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Abstract

The development of new 2D and 3D phenotypic screening assays combined with high-throughput genomic and proteomic technologies are well placed to advance a new era of molecular pathway informed Phenotypic Drug Discovery. We describe the application of Reverse Phase Protein Array (RPPA) technology to elucidate the mechanism-of-action of small molecules at the post-translational pathway level. We propose that profiling of phenotypic hits and lead molecules in increasingly more complex 3D *in vitro* and *ex vivo* models at the post-translational pathway network level represents an effective strategy to both triage and progress the preclinical development of phenotypic screening hits.

Introduction

Advances in new cell based assay technologies including primary patient-derived cell culture protocols, induced pluripotent stem cell technology, precise genome editing, 3-dimensional and microfluidic cell culture systems and automated high content imaging and image-informatics are converging to stimulate an exciting new era of phenotypic drug discovery [1, 2]. Such breakthroughs in cell culture technologies provide new opportunities to custom design phenotypic screening assays, which more accurately represent the genetic drivers and pathophysiology of diseased tissue [3]. The development of new cell based assay technologies promise to advance drug discovery into new disease areas, which have not previously been tractable to *in vitro* model systems [4]. While cell based assay technologies provide important functional assays to support target validation and subsequent testing of hit and lead molecules from target directed drug discovery strategies, their integration with automated liquid handling robotics and automated phenotypic data analysis pipelines provides new opportunities to incorporate more complex cell assays into phenotypic screening projects in the absence of molecular target hypotheses. In this article we highlight some of the challenges in de-convoluting the mechanism-of-action of small molecules identified as phenotypic hits at an individual molecular target level. We discuss how a high throughput antibody-based proteomics method called Reverse Phase Protein Array (RPPA) can help to profile compound mechanism-of-action at the post-translational pathway network level across dose-response and time-series studies performed in both 2D and 3D cell models to progress further preclinical development of phenotypic hits in an efficient manner.

Target deconvolution and mechanism-of-action profiling strategies in phenotypic drug discovery.

Phenotypic screening as defined in this article as "target agnostic screening and selection of hit molecules, lead compounds and approved drugs based on quantifiable phenotypic endpoints" has been proposed as an empirical drug discovery approach to identify new therapeutic targets or alternatively to accelerate target-agnostic drug discovery and development strategies [1, 2, 5]. In support of phenotypic-led discovery of new targets, cell based assay screening technologies are further complemented by advances in target deconvolution technologies, including chemical proteomics methods such as affinity mass spectrometry and thermostability shift assays, cDNA expression microarray technologies, haploid genetic/gene trap and whole genome CRISPR screening performed in parallel with pharmacological phenotypic assays among others [6-12].

However, the target deconvolution paradigm and associated methods described above make a number of assumptions, which are unlikely to be suitable for all phenotypic hits and disease models or the human disease indications which they represent. For example, the majority of target deconvolution strategies assume that the phenotypic hit or lead molecule exerts its phenotypic response through modulation of a single protein target, which does not account for compounds modifying phenotypes through multi-targeted polypharmacology or adaptation and reprogramming of transcriptional and post-translational pathway networks. For many complex disease and gain-of-function phenotypic assays it may also be unlikely that the one-drug/one-target paradigm will be sufficient to restore normal cell or tissue physiology and thus more complex multi-targeted approaches

will be required to reprogram cellular phenotypes. Furthermore, chemical proteomic strategies identify multiple proteins which bind to compounds, which necessitate substantial follow up with functional genomic studies to identify which protein binders represent the key therapeutic target responsible for the pharmacologically induced phenotype. Critical to the identification of true compound targets regulating cell phenotype over false positive non-specific binding interactions is the need to incorporate several negative control samples into the chemical proteomic workflow. Such negative control samples may include, inactive isomers of hit compounds, comparison of protein interaction profiles in extract from cells which show no or distinct phenotypic response to compound hits and negative (background control) samples using linker-bound affinity matrix (no compound) only samples. It is also unlikely that initial hits from a phenotypic screen will have sufficient potency or selectivity to support effective target deconvolution studies and thus further medicinal chemistry to improve potency and understand structure activity relationships with regards to phenotypic response will be required prior to instigating a target deconvolution program. Thus, there is a significant risk that poorly designed phenotypic screening and target deconvolution strategies may create expensive new drug discovery bottlenecks in target deconvolution and further investment of significant chemistry resources on poorly validated targets, which, do not directly address specific disease conditions and urgent unmet clinical needs.

We propose that target deconvolution should not be instigated early in a phenotypic drug discovery program. Rather, phenotypic leads should be carefully triaged through increasingly more complex and disease relevant secondary phenotypic assays to build further confidence in their translational potential and a deeper understanding of mechanism-of-action at transcriptome and post-translational pathway levels to support

