The treatment of breast cancer is based on the knowledge of estrogen (ER), progesterone (PR) and HER2/Neu status of a tumour (HER2). However, prediction of the effect of treatment based on these three biomarkers alone is somewhat limited, thus motivating the inclusion of additional molecular biomarkers. To this end, we developed and validated a multiplexing technology to allow multiple biomarkers to be imaged on the same tissue section and then analyzed by biomarker expression at the subcellular level (nuclear, membrane, and cytoplasmic).

Introduction
GE Global Research Center (GRC) has developed proof of concept instrumentation and the tools for labeling, multiplexing, imaging and analysis of onco-proteins in fixed tissues. This multiplexing platform enabled researchers at Sunnybrook Research Institute to study multiple biomarkers on the same tissue section. Specifically we aimed to 1) validate multiplexing technology by comparing to traditional histology and 2) investigate co-localization of 3 established biomarkers in breast cancer, namely, HER2/Neu, Estrogen receptor (ER), and progesterone receptor (PR) along with epithelial biomarker (cytokeratin), proliferation index biomarker (Ki67) and segmentation biomarker (NaKATPase).

Materials and Methods
An anonymized sample of triple positive breast cancer was retrieved from the files of Sunnybrook Health Science Centre Department of Anatomic Pathology. Multiplexing was performed using a proprietary microfluidic system (Fig. 2). Slides were stained with primary antibodies against HER2/neu and ER, and then visualized using a secondary antibody directly conjugated with fluorescent probes Cy3 and Cy5, respectively. After bleaching the sample, antibodies against PR and Ki67 and directly conjugated with Cy3 and Cy5 were subsequently used; and followed by cytokeratin and NaKATPase direct conjugates (Fig. 1).

Images of background fluorescence were acquired before and after the staining. Images were registered, background fluorescence removed, segmented and analyzed at the single cell level. Biomarker expression was quantified for subcellular compartments such as nucleus, cytoplasm and membrane.

Results and Discussion
Immunofluorescence images confirm the positivity of the ER/PR/HER2 as diagnosed by traditional DAB staining and confirmed by clinical pathologist at Sunnybrook (JZ). This validated the multiplex platform for the use in the clinical pathology lab. Next the co-localization of HER2/Neu, ER, PR along with Ki67, cytokeratin at specific subcellular compartment was investigated at the single cell level. As expected, ER, PR and Ki67 expression was localized to the nuclei, whereas cytokeratin and HER2 staining was membranous (Fig. 3).

The matrix plot of these 5 biomarkers is shown in Fig. 4. The correlation between PR and ER is very significant (R=0.77), although there are some cells with low ER, but high PR. This is consistent with the well-established correlation between ER and PR expression. Correlations between Ki67 and ER/PR are interesting. Cells cluster into two groups along the marker axes. Cells with low Ki67 expression have high ER or PR expression, and vice versa, suggesting that cells in the proliferative state (high Ki67 expression) tend to lose the ER/PR expression. There is a much less correlation between Ki67 and HER2, as the membranous biomarker expression is probably less dependent on the cell cycle.

Validation of the platform was performed using a proprietary microfluidic technology to allow multiple biomarkers to be imaged on the same tissue section and then analyzed by biomarker expression at the subcellular level (nuclear, membrane, and cytoplasmic).

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