Design, Optimization, and Validation of Multiplex Immunofluorescence Assay for Detecting Biomarker Expression on Circulating Tumor Cells in Breast Cancer

Tanya Kumar[1], Hariprasad Thangavel[1], Raksha Bhat[1], Noor Mazin Abdulkareem[1,2], Lacey E. Dobrolecki[3], Michael T. Lewis[3,4,5], Meghana V. Trivedi[1,2,3,4]

[1]Department of Pharmacy Practice and Translational Research, University of Houston; [2]Department of Pharmacological and Pharmaceutical Sciences, University of Houston; [3]Lester and Sue Smith Breast Center, Baylor College of Medicine; [4]Dan L. Duncan Comprehensive Cancer Center, Baylor College of Medicine; [5]Department of Molecular and Cellular Biology, Baylor College of Medicine

Contact:
Meghana V. Trivedi, Pharm.D., Ph.D., BCOP
Assistant Professor
Phone: 832-842-8335 | Email: mtrivedi@central.uh.edu

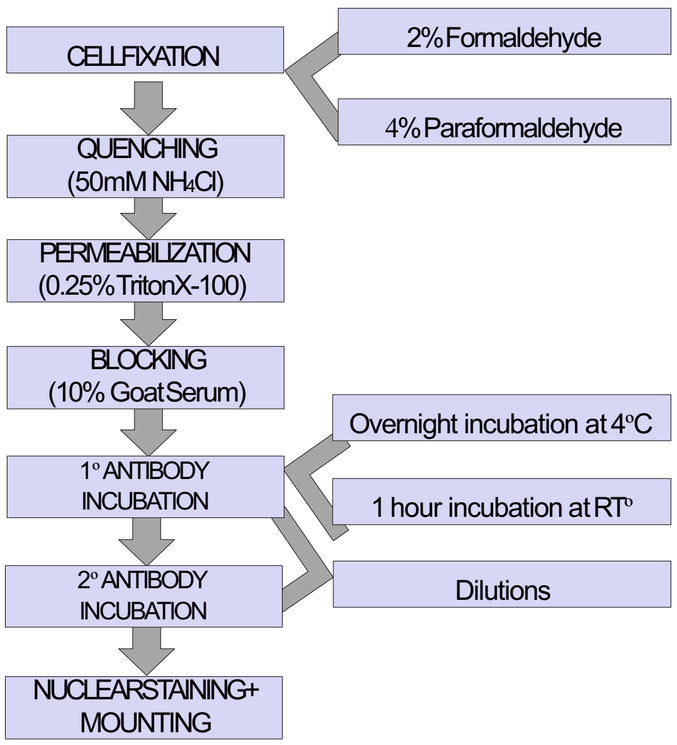
BACKGROUND

- Majority of breast cancer-related deaths occur due to metastasis. In the metastatic process, tumor cells travel from the primary site to distant organs.
- CTCs are tumor cells found in the bloodstream entering through either active intravasation or passive shedding of compromised vasculature.
- CTCs exist as epithelial, mesenchymal, or a hybrid of both. They can be found as individual cells or clusters; the latter has been shown to have increased metastatic potential.
- CTCs can serve as liquid biopsies as representative of the tumor bulk in patients since it is less-invasive, allowing more frequent and real-time monitoring of treatment response over conventional tissue biopsies.

OBJECTIVE

- The purpose of this study was to design, optimize and validate a multiplex immunofluorescence (IF) assay to identify CTCs and detect a panel of biomarkers on CTCs.

METHODOLOGY



CTCs were defined as pan-CK-positive, CD45-negative, and nuclear stain-positive cells.