both, subsequent target deconvolution activities or further preclinical development with or without knowledge of the target (Figure 1). This more broader view of profiling the mechanism-of-action of phenotypic hits beyond a single molecular target is facilitated by the development of new rapid and high-throughput genomic, proteomic and phenotypic profiling methodologies [13-15]. Recent advances in mechanism-of-action profiling technologies include high throughput gene transcription profiling [13, 16]. For example, the Connectivity Map program developed by the Broad Institute combines a public repository of gene expression profiles collected from large panels of compound perturbed samples with computational and statistical methods to support similarity profiling of gene expression patterns to infer compound MOA [13]. Further technical advances in higher throughput and more cost-effective gene-expression methods such as the L1000™ expression profiling platform which underpins the Library of Integrated Cellular Signatures (LINCS) NIH program, supports drug MOA profiling at the transcriptional level at scale [16-18]. The impact of higher throughput transcriptional profiling upon phenotypic drug discovery programs remains to be fully determined and will likely depend upon optimal experimental designs and inclusion within logical phenotypic drug discovery workflows to ensure these methods are applied to the most appropriate compounds and cell models. While many of the underlying causes of cancer occur at genetic and epigenetic levels, the direct targets of drugs are typically functional proteins, and thus drug MOA and the most appropriate pharmacodynamic biomarkers are likely to be discovered at the protein level. Also for those complex disease traits that do not represent single gene disorders, understanding the dynamic post-translational pathway networks that control and predict therapeutic response across a heterogeneous patient population and evolving disease progression are essential to progress effective drug discovery and development. Therefore, only by studying the

dynamic state of the proteome and its functional states we can obtain a clear understanding of the relationship between a drug's mechanism and disease to adequately inform biomarker discovery and drug development programs that embrace the complexities of disease.

Traditionally, functional proteomic methodology has relied on quantitative mass-spectrometry techniques such as ITRAQ (Isobaric Tags for Relative and Absolute Quantitation) and SILAC (Stable Isotope Labelling with Amino acids in Cell culture), which remain the standard approaches for *de novo* identification of post-translational biomarkers [19]. However, limitations in speed, cost, sensitivity and reproducibility of quantitative mass spectrometry approaches has restricted their routine application across multiple samples at scale. The evolution of antibody-based RPPA, combined with more sophisticated sample handling, optical detection and better quality (validated mono-specific) antibody reagents, provide an alternative approach enabling exquisite sensitivity and substantial sample throughput of functional proteomic analysis across multiple pathways [20, 21]. Analysis of protein abundance and post-translational activation states of pathways across dose-response and time series may be a more appropriate and informative methodology toward identification of pharmacodynamic or predictive biomarkers to progress phenotypic hits.

Reverse Phase Protein Array (RPPA) platforms

RPPA represents a high-throughput and miniaturized immunoassay methodology, which provides precise quantitative analysis of the abundance of total protein and post-translationally modified protein analytes across multiple biological samples, including preclinical and clinical samples [14, 20, 21]. In contrast to standard ELISA based formats, in

“reverse phase” protein arrays, the protein analytes as part of crude tissue or whole cell lysate are immobilized on the solid phase and subsequently probed with antibodies toward a specific protein or modified protein epitope. The recent evolution of RPPA combined with robust sample preparation, more sophisticated sample handling, optical detection and better quality affinity reagents provides exquisite sensitivity (down to marker detection from single cell equivalents) and high sample throughput (100s of samples in parallel in one assay) at a reasonable cost per sample. This facilitates large-scale multiplex analysis of multiple post-translational markers across samples from in vitro, preclinical, or clinical samples. Recent applications include: Biomarker discovery in preclinical and clinical sample cohorts to identify post-translational prognostic or predictive biomarkers which correlate with disease progression or therapeutic response [22, 23]; Profiling drug MOA by pathway network analysis across dose-response and time-series studies in biological samples following compound exposure and correlation with phenotypic effects to determine pharmacodynamic biomarkers and novel pathway interaction networks. [14, 24]. Profiling pathway network response in biological samples before and after compound exposure to determine the activation state of druggable pathways which correlate with drug insensitivity or drug resistance and which can be mapped to drug-target databases thus informing upon rational drug combination strategies[25, 26].

RPPA employs the following core processes: total protein extracts are prepared from cell culture (2D/3D), animal (e.g. Xenograft models) or human clinical tissue using quality assured procedures, and samples are spotted onto nitrocellulose or a hydrophobic-coated chip surface, in a miniaturized dot-blot manner, using automated sample printing systems. The extracted protein sample sets are printed across multiple distinct locations (sub-arrays) physically separated from each other. Immobilized protein sub-arrays are then incubated

with distinct mono-specific antibodies to detect individual proteins, or their post-translationally modified forms in each (one antibody on one individual sub-array). Protein samples are typically printed as a concentration series in replicates and multiple exposure and/or curve fitting parameters ensure measurement of protein abundance is conducted in the linear range to obtain a single value (e.g. in RFI = Relative Fluorescence Intensity units) which provides an absolute or relative measure of protein abundance across a sample set (Figure 2). Because the primary antibodies are physically separated from each other between sub-arrays there is no issue of cross-reactivity between antibody reagents enabling unlimited multiplexing of RPPA validated antibodies. The majority of RPPA platforms require nano-litres of protein lysate and picogram-to-femtogram quantities of protein, so permitting analysis of small preclinical and clinical samples for up to 100s of proteins of interest. The Zeptosens platform uses planar waveguide technology encompassing nano-structured glass protein array chips, further enhancing the read-out sensitivity (Figure 2) [20]. Excitation laser light is directed into the waveguiding layer by means of a nano-structured diffractive grating on the chip surface, orthogonally to the fluorescence emission path. The evanescent measurement of bound, labelled antibodies by the ZeptoREADER is confined to the sample surface, minimizing background interference from unbound antibodies or excitation light, and maximizing signal-to-noise ratios regardless of the low levels of individual proteins [20]. In combination with well validated antibodies and a user-friendly, easy-to-handle assay equipment, the Zeptosens platform provides exquisite sensitivity (zeptomole and single cell equivalent levels of protein abundance per spot), quantitative linear signal response and good assay robustness and reproducibility with CVs of approximately 4% in a direct low volume immunoassay that does not require further signal amplification steps [14, 20, 27]. The enhanced sensitivity provided by the advances in optical detection and protein

microarray design enables further miniaturization of sample detection (down to 400 pico-litres) and accordingly lower sample and reagent volumes (Figure 2). Thus, while RPPA offers similar levels of reproducibility to ELISA and automated western blotting assay methods, RPPA can provide increased sensitivity of several orders of magnitude for low abundant proteins relative to ELISA, Western and Mass Spectrometry across hundreds of proteins and hundred of samples simultaneously [14].

