The reported cause of death in over 90% of cases of breast cancer is due to metastasis of the primary tumor into secondary sites. Once dislodged from the primary tumor, metastatic cells may enter the bloodstream. Select metastatic cancer cells exhibit viability within the circulatory system and are potential precursors to secondary tumor formation. The presence of circulating tumor cells (CTCs) following the treatment of a primary tumor may indicate metastatic status or cancer recurrence. A current difficulty in measuring CTCs is their relative sparseness in the blood. Another issue of current technology, is dealing with the background level of circulating leukocytes detected in cell-sorting methods such as fluorescent-activated cell sorting (FACS). HER2+ breast cancer is easily detectable through immunohistological methods in both localized tumors and CTCs. This proposal will develop a compact, wearable device capable of measuring the presence of HER2+ CTCs in real time based on a positive fluorescent antibody marker for the HER2 receptor, and negative markers for the CD45 receptor unique to leukocytes. We propose the following objectives to test the efficacy of such a device:

AIM 1: **Quantify targeting of CTCs in vitro, using specific fluorescent-labeled antibodies.**
Hypothesis: Conventional FACS can distinguish HER2+ breast cancer CTCs from leukocytes, when the respective cell types are labeled with HER2 and CD45 fluorescent-labeled antibodies.
Method: AIM 1 will provide standard data for the calibration of the prototype, and indicate relative antibody binding efficiency in blood versus suspended culture, based on individual detection of each label via conventional FACS. We will mark a suspended culture of SKBR3 breast cancer cells with CD340-FITC antibodies as a positive control for HER2, and both CD45-FITC and CD45-PE-Cy5 antibodies as negative controls for the leukocyte marker CD45. We will repeat the control tests on SKBR3 cells mixed into human blood containing leukocytes.

AIM 2: **Develop an external wearable flow cytometry device to detect CTCs in the bloodstream over time in vivo.**
Hypothesis: A compact flow cytometry device, designed based on the BD FACSCalibur flow cytometer, can generate the same quality of raw data as the FACs device in vivo.
Method: AIM 2 will create a wireless, compact, wearable flow cytometry device containing a laser, sensor, power source, and memory storage and wireless communication equipment. Synthetic fistula will integrate the prototype device with the circulatory system in vivo.

AIM 3: **Calibrate the prototype and assess performance in vitro based on FACS results.**
Hypothesis: Empirical calibration will allow dual detection of fluorescent-labeled antibodies against HER2 and CD45, at a level of accuracy comparable to conventional FACS.
Method: AIM 3 will repeat the control tests from AIM 1 while circulating blood samples through the device, to calibrate the prototype and determine the minimum circulation time. In a new test, performed using both FACS and the prototype, dual detection of CD340-FITC and CD45-PE-Cy5 antibodies will distinguish CTCs from leukocytes in blood.

One long term goal of this proposal is the introduction of this device as a clinical means to measure quantitative changes in breast cancer CTC levels, which present prognostic and predictive value in the selection of targeted breast cancer therapies. The presence of HER2+ CTCs may direct treatment with HER2 inhibitors. Additionally, this technology will provide a vital learning tool for evaluating the clinical role of CTCs in metastasis and late cancer recurrence for other types of cancer. The series of controlled experiments will prove the concept of selectively detecting cells in vivo, in real time, based on immunostaining, for broad applications in the field of personalized medicine. The prototype will be adaptable to other diagnostic tests using a variety of fluorescent-conjugated antibody tags.

Significance

The National Cancer Institute reports breast cancer occurence in the United States at an estimated incidence of 125 per 100,000 women, with 5% presenting metastatic forms of the cancer. Approximately 90% of cancer mortality results from metastasis. The five-year mortality rate for breast cancer is approximately 25%. Breast cancer recurs at a rate of 7-13%, depending upon the stage of advancement, after 5 years of therapy [2].

Metastatic cancer is difficult to detect due to its unpredictable nature. The understood mechanism of metastasis, is that metastatic cells migrate through the bloodstream from a localized tumor site to nearby tissues and lymph nodes, in the form of circulating tumor cells (CTCs). Recent research has revealed a prognostic value in counting CTCs in blood samples [3]. Growing evidence supports a predictive value of the CTC count for patient outcome and chance of recurrence [4,5]. Clinical applications of this research are limited, due to the difficulty in detecting CTCs, which are are relatively rare in blood. CTCs are present at concentrations six orders of magnitude lower than the concentration of leukocytes. This is especially problematic because leukocytes are the primary confounders of CTC detection, due to their large size.