Target	1° antibody Host	2° antibody
Human Pan-CK	Mouse	Goat anti-mouse AF488
Mouse CD45	Rat	Goat anti-rat AF647
Human Bcl2	Rabbit	Goat anti-rabbit AF594
Human ERα	Rat	Goat anti-rat DyLight 755
Human Rb	Mouse	Goat anti-mouse AF700
Human pRb	Rabbit	Goat anti-rabbit AF555

Table 1: Target antibodies with matching secondary antibodies used in this research. **Abbreviations:** AF- alexa fluor; Bcl2- B-cell lymphoma 2; CK- cytokeratin; CD45- cluster of differentiation 45; ER- estrogen receptor; Rb- retinoblastoma protein; pRb- phosphorylated retinoblastoma protein

RESULTS

OPTIMIZATION OF FIXATIVE REAGENTS

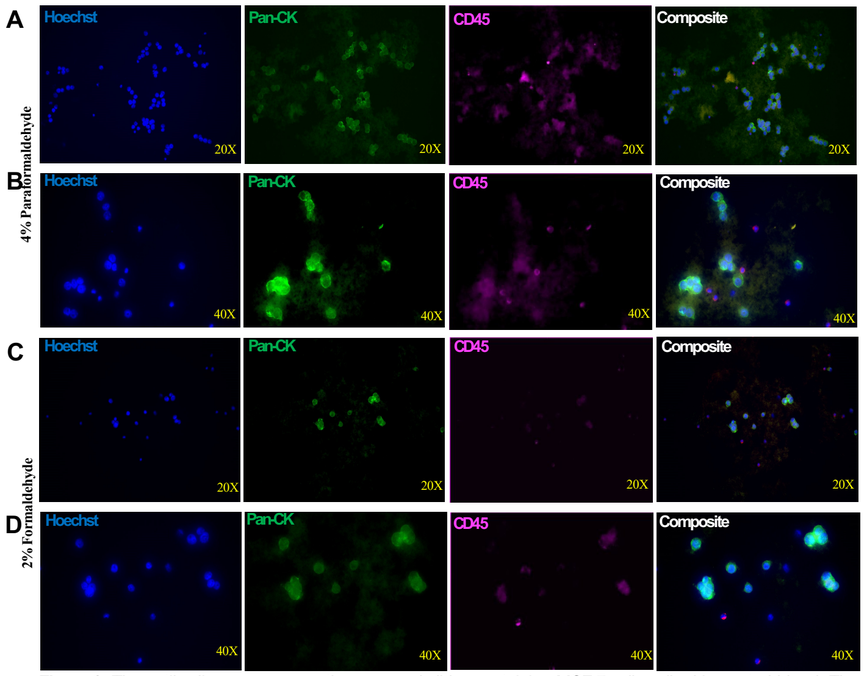


Figure 1: The antibodies were run on the prepared slides containing MCF-7 cells spiked-in control blood. The IF assay contained: anti-human pan-CK (AE1/AE3 and C-11) and anti-mouse CD45 in all four sets with the same primary (1:100) and secondary antibody (1:1000) dilutions. Hoechst was used as nuclear stain, pan CK as an epithelial cell marker, and CD45 as a leukocyte specific marker. **(A)** and **(B)** the cells were fixed using 4% paraformaldehyde; **(C)** and **(D)** the cells were fixed using 2% formaldehyde. Significant difference was not noticed between the two conditions; 2% formaldehyde will be used for future experiments since it aligns with the filtration-based protocol that is being tested for CTC and CTC cluster isolation and detection.

