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Multiphoton Imaging of Labeled Breast Cancer Cells to Quantify Intra and Extracellular Receptors

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Multiphoton Imaging of Labeled Breast Cancer Cells to Quantify Intra and Extracellular Receptors

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Abstract:

Every year 200,000 women in the United States are diagnosed with breast cancer. Of the cases diagnosed, 10% -15% are classified as triple negative breast cancer (TNBC) due to the absence of estrogen, progesterone, and HER-2/Neu receptors. This breast cancer sub-type is markedly more aggressive and twice as likely to develop in premenopausal women. TNBC is resistant to endocrine therapies and current targeted agents, making clinical need for the development of validated therapeutics for TNBC a pressing matter. To initiate drug development, the internalization of directly immunolabeled epidermal growth factor receptors (EGFR) in SK-BR-3 human breast adenocarcinoma cells was quantitated using live-cell multiphoton microscopy for 30 minutes over 5 minute intervals. EGFR targeting is of interest because its internalization triggers the signaling pathway that disrupts cell-cell adhesion and induces cell motility. The images acquired were processed using ImageJ and analyzed through line profiles. After measuring the full width half max at each time point of the 30-minute time series, it was determined that significant EGFR internalization did not occur.

Key words: Epidermal growth factor receptor, Triple negative breast cancer, Multiphoton microscopy

1. INTRODUCTION

Breast cancer currently accounts for 15% of cancer-related deaths in women and is the most common cancer in women worldwide. (1-2). In the United States alone, there are 200,000 invasive breast cancer cases and 50,000 in situ breast cancer cases diagnosed each year (1,3). Breast cancers can generally be categorized into three sub-types: hormone receptor positive tumors, HER-2/Neu amplified tumors, or triple negative tumors. The expression of estrogen receptors and progesterone receptors indicates hormone receptor positive tumors. HER-2/Neu is a member of the epidermal growth factor receptor (EGFR) family of tyrosine kinase receptors (RTK). Its overexpression indicates HER-2/Neu amplified tumors. With triple negative tumors, however, all three of these types of receptors are absent. Triple negative breast cancer (TNBC) makes up 10%-15% of breast cancer cases, with women under 40 years old being twice a likely to develop this subtype than women over 60 years old (1,4). There are 6 classifications of TNBC that have been identified: basal-like (BL1 and BL2), immunomodulatory (IM), mesenchymal (M), mesenchymal stem-like (MSL), and luminal androgen receptor (LAR). These classifications are determined through immunohistochemistry and fluorescence assessment using *in situ* hybridization technique. About 75% of TNBC cases diagnosed are basal-like (5). TNBC is typically diagnosed as having a higher histologic grade than other breast cancer types. This higher grade indicates high aggression, poor prognosis, increased recurrence three years after diagnosis, and high five-year mortality. Because TNBC lacks estrogen, progesterone, and HER-2/Neu receptors, the treatment options available are limited due to tumors not responding to endocrine therapies and current targeted agents (3-4).

In TNBC cells, the signaling leading to invasiveness and metastasis is known. When epidermal growth factor (EGF) binds to EGFR, the receptor dimerizes and trans-phosphorylates the Tyr1068 and Tyr1086 regions of the receptor. This causes GEP100, a guanine nucleotide effector protein, to be recruited and its pleckstrin homology domain to bind with the heavily phosphorylated regions of the EGFR. This interaction causes Arf6 to be activated and to be recruited. Arf6 regulates endocytosis and recycling of plasma membrane components and, when activated, causes cell metastasis, invasion, and proliferation. AMAP1, an effector protein of the activated Arf6, is recruited to interact with Arf6. This signaling cascade, shown in **Figure 1**, induces cells to develop a motile phenotype, in which E-cadherin-based cell-to-cell adhesion is lost and the tumor cells become invasive and metastatic (5-7). When a ligand binds to RTKs, they experience ligand-enhanced internalization after 15 minutes into the endocytic pathway and they continue signaling within endosomes (8-10).