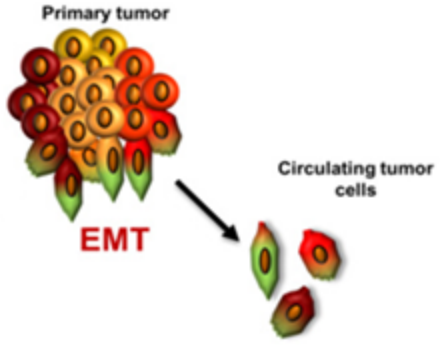

Figure 1: Schematic of CTC metastasis. EMT denotes epithelial-mesenchymal transition [1].

Detection of HER2+ breast cancer, through immunofluorescent staining of biopsy samples, merits targeted treatments such as trastuzumab and lapatinib. We hypothesize that the over-expression of HER2 can differentiate HER2+ breast cancer CTCs from leukocytes, the only other cells likely to express the marker normally. Leukocytes are differentiable by their unique expression of the CD45 surface marker. A live, accurate count of CTCs in the bloodstream of a patient, would indicate the metastatic prognosis of cancer, and provide therapeutic direction. HER2 status of CTCs is a strong predictive marker for the effectiveness of HER2-targeted therapies for both metastatic and adjuvant breast cancers [6].

Due to the heterogenous nature of cancer, HER2 discordance may exist between the primary tumor and CTCs, as both gains and losses of expression [7]. The proposed device will eliminate erroneous, ineffective treatment in cases where a metastatic loss of HER2 expression occurs. Conversely, if a patient presents a metastatic lesion along with high levels of HER2+ CTCs in the bloodstream, an accurate HER2+ CTC count will necessitate targeted HER2+ treatment. Real-time monitoring of CTC levels based on HER2 overexpression will indicate the metastatic risk of HER2+ breast cancers during adjuvant therapy and beyond. The device will enable oncologists to detect and classify metastases at early, treatable stages, increase the five-year patient survival rate, and treat relatively manageable post-therapy recurrences.

We will focus the preliminary experiments, design, and simulated diagnostic tests of our prototype device toward the specific problem of counting and distinguishing HER2+ breast cancer CTCs from leukocytes in the bloodstream. A series of controlled experiments will demonstrate general proof of concept to detect three fluorescent-conjugated antibodies, and determine possible rates of false positive detection. Alternate versions of the device could detect many combinations of immunofluorescent staining tags. The device could serve as a future diagnostic tool with broad applications in the field of personalized medicine.

Innovation

Personalized medicine technologies intended for point-of-care use often involve biopsies, or require specialized imaging or cell sorting equipment. The proposed CTC counting device presents two important advantages over existing targeted diagnostic technologies.

1. The portable, compact CTC counting device will interface with a wireless network, allowing patients to wear it continuously while continuing normal activities.

In vivo, targeted diagnostic methods require specialized equipment in a laboratory setting. Gee et al. have applied intravital laser scanning fluorescence microscopy to detect the HER2 biomarker in human breast cancer tumors implanted into mice [8]. This method of in vivo, targeted labeling has a significant drawback in its use of tomographic imaging methods. Our method could detect the HER2 biomarker in CTCs in a home setting, while sending the data to appropriate medical information systems.

2. The CTC counting device will detect individual cells in the bloodstream over a period of time, which eliminates the need for biopsy or blood sampling, and improves accuracy.

Raihi et al. have developed a microchannel-based microfluidics device to capture individual CTCs selectively from blood in single-cell chambers, based on the size and deformability of the CTCs [9]. The device enables genetic, immunological, and morphological characterization of a heterogeneous sample of CTCs at the cellular level, provided that the presence of CTCs in the patient is already known, and the analyzed blood contains a representative quantity of CTCs. Patients with unknown risk of metastasis cannot benefit from this technology.