OPTIMIZATION OF ANTIBODY DILUTIONS

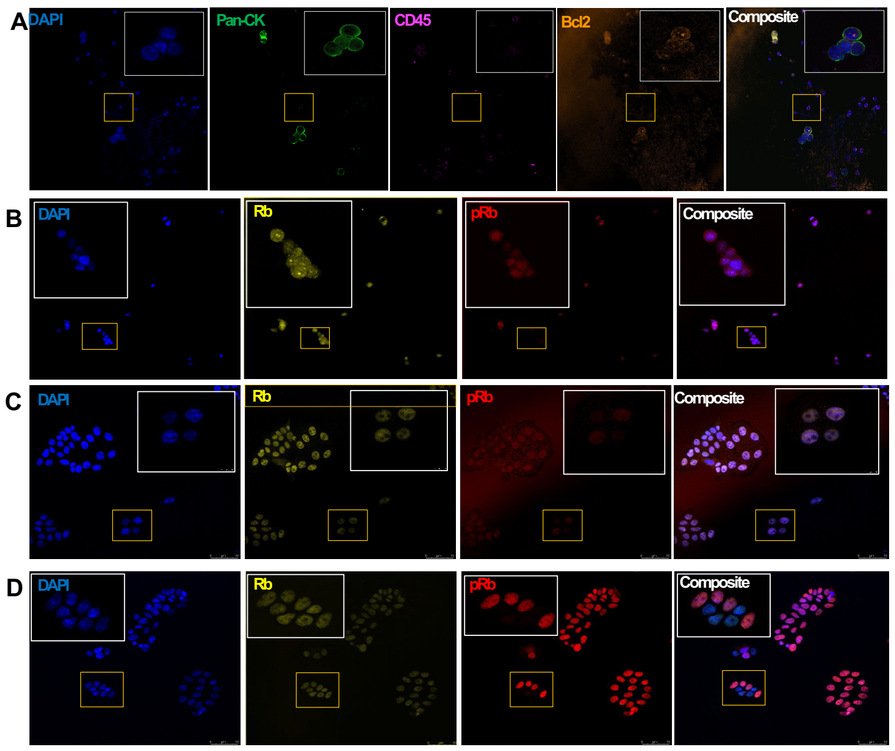


Figure 2: (A) Primary antibody dilutions: Pan-CK (1:200), CD45 (1:100), Bcl2 (1:100); Secondary Antibody dilution: 1:500. **(B)** Primary antibody dilutions for Rb and pRb (1:400); Secondary antibody dilution: 1:500. ERα was also run in set B, under the same conditions, but no staining was observed. **(C)** The original primary and secondary antibody combination as shown in Table 1: Rb(1:400)/AF700(1:500) + p-Rb(1:400)/AF555(1:500) was used. **(D)** The secondary antibody combination was varied: Rb(1:400)/AF488 (1:500) + p-Rb(1:400)/AF594(1:500). The second combination showed greater signal at lower exposure time.

OPTIMIZATION OF ANTIBODY INCUBATION TIME

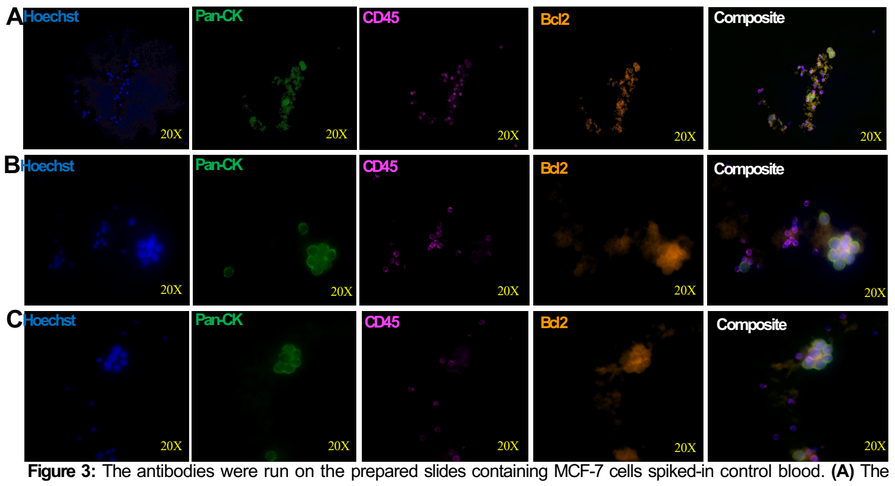


Figure 3: The antibodies were run on the prepared slides containing MCF-7 cells spiked-in control blood. **(A)** The images were obtained for slides incubated for 1-hour with the primary antibody (1:200) followed by 1-hour with the secondary antibody. There is very observable background, making it difficult to differentiate the epithelial cells and leukocytes. **(B)** and **(C)** The images were obtained for slides incubated overnight with the primary antibody (1:200) followed by 1-hour with secondary antibody. There is poor Bcl2 staining in both the overnight and 1-hour condition. Overall, the overnight incubation provides better staining and will be used for all future studies.