The modern generation of RPPA platforms provides a cost-effective solution for high throughput post-translational pathway analysis and biomarker research, supporting a variety of clinical and preclinical applications. An expanding set of validated mono-specific antibodies ensure RPPA methods can be used to profile broad pathway responses simultaneously. Pathways typically covered in RPPA studies include well-characterized canonical signalling pathways, multiple DNA repair, cell-cycle, growth and apoptosis-regulating proteins, transcription factors and multiple histone modifications. The technical advances in RPPA detection methodologies are complemented by developments in standard operating procedures, reagents, liquid handling equipment and best-practices for, preservation of proteins and post-translational modifications, reproducible sample printing and analysis, tailored to the needs of complex mixtures of cell- or tissue-derived protein extracts [14]. Environmentally controlled liquid handling instruments that create highly uniform arrays of complex protein samples have significantly advanced the throughput, sensitivity and reproducibility of protein/antibody array based proteomics (Figure 2).

RPPA profiling drug mechanism-of-action

An important area of modern drug discovery is the identification of predictive and pharmacodynamic biomarkers that direct dosing, patient stratification and/or inform on appropriate drug combination strategies to enhance efficacy and counteract anticipated drug resistance mechanisms. Separate studies by Cardnell *et al* and Ummanni *et al.* correlated drug sensitivity across a panel of cancer cell lines with basal levels of protein and post-translational modifications determined via RPPA to identify minimal sets of protein markers that predict drug sensitivity and resistance [25, 28]. Thus, RPPA can be used to identify therapeutic response markers that might be readily suitable for the development of antibody-based diagnostic tests to select patients for treatment studies. RPPA has also been used to uncover unanticipated MOA of drugs already in clinical use. Retrospective analysis of esophageal adenocarcinoma patients who were also being treated with the biguanide small molecule Metformin for diabetes demonstrated a better response to chemoradiation therapy compared with patients who were not receiving Metformin [29]. However, the MOA of Metformin in esophageal cancer was unknown. RPPA analysis applied to esophageal cancer cells treated with Metformin revealed inhibition of PI3K/mTOR signaling pathway, which correlated with reduced cell growth and increased apoptosis [30]. Using a similar approach, retrospective analysis comparing recurrence rates for breast cancer demonstrated a significant reduction in recurrence rates in breast cancer patients that were users of the lipid-lowering drug Simvastatin [31]. RPPA analysis of triple-negative breast cancer cell lines following Simvastatin treatment demonstrated decreased phosphorylation of FOXO3a. Subsequent knockdown of FOXO3a attenuated the effect of Simvastatin on suppression of 3D *in vitro* mammosphere formation and migration [32]. Corilagin, which has recently been identified as a major active component in a well-known herbal medicine (*Phyllanthus niruri L.*) elcits antitumor activity although an unknown mechanism. RPPA

analysis of a panel of ovarian cancer cell lines treated with Corilagin demonstrated suppression of canonical Smad and noncanonical ERK/AKT pathways, which correlated with inhibition of TGF-β secretion and TGF-β pathway activation [33].

Another study combined RPPA analysis and whole genome RNA sequencing over a time series following compound exposure to evaluate the MOA of novel potent organometallic compounds in ovarian cancer cell lines [34]. RPPA analysis revealed that the organometallic compound induced a DNA damage signaling response with upregulation of p21, p53 and ATM proteins detected by RPPA [34]. Investigation into the signaling pathways mediated downstream of the therapeutic target FLT1 included RPPA analysis of bone marrow derived macrophages following exposure to the FLT1-neutralizing antibody MF1 [35]. These studies indicated that Focal Adhesion Kinase (FAK) signaling may represent the mechanistic link between FLT1 activation to downstream gene expression and thus represents an appropriate pharmacodynamic biomarker for FLT-1 targeting therapies [35]. Evaluation of a collection of small molecule Raf inhibitors, tested across a panel of patient derived melanoma cell lines which exhibit resistance to the approved BRAF inhibitor Vemurafenib identified two novel pan-Raf inhibitors, CCT196969 and CCT241161 which inhibited the growth of the Vemurafenib resistant melanoma lines [36]. RPPA analysis revealed that inhibition of the phosphorylation of Src by CCT196969 and CCT241161 correlated with sensitivity in Vemurafenib resistant cell lines demonstrating that concurrent inhibition of Raf and Src family kinases co-operate to inhibit the growth of cells that are resistant to BRAF-selective inhibitors [36]. RPPA analysis following treatment of a panel of AML (Acute Myeloid Leukaemia) cell lines with the small molecule pan-Pim kinase inhibitor AZD1208 detected suppression of phosphorylation of mTOR (Ser2448), p70S6K (Thr389), S6 (Ser235/236), and 4E-BP1 (Ser65) consistent with a reduction in protein synthesis which

