer-Based Receptoromic Screening: A High-throughput Quantification Approach Informing Biomarker Selection and Nanosensor Development," *Submiss.*, 2016.
- [52] S. Pan, R. Aebersold, R. Chen, J. Rush, D. R. Goodlett, M. W. McIntosh, J. Zhang, and T. A. Brentnall, "Mass spectrometry based targeted protein quantification: methods and applications.," *J. Proteome Res.*, vol. 8, no. 2, pp. 787–97, Feb. 2009.
- [53] J. P. Nolan and F. Mandy, "Multiplexed and microparticle-based analyses: Quantitative tools for the large-scale analysis of biological systems," *Cytom. Part A*, vol. 69A, no. 5, pp. 318–325, May 2006.
- [54] C. S. H. Tan and R. Linding, "Experimental and computational tools useful for (re)construction of dynamic kinase-substrate networks.," *Proteomics*, vol. 9, no. 23, pp. 5233– 42, Dec. 2009.
- [55] P. Picotti, O. Rinner, R. Stallmach, F. Dautel, T. Farrah, B. Domon, H. Wenschuh, and R. Aebersold, "High-throughput generation of selected reaction-monitoring assays for proteins and proteomes.," *Nat. Methods*, vol. 7, no. 1, pp. 43–6, Jan. 2010.
- [56] J. a Hewel, J. Liu, K. Onishi, V. Fong, S. Chandran, J. B. Olsen, O. Pogoutse, M. Schutkowski, H. Wenschuh, D. F. H. Winkler, L. Eckler, P. W. Zandstra, and A. Emili, "Synthetic peptide arrays for pathway-level protein monitoring by liquid chromatography-tandem mass spectrometry.," *Mol. Cell. Proteomics*, vol. 9, no. 11, pp. 2460–73, Nov. 2010.
- [57] E. A. Kislinger T, Gramolini AO, Maclennan DH, "Multidimensional protein identification technology (MudPIT): technical overview of a profiling method optimized for the comprehensive proteomic investigation of normal and diseased heart tissue.," *J Am Soc Mass Spectrom*, vol. 16, pp. 1207–20, 2005.
- [58] D. B. Kell, M. Brown, H. M. Davey, W. B. Dunn, I. Spasic, and S. G. Oliver, "Metabolic footprinting and systems biology: the medium is the message.," *Nat. Rev. Microbiol.*, vol. 3,

no. 7, pp. 557–65, Jul. 2005.