The proposed device is not an imaging or capture device, but a counting device. Our prototype will indicate whether a significant number of CTCs are present in the bloodstream, based on the total number of signals detected from fluorescent probes. The intended application is to assess risk of metastasis based on the number of CTCs detected. Detailed analysis of individual CTCs, by means such as the Raihi device, could follow this initial screening.

Approach

AIM 1: **Quantify targeting of CTCs in vitro, using specific fluorescent-labeled antibodies.**
Hypothesis: Conventional FACS can distinguish HER2+ breast cancer CTCs from leukocytes, when the respective cell types are labeled with HER2 and CD45 fluorescent-labeled antibodies.

1A. Preliminary FACS screening of one positive control, and two negative controls, in suspended cultures of SKBR3 cells will provide evidence for proof of concept in vitro.

Conventional FACS on suspended cultures using a BD FACSCaliber flow cytometer will prove the concept of selectively labeling and detecting SKBR3 cells, and quantify the background detection of unbound antibodies. Following a published protocol, we will grow cultures of SKBR3 cells, before marking the cells with a 50:1 ratio of antibody marker per cell [9]. Table 1 details the antibodies and FACS settings applied in each test.

Because SKBR3 breast cancer cells are HER2+ and CD45-, we expect the cells to bind CD340/HER2 antibodies, but not CD45 antibodies. We have selected two fluorescent tags from BD Biosciences which excite at the same blue laser setting, but emit distinct signal colors at comparable intensities. Control tests will implement CD45/PE-Cy5, CD45/FITC, and FDA-approved CD340/FITC fluorescent-conjugated antibodies (BD Biosciences, San Jose, CA).

Table 1: Experimental strategy for proof of concept by FACS detection of fluorescent-conjugated antibodies. "GMP status" denotes FDA approval for Good Manufacturing Practices.

Fluorescent Tag	**Absorbance/Emission Wavelengths: Filter, Intensity, Signal Color**	**Conjugated Antibody, Cell Type: Cat. No. *GMP Status***	**Control Test**
FITC	488 nm / 528 nm: 530/30 nm, moderate, green	CD340 (HER2), SKBR3: 340553 *GMP status*	Positive
PE-Cy5	488 nm / 667 nm: 670/14 nm, bright, red	CD45, leukocyte: 560974 *Non-GMP status*	Negative
FITC	488 nm / 528 nm: 530/30 nm, moderate, green	CD45, leukocyte: 560976 *Non-GMP status*	Negative

1B. FACS tests on blood mixtures containing varied concentrations of pre-labeled and post-labeled SKBR3 cells, will provide a basis to assess the accuracy of the prototype.

We will prepare a set of mixtures of SKBR3 cells, pre-labeled with the CD340/FITC marker, in human blood following a published protocol [9]. Briefly, we will culture SKBR3 cells for four days, label with a 50:1 ratio of antibodies per cell, mix known quantities of labeled cells into blood, incubate the mixtures for 10 minutes, and analyze each sample via FACS. The prototype must accurately count CTCs at levels as low as 1 per million, to 1 per billion blood cells [9]. We will test CTC concentrations of 1 per billion, 1 per million, 1 per thousand, and 1 per hundred red blood cells. We will then simulate in vivo labeling by administering each marker individually, at a 50:1 ratio per SKBR3 cell, to blood mixtures containing unlabeled SKBR3 cells. These post-labeled tests serve three purposes:

1. Determine antibody binding efficiencies in blood. Results for the CD340/FITC marker will indicate the efficiency of HER2 antibody binding for CTCs in blood, which may be lower than the binding efficiency in suspended culture determined from the preliminary control experiments in Aim 1A. Results for the CD45/FITC and CD45/PE-Cy5 markers will indicate the binding efficiency for leukocytes, after accounting for the unbound antibody background detection rates determined in Aim 1A.

2. Determine the rate of false positive CTC detection. The CD45/FITC negative controls in blood are critical to assess the rate of false positive detection. We assume that HER2+ cells, such as the SKBR3 cell line, will bind the HER2 antibody to a significantly greater extent than any other cells which express the HER2 receptor at normal levels. The CD45 receptor is normally expressed on leukocytes present in human blood [9]. After deducting any background measured in the pre-labeled CD45/FITC negative control, the level of detection in the post-labeled CD45/FITC negative control will simulate one possible rate of false positive detection for an FITC-conjugated antibody. Because all leukocytes express the CD45 receptor, we expect that this test will represent a maximal rate of false positive detection. We expect that the binding efficiency of CD45/FITC onto leukocytes in blood will be much lower than that of CD340/FITC onto CTCs in blood. Otherwise, false positives may skew the CTC count.