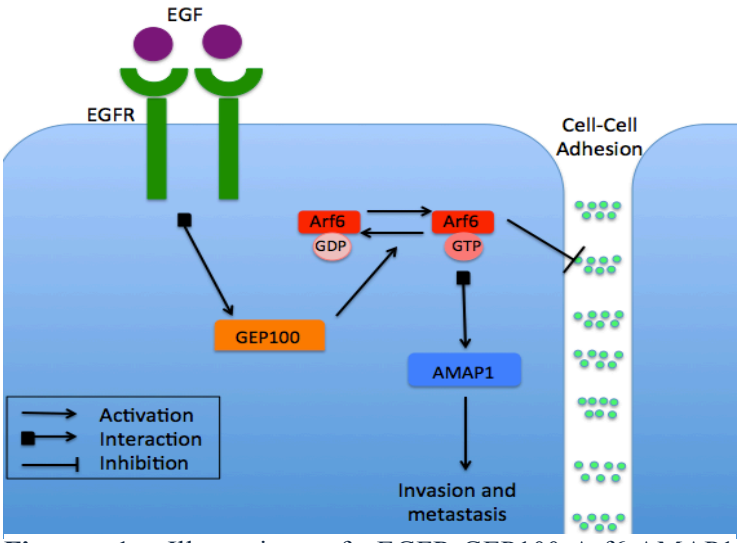

Figure 1: Illustration of EGFR-GEP100-Arf6-AMAP1 signaling cascade seen in breast cancer leading to invasion and metastasis. When EGF binds to EGFR, the Tyr1068 and Tyr1086 regions of the receptor highly phosphorylate and bind the pleckstrin homology domain of GEP100. Through this binding, GEP100 activates and recruits Arf6 and downstream signaling employs AMAP1 as its effector. This signaling pathway causes E-cadherin-based cell-cell adhesion to be disrupted.

The absence of well-defined molecular targets and the heterogeneity of the disease make the clinical need for TNBC specific therapies imperative (11). To begin to

develop validated therapeutic targets for TNBC, the cell biology behind EGFRs must be clarified. To initiate this task, EGFR was fluorescently labeled in SK-BR-3 human breast adenocarcinoma cells to quantify the amount of extra- and intracellular EGFR using live-cell multiphoton microscopy.

2. MATERIALS AND METHODS

2.1 Cell Culture

SK-BR-3 cells, obtained from the American Type Culture Collection (ATCC), were cultured in DMEM (ATCC) consisting of 10% fetal bovine serum (ATCC) and 5% penicillin/streptomycin (Gibco) at 37°C in 5% CO_2. Cells were passaged up to P4 to maintain EGFR expression (12). At least 12 hours prior to image acquisition, the cells were cultured onto 25 mm round coverslips (VWR) with 100,000 cells per mL under serum-starved conditions in DMEM (ATCC) consisting of 10% charcoal stripped fetal bovine serum (Gibco, cat. 12676-029) and 5% penicillin/streptomycin at 37°C in 5% CO_2 (8).

2.2 Direct Immunolabeling

Prior to image acquisition, the serum-starved cells on the cover slip were directly labeled with 10μg/mL Alexa Fluor 488-conjugated mouse anti-human ErbB2 monoclonal antibodies (mAb) from Biolegend (clone: AY13, cat. 352907) or 10μg/mL Alexa Fluor mouse mAb IgG1 control (clone: MOPC-21, cat. 400132) from Biolegend diluted in 1x PBS with and DAPI for 30 minutes on ice in the dark. After incubation, the cells were washed with 1x PBS three times. Once labeled, the cover slip was placed into an adapter and 2mL of Tyrode's buffer solution was added.

2.3 Image Acquisition

Live-cell multiphoton imaging was performed using a Mai Tai femtosecond pulsing Ti:Sapphire laser (Spectra Physics) and a 40x water immersion objective with a 0.8 NA (Nikon). An overview of the multiphoton microscope set-up used can be seen in **Figure 2**.