correlated with cell size reduction and growth inhibition following AZD1208 treatment [37]. Thus RPPA analysis reveals that mTOR pathway inhibition contributes to the MOA of AZD1208 [37]. While the majority of exemplar studies describing RPPA applications in drug MOA analysis have been applied to late-stage or approved drugs many of which have come from target-directed drug discovery, the success of this approach in revealing unanticipated drug MOA indicates this will also be a useful method for uncovering the MOA of hits and lead compounds derived from phenotypic screens. Phenotypic screening with non-ribosomal peptide synthase (NRPS)-derived natural products has potential to provide a wealth of therapeutic leads, which explore broad areas of novel target biology owing to the extensive chemical and structural diversity that they encompass. Evaluation of the linear peptide bisebromoamide (BBA), isolated from a marine cyanobacterium in HCT116 colorectal carcinoma cells demonstrated anti-growth and modulation of the actin cytoskeleton phenotypes [38]. Subsequent RPPA analysis of HCT116 pathway responses suggests BBA has a selective MOA with dose-dependent activity upon inhibition of protein levels of the oncogenic signaling protein, insulin receptor substrate (IRS-1) [38].

RPPA has also been used to identify new, unexpected mechanisms of targeted therapy resistance and compensatory signalling pathways. In such work the ability to quantitatively measure the activation state of many dozens of signalling proteins simultaneously over time identifies new PD biomarker and drug combination strategies which monitor and target adaptive resistance mechanisms [26]. The identification of feedback loop mechanism pathway markers of drug resistance can be directly cross-referenced to approved drug or broader drug-target databases to build rational drug combination hypotheses for further testing [39]. While recent advances in RPPA technology have improved the sensitivity and fidelity of proteomic analysis across complex biological samples a number of limitations

remain. For example RPPA is dependent upon the availability and validation of high-quality monospecific antibody reagents that can detect with high affinity and specificity a protein or post-translationally modified protein on a solid matrix. The challenges associated with identifying and validating suitable antibody reagents limits most RPPA studies to analysis of a few hundred protein analytes, thus RPPA proteomic analysis is inherently biased to the pathways for which suitable antibody reagents are available. RPPA represents a homogeneous assay on complex cell lysates and thus does not provide sufficient spatial information of protein expression across cell subpopulations from heterogeneous samples such a co-culture assays or tissue samples. RPPA methods are currently not standardized between laboratories due to the broad variety of platforms, protein sample preparation protocols and analysis approaches. Despite these limitations the RPPA advances described in this article are poised to complement alternative genomic and mass spectrometry technologies by enabling rapid and cost-effective profiling of post-translational pathway network dynamics following compound exposure in biological systems.

RPPA profiling for drug mechanism-of-action in 3D vs 2D model systems

New advances in *in vitro* cell culture assay formats provide a variety of 3D-cell cultures systems which attempt to better mimic the extracellular composition and architecture of *in vivo* tissues. 3D *in vitro* models which utilize both natural and synthetic biomaterials are available in multiwell formats which have been developed specifically for medium- to high-throughput phenotypic screening [40]. Specific focus and substantial research efforts have been placed on the development and application of 3D tumor spheroid assay systems which simulate the multicellular, 3D architecture and hypoxic characteristics of the *in vivo* tumor

microenvironment [41, 42]. 3D tumor spheroid models are readily amenable to RPPA profiling of drug and compound mechanism-of action at the molecular pathway level within such 3D microenvironments. As an example, Figure 3 shows that RPPA could successfully elucidate distinct molecular pathway response upon treatment with Torin 2 (shown here), one out of a number of compounds, studied in more detail from a phenotypic compound library screen: heterotypic 3D microtumors (3D HEY/NIH3T3 cell cultures, 500 µm diameter, kindly provided by Jens M. Kelm, InSphero AG, Schlieren, Switzerland) were treated at IC50 concentration at two different time points (1 h and 48 h) and subsequently analyzed with RPPA and almost 50 antibodies covering key nodes of different pathways. Log2 fold changes (ratios of treatment to vehicle control signals, in triplicate) revealed a clear inhibition of the PI3K/Akt/mTOR (Figure 3), with prominent down-regulation of downstream S6 ribosomal protein phosphorylated at Ser240/244, Ser235/236, and to a minor extent also for other markers, indicating a strong inhibitory effect on translation and cell growth control; no inhibition or cross-compensatory effects could be observed in the other pathways studied.

Ex vivo culture of 3D human tissues or established cell lines seeded on human *ex vivo* tissue scaffolds potentially provide a more physiologically relevant tissue substrate albeit within a non-physiological environment with limited throughput for screening. One such example is represented in Figure 4 which represents an *ex vivo* culture of a green fluorescent protein (GFP) labelled human pancreatic cancer cell line (PANC-1) on a human 3D retroperitoneal tissue slice culture. Human abdominal peritoneal and retroperitoneal (behind the peritoneum) tissue is readily available from routine abdominal surgery and are composed of layers of epithelial mesothelium and connective tissue. The retroperitoneal tissue