OPTIMIZED MULTIPLEX IMMUNOFLUORESCENCE ASSAY

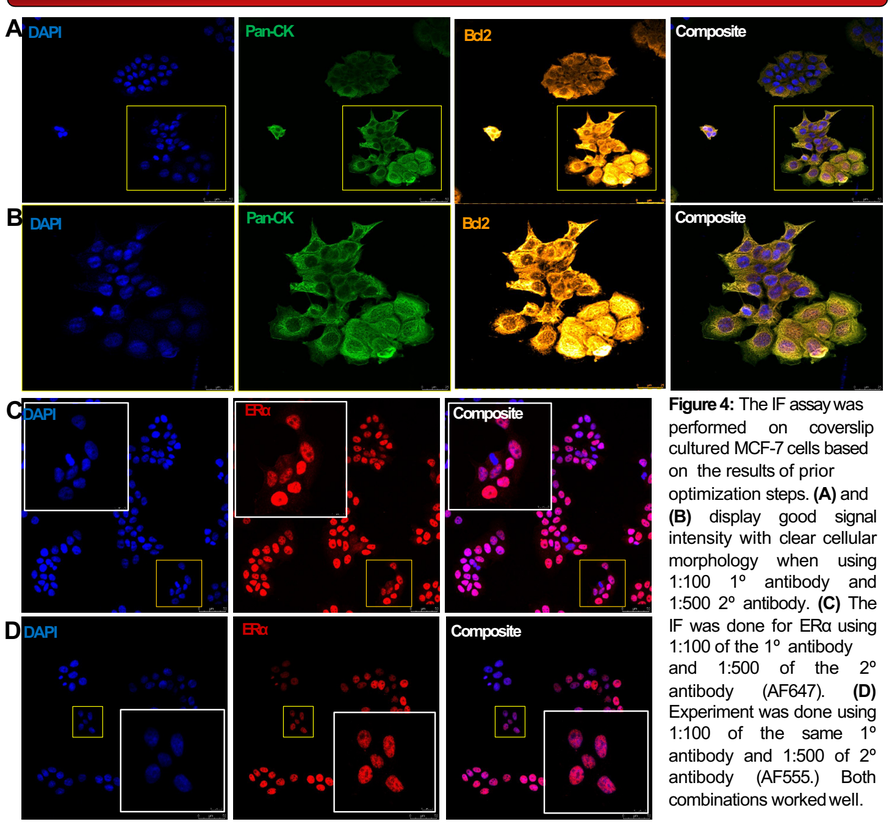


Figure 4: The IF assay was performed on coverslip cultured MCF-7 cells based on the results of prior optimization steps. **(A)** and **(B)** display good signal intensity with clear cellular morphology when using 1:100 1° antibody and 1:500 2° antibody. **(C)** The IF was done for ERα using 1:100 of the 1° antibody and 1:500 of the 2° antibody (AF647). **(D)** Experiment was done using 1:100 of the same 1° antibody and 1:500 of 2° antibody (AF555.) Both combinations worked well.

CONCLUSIONS AND FUTURE DIRECTION

- We have optimized a multiplexed IF assay (DAPI/CK/CD45/ERα/Bcl2) to detect expression of Bcl2 on CTCs.
- Future directions include (1) optimization of RBC lysis protocol to remove background on IF staining and (2) development of another multiplexed assay to detect expression of ERα/Rb/pRb/CK/CD45 on CTCs.
- The optimized assays will be used for future therapeutic studies in patient-derived xenograft models and patients.