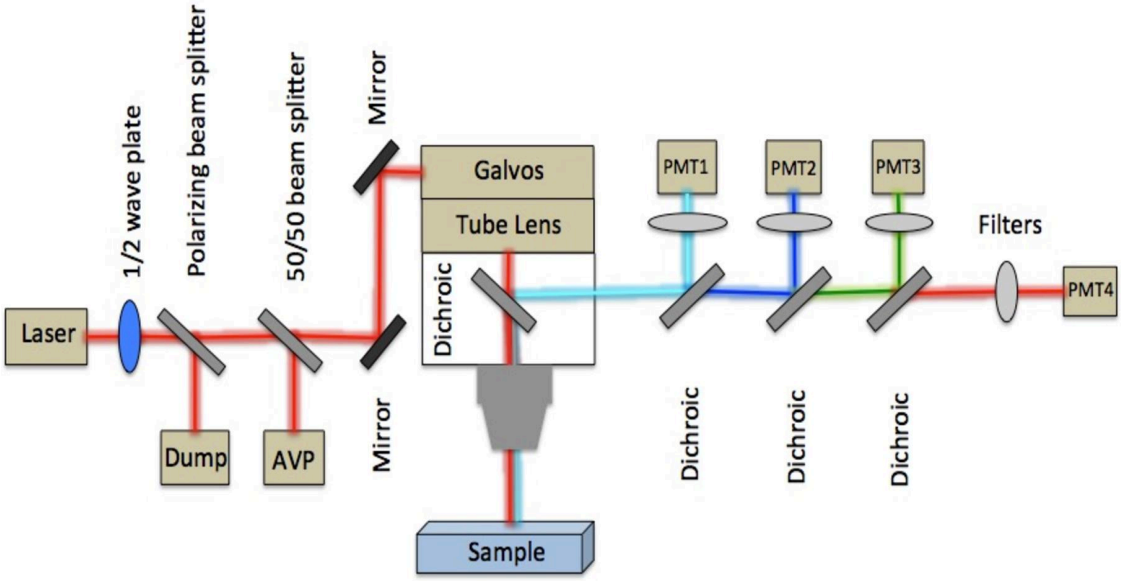

Figure 2: Schematic overview of multiphoton microscope with a femtosecond pulsing Ti:Sapphire laser and a 40x water immersion 0.8 NA objective.

All images were captured with an incident laser power of 20mW. Initially, the DAPI stained nuclei were capture at a wavelength of 720nm at 200 frames per second and 50% of maximum gain of photomultiplier tubes to properly align cells. Then, for the live-cell study to capture the Alexa Fluor 488-conjugated mouse anti-human EGFR mAb being internalized, a time series was established to image every 300 seconds for a total of 6 measurements (total time of 30 minutes). The images were acquired at 200 frames per second with a laser wavelength of 930nm, using 50% of maximal gain of the

photomultiplier tubes, and with a 512µm x 512µm acquisition field of view. Once the time series was completed, the DAPI was imaged a second time with the same parameters used initially at 720nm.

2.4 Image Analysis

Following acquisition, the images were loaded into ImageJ for analysis. The images of the 3 time series captured were stacked and merged with their corresponding DAPI image. This served to visualize the trafficking of EGFR and qualitatively determine if internalization occurred. Then, lines were manually drawn through the centers of 6 imaged cells to develop a line profile of EGFR location at each of the 6 time points within the 30 minutes of the live-cell image capture at 930nm. The line profile provided data for gray value of the image verses the length of the line drawn in microns. From the graphs generated with this data, the full width half max (FWHM) was measured from the peaks present. The average FWHM from the 6 cells at each time point was then calculated and at t-test ($p=0.05$) was conducted between 5-minute and 30-minute time point averages to determine if a statistical difference existed.