represents a major site of pancreatic cancer metastasis [43]. Fresh retroperitoneal tissue available from human surgery was dissected by tissue slice and placed in the upper chamber of a transwell insert to form a 3D tissue substrate for *in vitro* cell culture (Figure 4). Protein extracts for RPPA analysis were prepared from the pancreatic cancer cell line, PANC-1 cultured on a standard 2D cell culture plastic substrate and the 3D retroperitoneal tissue following 24hrs treatment with a 3-point log-dose response of Gemcitibine (1, 0.1 and 0.01μM) and DMSO control. Each treatment was performed in duplicate across all samples and protein extracts prepared from each sample were spotted onto the Zeptosens RPPA microarray chip at 4 separate dilutions (0.2; 0.15; 0.1 and 0.75mg/ml). A single relative fluorescence intensity (RFI) value relating to protein abundance is obtained for each pathway analyte by a weighted linear least squares fit through each dilution series and a standard deviation value is calculated from the fit and Sahpiro-Wilk statistical test of intensity distributions across all 8 data-points/replicate spots for each treatment. RPPA analysis of the pathway signalling response of PANC-1 cells following exposure to the nucleoside analogue, Gemcitibine clearly shows a suppressed induction of the p53 DNA damage response and enhanced signalling through the SRC/FAK pathway in PANC-1 cells cultured on 3D retroperitoneal *ex vivo* tissue relative to 2D cell culture plastic substrates (Figure 4). This data demonstrates that the tissue microenvironment of retroperitoneal tissue can alter the intracellular signalling response of pancreatic cancer cells to Gemcitabine, including induction of cell growth and survival signalling and reduced activation of the p53 tumour suppressor pathway, which may contribute to drug resistance. In contrast to potent cell killing activity observed in standard 2D *in vitro* pancreatic cancer cell viability assays, drug resistance and poor efficacy response to Gemcitabine was observed *in vivo* and in clinical studies [44] [45]. These experiments were performed on

retroperitoneal tissue taken from a single patient, further studies are underway to determine the reproducibility or heterogeneity in pancreatic cancer cell response when cultured on genetically- and physiologically-distinct retroperitoneal tissue samples from different patients. Thus characterizing MOA in more disease relevant, heterogeneous, 3D *ex vivo* assays will help triage the most promising lead molecules or drug candidates and biomarkers to inform further investments in preclinical development or target deconvolution.

Conclusions

Advances in high-throughput genomic and proteomic technologies such as RPPA supports informed mechanistic classification and triaging of phenotypic hits to further assist target deconvolution and preclinical development. Such RPPA profiling of post-translational pathways can facilitate the selection of the most appropriate phenotypic hits to take forward into further preclinical development, identify new predictive and pharmacodynamic biomarkers or new assay endpoints to support further hit-to-lead chemical optimization, provide corroborative evidence for target deconvolution studies and support further preclinical development and translation towards clinical studies with or without conclusive target identification. This more in-depth biological investigation of drug MOA at pathway levels in complex 3D and *ex vivo* models are well placed to shift the PDD bottleneck from target deconvolution towards increased disease relevance and hopefully improved efficacy and drug discovery productivity.

Figure Legends

Figure 1. Phenotypic Drug Discovery Operating Model. Our proposal for more informative and productive phenotypic drug discovery includes the progression of phenotypic hits or lead compound identified from phenotypic screening assays through increasingly more complex and disease relevant preclinical models to build increased confidence in their translational potential and a deeper understanding for drug mechanism-of-action at the transcriptomic and post-transational pathway network levels to supper subsequent target deconvolution and/or further preclinical and clinical development with our without a single molecular target hypothesis.

Figure 2. Reverse Phase Protein Array (RPPA) Procedure – Schematic describing the major steps in the RPPA experimental and analysis procedure.

Figure 3. (A.) An example of the application of RPPA profiling of hit compounds identified from a 3Dtumour spheroid phenotypic assay. (B.) Elucidation of compound mechanism-of-action (shown here for Torin 2) with heterotypic 3D microtumours (treated HEY/NIH3T3 cultures, kindly prepared and provided by InSphero AG), tested with RPPA across PI3K/Akt/mTOR pathway and others (not shown). FC = fold change of treatment-to-control signals, N=3 replicates. Data show prominent down-regulation of S6 ribosomal protein phosphorylated at Ser240/244 and Ser235/236, indicating strong inhibition on translation and cell growth.

Figure 4. RPPA applied to 3D *ex vivo* tissue culture assays. The human pancreatic cancer cell line PANC-1 expressing green fluorescent protein (GFP) was cultured on standard 2D tissue culture plastic substrates (A.) or 3D human retroperitoneal (RP) *ex vivo* tissue obtained from general surgery (B.). Exposure of PANC-1 cells cultured in 2D cell culture to Gemcitibine

induced a clear induction in total and phosphorylated p53 levels (C.) and a modest induction of p38MAPK protein levels (D.). Culture of PANC-1 cells on 3D retroperitoneal (RP) ex-vivo tissue results in a dramatic reduction in Gemcitibine stimulation of p53 tumour suppressor and p38MAPK stress protein response (C. and D.). Culture of PANC-1 cells on 3D retroperitoneal (RP) *ex-vivo* tissue also promoted increased stimulation of Focal Adhesion Kinase (FAK) (E.) and Src kinase (F.) signalling in response to Gemcitibine exposure relative to PANC-1 cells cultured on 2D plastic. All RPPA values represent abundance of protein analytes normalized to DMSO control treatments for each culture substrate (2D and 3D retroperitoneal tissue) with standard deviation values across samples replicates.