- [59] P. I. Imoukhuede and A. S. Popel, "Quantification and cell-to-cell variation of vascular endothelial growth factor receptors," *Exp. Cell Res.*, vol. 317, no. 7, pp. 955–965, 2011.
- [60] P. I. Imoukhuede and A. S. Popel, "Expression of VEGF receptors on endothelial cells in mouse skeletal muscle," *PLoS One*, vol. 7, p. e44791, 2012.
- [61] P. I. Imoukhuede, A. O. Dokun, B. H. Annex, and A. S. Popel, "Endothelial cell-by-cell profiling reveals the temporal dynamics of VEGFR1 and VEGFR2 membrane localization after murine hindlimb ischemia.," *Am. J. Physiol. Heart Circ. Physiol.*, vol. 304, no. 8, pp. H1085-93, Apr. 2013.
- [62] P. I. Imoukhuede and A. S. Popel, "Quantitative fluorescent profiling of VEGFRs reveals tumor cell and endothelial cell heterogeneity in breast cancer xenografts.," *Cancer Med.*, vol. 3, no. 2, pp. 225–44, Apr. 2014.
- [63] J. P. Nolan, D. Condello, E. Duggan, M. Naivar, and D. Novo, "Visible and near infrared fluorescence spectral flow cytometry.," *Cytometry*. *A*, vol. 83, no. 3, pp. 253–64, Mar. 2013.
- [64] A. M. Smith, H. Duan, A. M. Mohs, and S. Nie, "Bioconjugated quantum dots for in vivo molecular and cellular imaging," *Adv. Drug Deliv. Rev.*, vol. 60, no. 11, pp. 1226–1240, 2008.
- [65] F. T. Lee-Montiel and P. I. Imoukhuede, "Engineering quantum dot calibration standards for quantitative fluorescent profiling," *J. Mater. Chem. B*, vol. 1, p. 6434, 2013.
- [66] F. T. Lee-Montiel, P. Li, and P. I. Imoukhuede, "Quantum dot multiplexing for the profiling of cellular receptors.," *Nanoscale*, vol. 7, no. 44, pp. 18504–14, Nov. 2015.
- [67] P. Aloy and R. B. Russell, "Structural systems biology: modelling protein interactions," *Nat. Rev. Mol. Cell Biol.*, vol. 7, no. 3, pp. 188–197, Mar. 2006.
- [68] A. Pujol, R. Mosca, J. Farrés, and P. Aloy, "Unveiling the role of network and systems biology in drug discovery," *Trends Pharmacol. Sci.*, vol. 31, no. 3, pp. 115–123, 2010.
- [69] A. Brückner, C. Polge, N. Lentze, D. Auerbach, and U. Schlattner, "Yeast two-hybrid, a powerful tool for systems biology.," *Int. J. Mol. Sci.*, vol. 10, no. 6, pp. 2763–88, Jun. 2009.
- [70] J. J. Hughey, T. K. Lee, and M. W. Covert, "Computational modeling of mammalian signaling networks.," *Wiley Interdiscip. Rev. Syst. Biol. Med.*, vol. 2, no. 2, pp. 194–209, 2010.
- [71] Å. Frostell, L. Vinterbäck, and H. Sjöbom, "Protein-Ligand Interactions Using SPR

Systems," 2013, pp. 139–165.

- [72] I. G. Khalil and C. Hill, "Systems biology for cancer," pp. 44–48, 2005.
- [73] H. Yu, I. Meyvantsson, I. a Shkel, and D. J. Beebe, "Diffusion dependent cell behavior in microenvironments.," *Lab Chip*, vol. 5, no. 10, pp. 1089–95, Oct. 2005.
- [74] R. B. Rutherford and R. Ross, "Platelet factors stimulate fibroblasts and smooth muscle cells quiescent in plasma serum to proliferate," *J Cell Biol*, vol. 69, no. 14, pp. 196–203, 1976.
- [75] C. J. Jackson and M. Nguyen, "Human microvascular endothelial cells differ from macrovascular endothelial cells in their expression of matrix metalloproteinases.," *Int J Biochem Cell Biol*, vol. 29, no. 10, pp. 1167–77, Oct. 1997.
- [76] B. A. Bryan and P. A. D'Amore, "Pericyte isolation and use in endothelial/pericyte coculture models.," *Methods Enzymol.*, vol. 443, pp. 315–31, Jan. 2008.
- [77] J. Jedelská, B. Strehlow, U. Bakowsky, A. Aigner, S. Höbel, M. Bette, M. Roessler, N. Franke, A. Teymoortash, J. A. Werner, B. Eivazi, and R. Mandic, "The chorioallantoic membrane assay is a promising ex vivo model system for the study of vascular anomalies.," *In Vivo*, vol. 27, no. 6, pp. 701–5, 2013.
- [78] I. Arnaoutova and H. K. Kleinman, "In vitro angiogenesis: endothelial cell tube formation on gelled basement membrane extract.," *Nat. Protoc.*, vol. 5, no. 4, pp. 628–35, Apr. 2010.
- [79] Z. Shao, M. Friedlander, C. G. Hurst, Z. Cui, D. T. Pei, L. P. Evans, A. M. Juan, H. Tahir,
 F. Duhamel, J. Chen, P. Sapieha, S. Chemtob, J.-S. Joyal, and L. E. H. Smith, "Choroid sprouting assay: an ex vivo model of microvascular angiogenesis.," *PLoS One*, vol. 8, no. 7, p. e69552, Jan. 2013.
- [80] C. a Staton, M. W. R. Reed, and N. J. Brown, "A critical analysis of current in vitro and in vivo angiogenesis assays.," *Int. J. Exp. Pathol.*, vol. 90, no. 3, pp. 195–221, Jun. 2009.
- [81] S. J. Maerkl, "Next generation microfluidic platforms for high-throughput protein biochemistry.," *Curr. Opin. Biotechnol.*, vol. 22, no. 1, pp. 59–65, Feb. 2011.
- [82] C. L. Stokes and D. a Lauffenburger, "Analysis of the roles of microvessel endothelial cell random motility and chemotaxis in angiogenesis.," *J. Theor. Biol.*, vol. 152, no. 3, pp. 377– 403, Oct. 1991.
- [83] A. a. De Graaf, A. P. Freidig, B. De Roos, N. Jamshidi, M. Heinemann, J. a C. Rullmann,