3. RESULTS AND DISCUSSION

After the images of the DAPI captured at 720nm and the time series images of anti-EGFR captured at 930nm were acquired, they were merged to form a single image for each time point within ImageJ. By observation of the cells in these merged images, it appeared that the band of EGFR at the surface of the cell was thinner at the 5-minute time point than it was at the 30-minute time point (**Figure 3**). It was believed this band thickening was qualitative evidence of EGFR internalization.

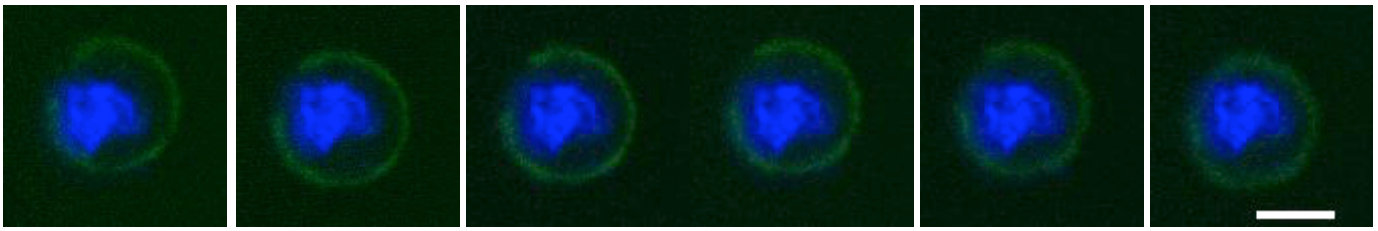

Figure 3: Time series images of a live SK-BR-3 cell over a 30-minute period, showing 5 intervals. Cells were labeled with DAPI and anti-EGFR and images were acquired with an excitation wavelength of 720nm and 930nm, respectively. Scale bar represents 10 microns.

To quantitate EGFR internalization, the line profiles of 6 cells imaged at 930nm were collected for each time point. As seen in **Figure 4 (A)**, there were 2 peaks at every time point signifying the locations of the cell surface along the center-line drawn where the EGFR was located. If internalization were occurring these peaks would theoretically widen as time progressed. This widening was thought to have been qualitatively seen in **Figure 3**. The data from the line profiles was collected in the form of gray value verses distance. Once plotted, the FWHM was determined for the peaks present at all 6 time points and averaged for each time point.

With all of the FWHM determined for each time point in 6 imaged cells, they were then averaged and plotted in **Figure 4 (B).** A t-test was run to determine if there a significant difference in the cell-surface EGFR band at 5 minutes and at 30 minutes. It was determined that their widths were not significally different ($p=0.05$), thus there was not a significant amount of EGFR internalized.