Acknowledgements

M.P. Acknowledges support from NMI TT and NMI Natural and Medical Sciences Institute, Reutlingen. M.P. also thanks Jens M. Kelm and Zoe Weydert (both InSphero AG, Schlieren, Switzerland) for preparation and provision of treated 3D microtumor samples of high quality for RPPA analyses, and acknowledges intense discussions with Jens M. Kelm and Christian Schmees (NMI) about phenotypic screening of 3D models and its combination with down-stream genomic and proteomic profiling. N.O.C. Acknowledges previous support from RCUK for an academic fellowship, support from the Cancer Research UK Edinburgh Centre and also thanks M. Duxbury (University of Edinburgh) for assistance with retroperitoneal tissue culture.

Conflicts of Interest

The author(s) have no conflict of interest to declare.

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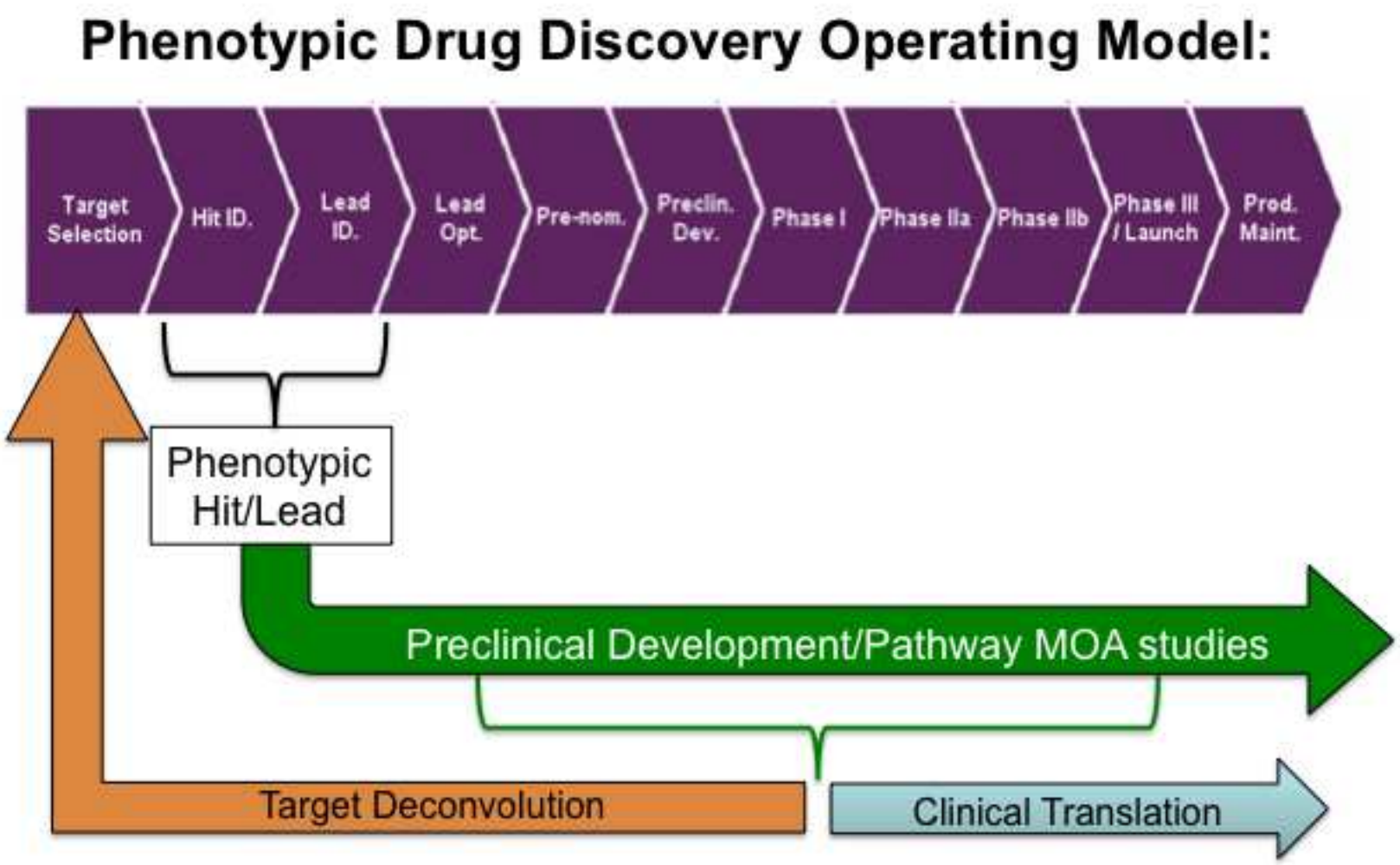