K. D. Hall, M. Adiels, and B. Van Ommen, "Nutritional systems biology modeling: From molecular mechanisms to physiology," *PLoS Comput. Biol.*, vol. 5, no. 11, p. e1000554, Nov. 2009.

- [84] Z. Qu, A. Garfinkel, J. N. Weiss, and M. Nivala, "Multi-scale modeling in biology: how to bridge the gaps between scales?," *Prog. Biophys. Mol. Biol.*, vol. 107, no. 1, pp. 21–31, Oct. 2011.
- [85] L. A. Chylek, E. C. Stites, R. G. Posner, and W. S. Hlavacek, "Systems Biology," pp. 273– 300, 2013.
- [86] W. W. Chen, B. Schoeberl, P. J. Jasper, M. Niepel, U. B. Nielsen, D. A. Lauffenburger, and P. K. Sorger, "Input–output behavior of ErbB signaling pathways as revealed by a mass action model trained against dynamic data," *Mol. Syst. Biol.*, vol. 5, p. 239, Jan. 2009.
- [87] M. W. Covert, N. Xiao, T. J. Chen, and J. R. Karr, "Integrating metabolic, transcriptional regulatory and signal transduction models in Escherichia coli.," *Bioinformatics*, vol. 24, no. 18, pp. 2044–50, Sep. 2008.
- [88] K. Bettenbrock, S. Fischer, A. Kremling, K. Jahreis, T. Sauter, and E.-D. Gilles, "A quantitative approach to catabolite repression in Escherichia coli.," *J. Biol. Chem.*, vol. 281, no. 5, pp. 2578–84, Mar. 2006.
- [89] H. N. Hayenga, B. C. Thorne, S. M. Peirce, and J. D. Humphrey, "Ensuring congruency in multiscale modeling: towards linking agent based and continuum biomechanical models of arterial adaptation.," *Ann. Biomed. Eng.*, vol. 39, no. 11, pp. 2669–82, Nov. 2011.
- [90] B. He, R. Baird, R. Butera, A. Datta, S. George, B. Hecht, A. Hero, G. Lazzi, R. Lee, J. Liang, M. Neuman, G. C. Y. Peng, E. Perreault, M. Ramasubramanian, M. Wang, J. Wikswo, G.-Z. Yang, and Y.-T. Zhang, "Grand challenges in interfacing engineering with life sciences and medicine," *IEEE Trans. Biomed. Eng.*, vol. 60, no. 3, pp. 589–598, 2013.
- [91] C. S. Greene, J. Tan, M. Ung, J. H. Moore, and C. Cheng, "Big Data Bioinformatics," J. Cell. Physiol., p. n/a-n/a, 2014.
- [92] M. Gerstein, "Genomics: ENCODE leads the way on big data," *Nature*, vol. 489, no. 7415, p. 208, 2012.
- [93] S. F. Martin, H. Falkenberg, T. F. Dyrlund, G. A. Khoudoli, C. J. Mageean, and R. Linding, "PROTEINCHALLENGE: Crowd sourcing in proteomics analysis and software

development," J. Proteomics, vol. 88, no. 0, pp. 41-46, 2013.