3. Provide a basis of calibration for the prototype device. The known concentrations and FACS detection rates of SKBR3 cells labeled after preparing the blood mixture, are necessary to calibrate the CD340/FITC detection mode for the prototype. We will later analyze each blood sample using the prototype. The number of leukocytes measured in each blood sample using FACS, will provide a basis to calibrate the CD45/FITC and CD45/PE-Cy5 detection modes for the prototype.

AIM 2: **Develop an external wearable flow cytometry device to detect CTCs in the bloodstream over time in vivo.**

Hypothesis: A compact flow cytometry device, designed based on the BD FACSCalibur flow cytometer, can generate the same quality of raw data as the FACS device in vivo.

2A. Develop a computational method to count cells based on raw flow cytometry data.

FACS is a type of flow cytometry which can analyze a large number of mixed cell types based on the fluorescent properties of the sample [10]. We will develop a compact, FACS-based sensor system to count the number of cells labeled with FITC and PE-Cy5 tags.

Figure 2 illustrates a generic FACS system. In conventional FACS, fluorescent-tagged cells pass through a small chamber, where they absorb energy from a laser beam and emit light of a distinct wavelength. A photomultiplier tube amplifies the signal before a computer receives and analyzes the signal. Based on this result, the system generates an electrical charge to direct the cell into the appropriate chamber by the force of an electrical field. FACS can analyze up to 30,000 cells/sec [11].

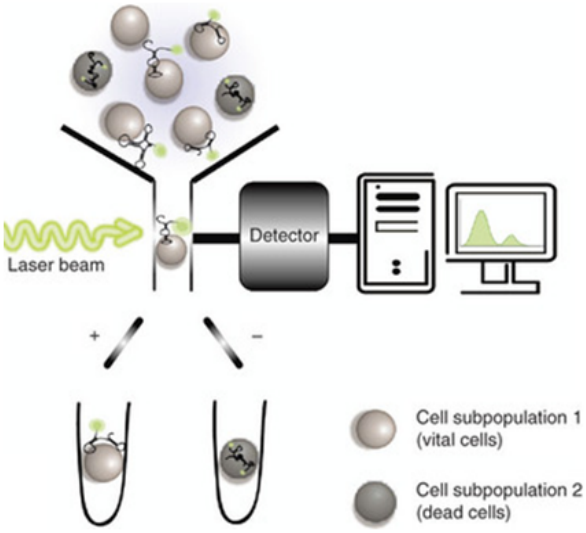

Figure 2: Schematic of a generic FACS system [12].

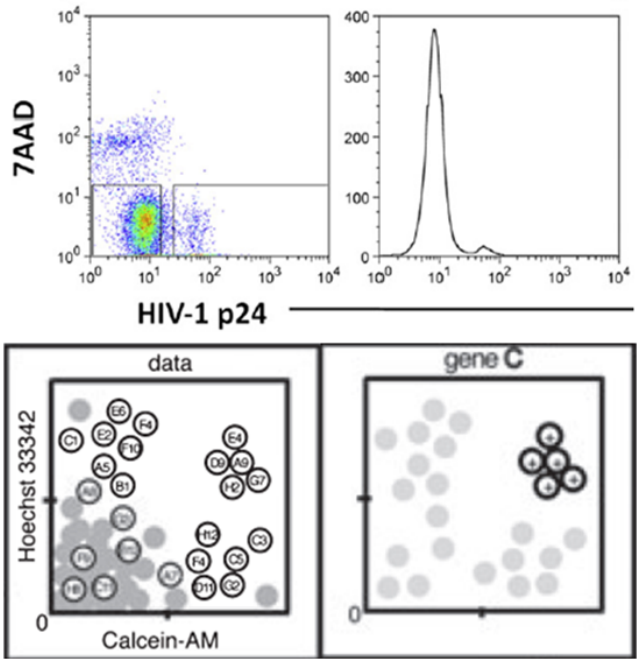

Figure 3: Top row shows a dual-marker density plot (left) and an example plot of frequency versus wavelength (right) for a FACS investigation of HIV [13]. Bottom row illustrates the identification of a single marked entity (right) from a dual-marker density plot [14]

Proper selection of fluorescent tags, with appropriate absorbance and emission spectra for the FACS system, enables simultaneous detection of multiple tags. When the tags emit distinct colors, the photo-detector and computer system can differentiate multiple signals. We have selected a pair of tags to distinguish CTCs from leukocytes in blood. This capability is essential for the clinical application of a portable FACS-based detection system, in order to detect cells of interest selectively, based on positive and negative markers.