Figure 1

Figure2

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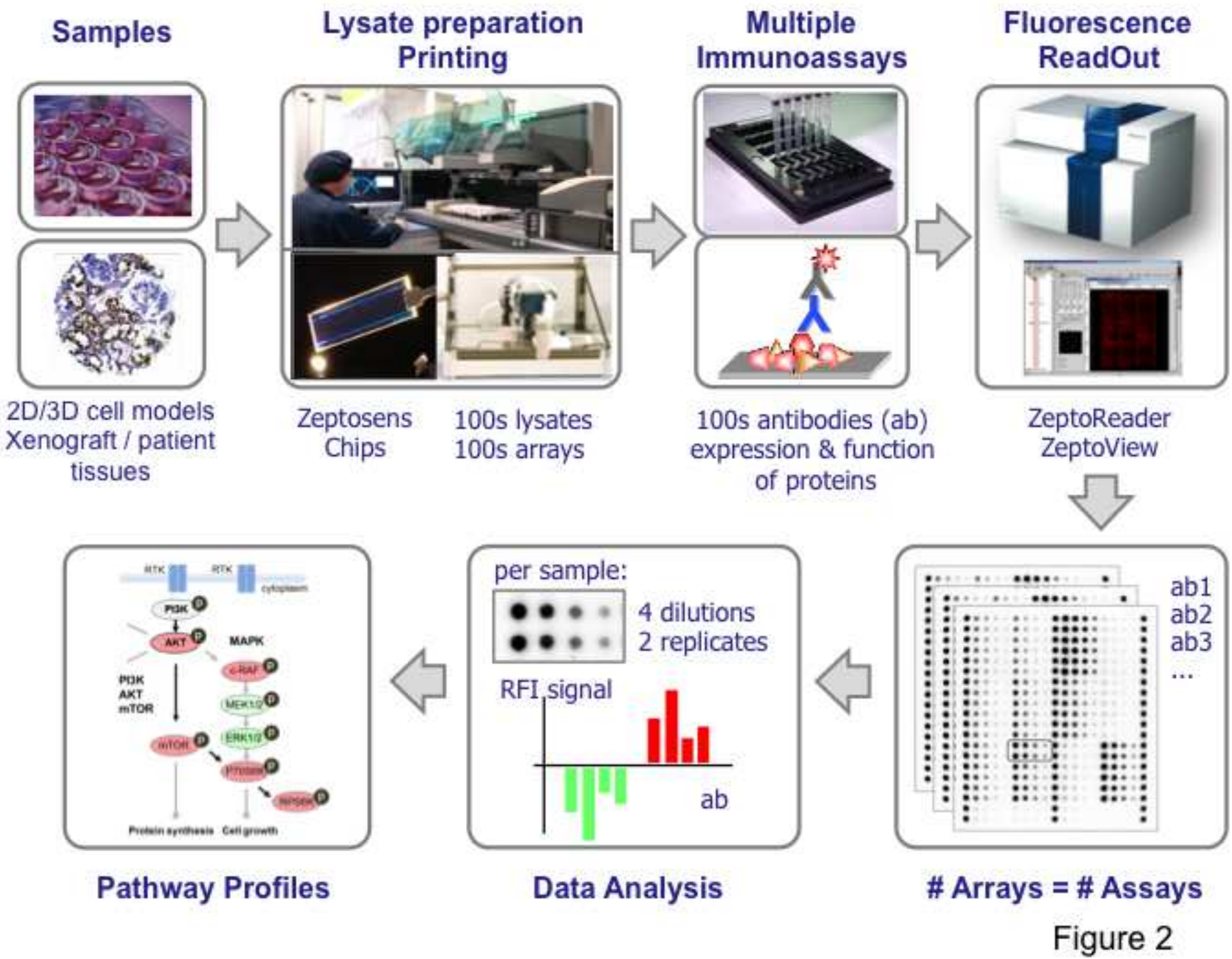

Figure 2

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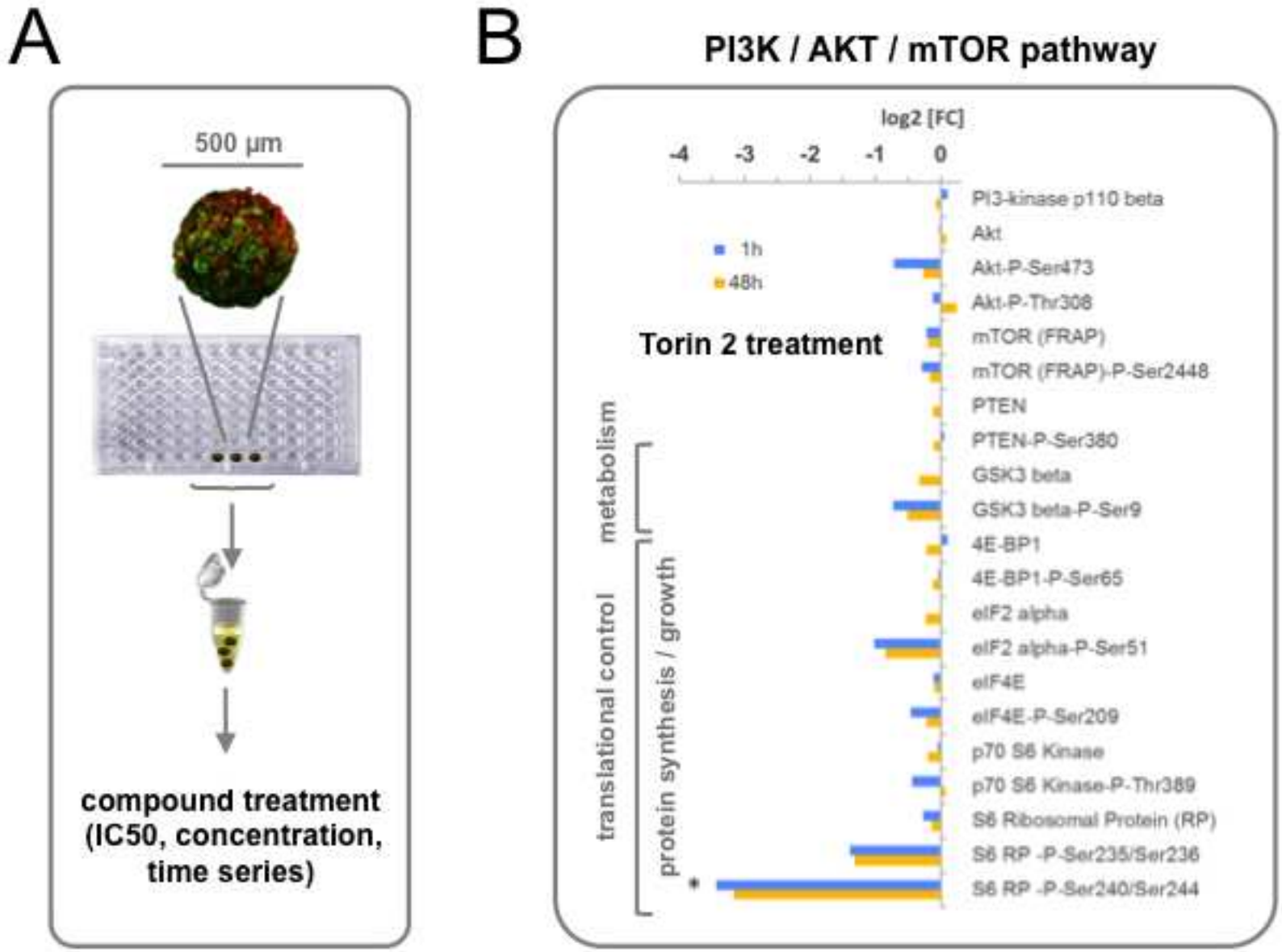