- [94] A. J. Lusis, A. D. Attie, and K. Reue, "Metabolic syndrome: from epidemiology to systems biology.," *Nat. Rev. Genet.*, vol. 9, no. 11, pp. 819–30, Nov. 2008.
- [95] Q. Meng, V.-P. Mäkinen, H. Luk, and X. Yang, "Systems Biology Approaches and Applications in Obesity, Diabetes, and Cardiovascular Diseases.," *Curr. Cardiovasc. Risk Rep.*, vol. 7, no. 1, pp. 73–83, Mar. 2013.
- [96] S. Pasinetti, Giulio M, Hiller-Sturmhofel, "Systems biology in the study of neurological disease: focus on Alzheimer's disease," *Putting Systems Biology Approaches into Practice*, 2008.
- [97] G. Juhász, I. Földi, and B. Penke, "Systems biology of Alzheimer's disease: how diverse molecular changes result in memory impairment in AD.," *Neurochem. Int.*, vol. 58, no. 7, pp. 739–50, Jun. 2011.
- [98] S. Pepke, T. Kinzer-Ursem, S. Mihalas, and M. B. Kennedy, "A dynamic model of interactions of Ca2+, calmodulin, and catalytic subunits of Ca2+/calmodulin-dependent protein kinase II.," *PLoS Comput. Biol.*, vol. 6, no. 2, p. e1000675, Mar. 2010.
- [99] P. K. Kreeger and D. a Lauffenburger, "Cancer systems biology: a network modeling perspective.," *Carcinogenesis*, vol. 31, no. 1, pp. 2–8, Jan. 2010.
- [100] M. R. Birtwistle, M. Hatakeyama, N. Yumoto, B. A. Ogunnaike, J. B. Hoek, and B. N. Kholodenko, "Ligand-dependent responses of the ErbB signaling network: experimental and modeling analyses.," *Mol. Syst. Biol.*, vol. 3, no. 144, p. 144, Jan. 2007.
- [101] S. Sanga, H. B. Frieboes, X. Zheng, R. Gatenby, E. L. Bearer, and V. Cristini, "Predictive oncology: a review of multidisciplinary, multiscale in silico modeling linking phenotype, morphology and growth.," *Neuroimage*, vol. 37 Suppl 1, pp. S120-34, Jan. 2007.
- [102] A. M. Gonzalez-Angulo, B. T. J. Hennessy, and G. B. Mills, "Future of personalized medicine in oncology: a systems biology approach.," *J. Clin. Oncol.*, vol. 28, no. 16, pp. 2777– 83, Jun. 2010.
- [103] R. A. Weinberg, *The biology of cancer*. 2007.
- [104] A. J. Guidi, S. J. Schnitt, L. Fischer, K. Tognazzi, J. R. Harris, H. F. Dvorak, and L. F. Brown, "Vascular permeability factor (vascular endothelial growth factor) expression and angiogenesis in patients with ductal carcinoma in situ of the breast.," *Cancer*, vol. 80, no. 10,

pp. 1945–53, Nov. 1997.