Multiple strategies exist to analyze raw data from the sensors. Signal intensity correlates with the number of markers detected. Plotting intensity versus wavelength, is not useful to detect cell types that may bind at least two fluorescent tags. A more appropriate method, is to plot the intensity of one signal of a particular wavelength on the x-axis, and another intensity for a signal of a different wavelength on the y-axis, as in

Figure 3. Each point represents one detected cell. Cells labeled with both markers appear in the upper right quadrant. Cells labeled with each marker alone, appear in the upper left and lower right quadrants. A computer can generate these plots and count each type of cell based on the raw data from the sensors. This method of data analysis is the basis for this prototype.

2B. Design a compact fluorescent-activated cell detection system based on the BD FACSCalibur flow cytometer.

A FACS-based compact, wearable device could measure the level of CTCs in a patient's bloodstream to assess risk of metastasis. Figure 4 is a schematic of the prototype. This device will interface with the circulatory system through a synthetic fistula derived from existing vascular graft technology. Blood will pass through a channel where a laser will excite bound tags on cells labeled with fluorescent-conjugated antibodies. We will assemble the prototype based on the operating parameters and components of the FACS system implemented in AIM 1. The assembled prototype must be sufficiently small and lightweight for a patient to wear comfortably and securely around the waist, arm, or leg in nearest proximity to the tumor. The device must also access a prominent blood vessel through the fistula without obstructing circulation or resulting in thrombosis. These criteria led to the selection of the following system components:

- Gore-Tex PTFE fistula, as used in hemodialysis
- Thorlabs DET100A photodetector 400 nm -1100 nm
  Weight: 0.2 lbs, Dimensions: 2.1" x 2.8" x 1.9"
- 488 nm Matchbox Laser
  Weight: 290 grams, Dimensions: 50 mm x 45 mm x 18 mm
- Rasberry Pi with SD data card, USB wireless adapter, CPU
  Weight: 45 grams, Dimensions: 85.6 mm x 56 mm x 21 mm
- Generic cell phone battery power source
- Plastic housing and belt/legband/armband
- FACS flow chamber, purchased as a replacement part

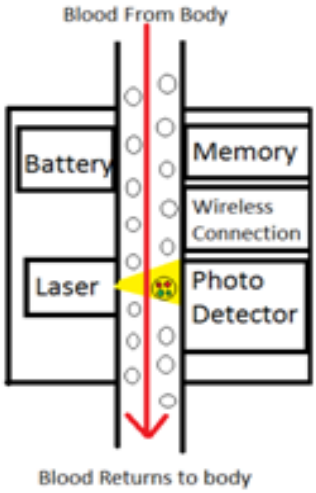

Figure 4: Schematic of prototype compact CTC detection system.

The essential component of this device is the Rasberry Pi, which will function as the brain of the device connecting and running all other components. The Rasberry Pi contains a CPU, which executes the code to operate the laser and photodetector. The prototype will store data on an SD card interfaced with the Rasberry PI. A USB adapter, also interfaced with the Rasberry Pi, will enable connection to wireless communication equipment. A generic cell phone battery will provide a compact, extended power supply. The 488 nm laser, directed across the diameter of the flowchamber, will excite both fluorescent tags implemented in our studies. The 400 nm - 1100 nm detection range of the Thorlabs photosensor, positioned opposite the laser, ecompasses the emisson wavelengths of both tags. A custom PTFE fistula will split the patient bloodstream and circulate a sufficiently narrow stream of blood through the flow chamber. We will determine the diameter of the smaller split segment of the fistula experimentally, to provide the correct flow rate of blood through the flow chamber. We will model the bloodstream as an artificially circulating loop of sample blood. We assume that the fraction of blood analyzed accurately represents CTC levels in patient bloodstream.