Figure 3

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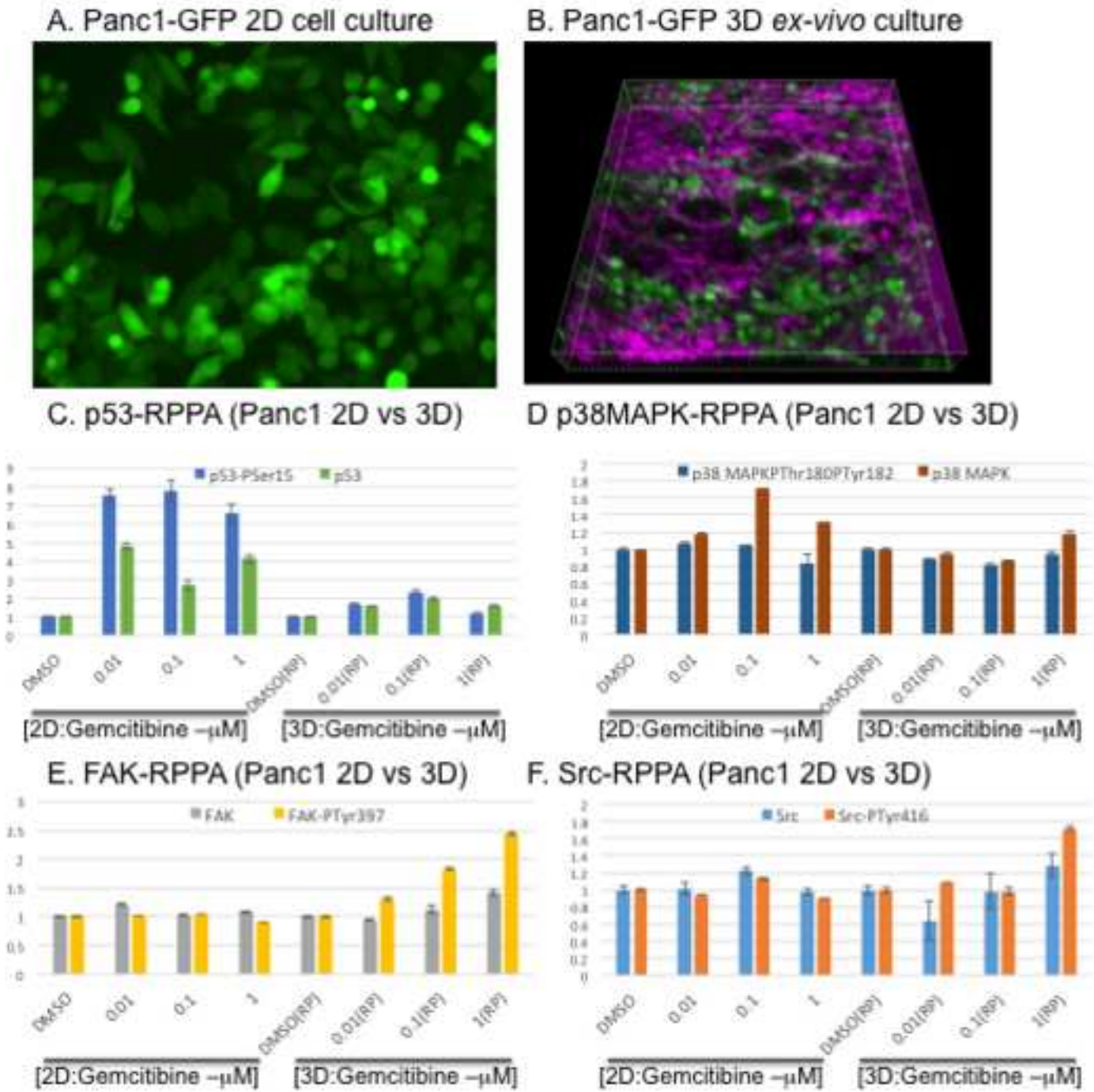

Figure 4