- [105] V. W. Rebecca, E. Wood, I. V Fedorenko, K. H. Paraiso, H. E. Haarberg, Y. Chen, Y. Xiang, A. Sarnaik, G. T. Gibney, V. K. Sondak, J. M. Koomen, and K. S. Smalley, "Evaluating melanoma drug response and therapeutic escape with quantitative proteomics," *Mol Cell Proteomics*, vol. 13, no. 7, pp. 1844–1854, 2014.
- [106] S. NA, F. Simpson, T. EW, H. MM, L. Endo-Munoz, G. Leggatt, M. RF, and A. Guminski, "Role of intratumoural heterogeneity in cancer drug resistance: molecular and clinical perspectives," *EMBO Mol Med*, vol. 4, pp. 675–684, 2011.
- [107] G. Bergers and D. Hanahan, "Modes of resistance to anti-angiogenic therapy," Nat Rev Cancer, vol. 8, no. 8, pp. 592–603, 2008.
- [108] A. B. Sandler, "Clinical trials comparing carboplatin/paclitaxel with or without bevacizumab in patients with metastatic NSCLC," *Lung Cancer Updat.*, vol. 2, pp. 6–10, 2005.
- [109] H. Hurwitz, "Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer," N Engl J Med, vol. 350, pp. 2335–2342, 2004.
- [110] B. P. Schneider and G. W. Sledge, "Drug insight: VEGF as a therapeutic target for breast cancer.," *Nat. Clin. Pract. Oncol.*, vol. 4, no. 3, pp. 181–9, Mar. 2007.
- [111] R. Erber, A. Thurnher, A. D. Katsen, G. Groth, H. Kerger, H.-P. Hammes, M. D. Menger, A. Ullrich, and P. Vajkoczy, "Combined inhibition of VEGF and PDGF signaling enforces tumor vessel regression by interfering with pericyte-mediated endothelial cell survival mechanisms.," *FASEB J.*, vol. 18, no. 2, pp. 338–40, Feb. 2004.
- [112] O. Casanovas, D. J. Hicklin, G. Bergers, and D. Hanahan, "Drug resistance by evasion of antiangiogenic targeting of VEGF signaling in late-stage pancreatic islet tumors," *Cancer Cell*, vol. 8, no. 4, pp. 299–309, Oct. 2005.
- [113] H. Iwata, S. Imamura, A. Hori, M. S. Hixon, H. Kimura, and H. Miki, "Biochemical characterization of TAK-593, a novel VEGFR/PDGFR inhibitor with a two-step slow binding mechanism," *Biochemistry*, vol. 50, no. 5, pp. 738–751, Feb. 2011.
- [114] A. Schwandt, V. E. von Gruenigen, R. M. Wenham, H. Frasure, S. Eaton, N. Fusco, P. Fu, J. J. Wright, A. Dowlati, and S. Waggoner, "Randomized phase II trial of sorafenib alone or in combination with carboplatin/paclitaxel in women with recurrent platinum sensitive

epithelial ovarian, peritoneal, or fallopian tube cancer.," *Invest. New Drugs*, vol. 32, no. 4, pp. 729–38, Mar. 2014.

- [115] R. Mabry, D. G. Gilbertson, A. Frank, T. Vu, D. Ardourel, C. Ostrander, B. Stevens, S. Julien, S. Franke, B. Meengs, J. Brody, S. Presnell, N. B. Hamacher, M. Lantry, A. Wolf, T. Bukowski, R. Rosler, C. Yen, M. Anderson-Haley, K. Brasel, Q. Pan, H. Franklin, P. Thompson, M. Dodds, S. Underwood, S. Peterson, P. V Sivakumar, and M. Snavely, "A dual-targeting PDGFRbeta/VEGF-A molecule assembled from stable antibody fragments demonstrates anti-angiogenic activity in vitro and in vivo.," *MAbs*, vol. 2, no. 1, pp. 20–34, 2010.
- [116] V. W. Rebecca, E. Wood, I. V. Fedorenko, K. H. T. Paraiso, H. E. Haarberg, Y. Chen, Y. Xiang, A. Sarnaik, G. T. Gibney, V. K. Sondak, J. M. Koomen, and K. S. M. Smalley, "Evaluating Melanoma Drug Response and Therapeutic Escape with Quantitative Proteomics," *Mol. Cell. Proteomics*, vol. 13, no. 7, pp. 1844–1854, Apr. 2014.
- [117] E. A. Logsdon, S. D. Finley, A. S. Popel, and F. Mac Gabhann, "A systems biology view of blood vessel growth and remodelling," *J. Cell. Mol. Med.*, vol. 18, no. 8, pp. 1491–1508, Nov. 2014.
- [118] F. Mac Gabhann and A. S. Popel, "Targeting neuropilin-1 to inhibit VEGF signaling in cancer: Comparison of therapeutic approaches," *PLoS Comput. Biol.*, vol. 2, no. 12, pp. 1649– 1662, Dec. 2006.
- [119] S. D. Finley, M. O. Engel-Stefanini, P. Imoukhuede, and A. S. Popel, "Pharmacokinetics and pharmacodynamics of VEGF-neutralizing antibodies," *BMC Syst. Biol.*, vol. 5, no. 1, p. 193, 2011.
- [120] S. D. Finley and A. S. Popel, "Predicting the effects of anti-angiogenic agents targeting specific VEGF isoforms.," AAPS J., vol. 14, no. 3, pp. 500–9, 2012.
- [121] J. C. Weddell and P. I. Imoukhuede, "Quantitative characterization of cellular membranereceptor heterogeneity through statistical and computational modeling," *PLoS One*, vol. 9, 2014.
- [122] H. F. Dvorak, V. M. Weaver, T. D. Tlsty, and G. Bergers, "Tumor microenvironment and progression.," J. Surg. Oncol., vol. 103, no. 6, pp. 468–74, May 2011.
- [123] P. Lu, V. M. Weaver, and Z. Werb, "The extracellular matrix: a dynamic niche in cancer