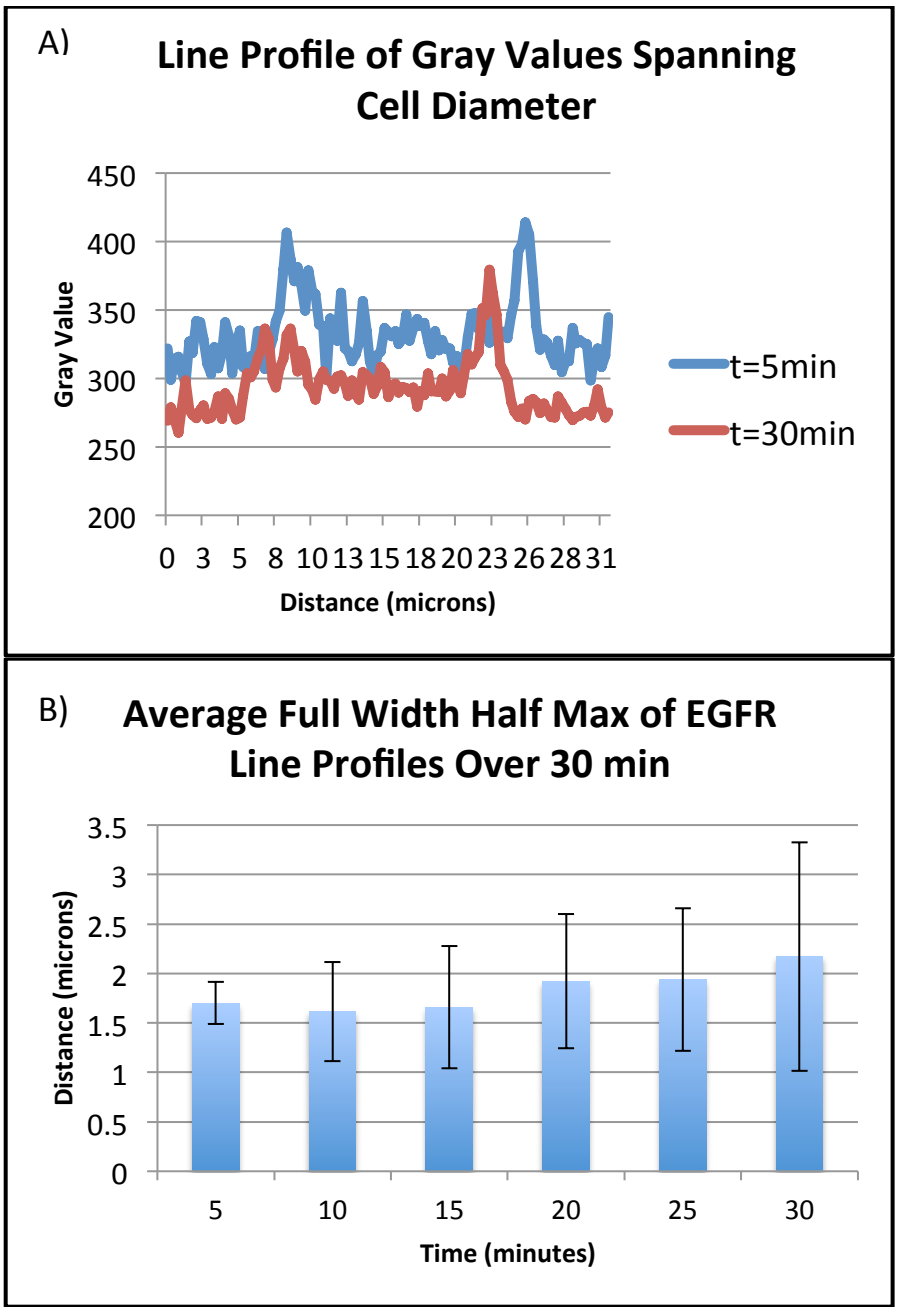

Figure 4: A) Example of line profile of an image acquired at 930nm at the 5-minute time point and the 30-minute time point. Peaks display EGFR initial and final location within cell. B) Plot of the average FWHM of 6 cells over 30 minutes. The FWHM at 5 minutes and 30 minutes were not significantly different (p=0.05).

4. CONCLUSIONS

Triple negative breast cancer is an invasive breast cancer sub-type that currently lacks targeted agents. Aiming to aid in the development of TNBC therapeutics, the internalization of EGFR was quantified using live-cell multiphoton microscopy on SK-BR-3 cells over 30 minutes. The cells were directly immunolabeled with Alexa Fluor 488-conjugated mouse anti-EGFR mAb and DAPI and imaged at 930nm and 720nm, respectively. The resulting time series images qualitatively showed the internalization of EGFR through the thickening of the EGFR band on the surface of the cell. When the FWHM was taken for each time point within the series, however, it was quantitatively shown that no significant amount of EGFR was internalized between the 5-minute and 30-minute time point.

5. FUTURE DIRECTIONS

Moving forward, this experiment will be duplicated using a higher incident power during image acquisition. It is believed this will enable better visualization of EGFR trafficking. In addition, MDA-MB-468 cells, a TNBC cell line with EGFR overexpression, will be used to quantify EGFR internalization. The MDA-MB-468 results will be compared to the SK-BR-3 results to further determine the validity of EGFR as a TNBC therapeutic target.

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