progression.," J. Cell Biol., vol. 196, no. 4, pp. 395–406, Mar. 2012.

- [124] M. Egeblad, M. G. Rasch, and V. M. Weaver, "Dynamic interplay between the collagen scaffold and tumor evolution.," *Curr. Opin. Cell Biol.*, vol. 22, no. 5, pp. 697–706, Oct. 2010.
- [125] O. Maller, C. C. DuFort, and V. M. Weaver, "YAP forces fibroblasts to feel the tension.," *Nat. Cell Biol.*, vol. 15, no. 6, pp. 570–2, 2013.
- [126] G. Haralabopoulos, "Inhibitors of basement membrane collagen synthesis prevent endothelial cell alignment in matrigel in vitro and angiogenesis in vivo.," ..., a J. Tech. ..., 1994.
- [127] R. O. Hynes and A. Naba, "Overview of the matrixome--an inventory of extracellular matrix constituents and functions.," *Cold Spring Harb. Perspect. Biol.*, vol. 4, no. 1, p. a004903, Jan. 2012.
- [128] M. Kanapathipillai, A. Mammoto, T. Mammoto, J. H. Kang, E. Jiang, K. Ghosh, N. Korin, A. Gibbs, R. Mannix, and D. E. Ingber, "Inhibition of mammary tumor growth using lysyl oxidase-targeting nanoparticles to modify extracellular matrix.," *Nano Lett.*, vol. 12, no. 6, pp. 3213–7, Jun. 2012.
- [129] H. Fang and Y. a Declerck, "Targeting the tumor microenvironment: from understanding pathways to effective clinical trials.," *Cancer Res.*, vol. 73, no. 16, pp. 4965–77, Aug. 2013.
- [130] R. Kalluri, "Basement membranes: structure, assembly and role in tumour angiogenesis.," *Nat. Rev. Cancer*, vol. 3, no. 6, pp. 422–33, Jun. 2003.
- [131] S. Huang, K. Shao, Y. Kuang, Y. Liu, J. Li, S. An, Y. Guo, H. Ma, X. He, and C. Jiang, "Tumor targeting and microenvironment-responsive nanoparticles for gene delivery.," *Biomaterials*, vol. 34, no. 21, pp. 5294–302, Jul. 2013.
- [132] A. Toma, A. Mang, T. A. Schuetz, S. Becker, and T. M. Buzug, "A novel method for simulating the extracellular matrix in models of tumour growth," *Comput. Math. Methods Med.*, vol. 2012, 2012.