AIM 3: **Calibrate the prototype and assess performance in vitro based on FACS results.**
Hypothesis: Empirical calibration will enable dual detection of fluorescent-labeled antibodies against HER2 and CD45, at a level of accuracy comparable to conventional FACS.

3A. Repeat the post-labeled tests from AIM 1 to calibrate the prototype.

The post-labeled tests provide standard FACS readings, which serve as a basis to calibrate the prototype for both FITC and PE-Cy5 detection modes. FACS results for the CD340/FITC and CD45/FITC markers will provide readings to calibrate FITC signal detection by the prototype. Additionally, the CD45/FITC marker test will simulate false positive CTC detection by the prototype. FACS results for the CD45/PE-Cy5 marker will serve as a calibration curve for PE-Cy5 signal detection by the prototype.

3B. Simulate a dual-marker diagnostic test to distinguish CTCs from leukocytes in blood, and assess accuracy of the prototype against FACS.

We will prepare mixtures of unlabeled SKBR3 cells in blood at concentrations of 1 per billion, 1 per million, 1 per thousand, and 1 per hundred red blood cells. We will treat each sample with equivalent amounts of CD340/FITC and CD45/PE-Cy5 antibodies, at a 50:1 ratio per SKBR3 cell. We will generate the plots described in Aim 2A using both FACS and the prototype. The intensity of the 667 nm signal will indicate the number of leukocytes. The intensity of the 530 nm signal will indicate the number of CTCs, with some potential false positives. Regions where we expect each type of cell to appear, are labeled in Figure 5.

HER2+ cells should bind more antibodies, and fluoresce at greater intensities, than any false positives expressing HER2 at normal levels. Actual positive signals will appear in the upper left quadrant. Leukocytes will appear in the lower right quadrant. The upper right quadrant should be empty as no SKBR3 cells express the CD45 receptor. The lower left quadrant will contain signals from dual-labeled cells, which are leukocytes that express HER2 normally. These signals represent successfully eliminated false positives, which may prove the concept of distinguishing non-CTC cells based on a negative marker. The lower half of the left axis will contain uneliminated, CD45- false positives, which are either non-leukocyte cells expressing HER2 normally, or insufficiently labeled HER2+ cells.

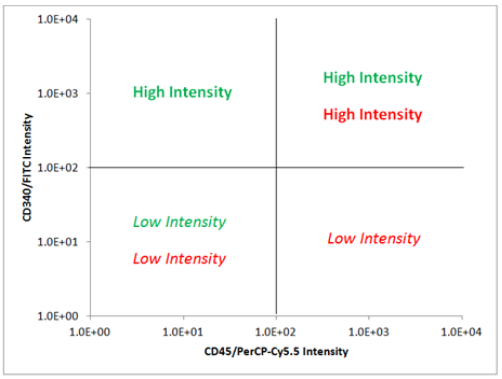

Figure 5: Expected dual-marker plot of CD340/FITC intensity on the y-axis, CD45/PerCP-Cy5.5 intensity on the x-axis.

We will calculate measured concentrations of CTCs, leukocytes, dual-labeled leukocytes, and false positives from these plots. We will compare the known number of CTCs with the measured level of CTCs. Additionally, based on the results of AIM 1B.2, we will compare a scaled estimate for the expected number of false positives, to the actual number of false positives. We expect that the actual rate of false positives will be lower than the inflated rate which we simulated in AIM 1B.2. This assumption follows from the fact that not all leukocytes express HER2 normally, whereas all leukocytes express CD45 normally.

We will perform a statistical analysis to assess the accuracy of the prototype against FACS. Our acceptance criteria requires that the prototype results for corrected FITC-tagged CTC levels, PE-Cy5-tagged leukocyte levels, and dual-tagged leukocyte levels, are consistent with FACS results within an 80% confidence interval. Then further improvements could close this gap. Our acceptance criteria also requires that the number of false positives detected by the prototype, is less than or equal to the number of false positives detected by FACS.

[1] Markiewicz, A., Książkiewicz, M., Seroczyńska, B., Skokowski, J., Szade, J., Wełnicka-Jaśkiewicz, M., & Zaczek, A. J. *Heterogeneity of Mesenchymal Markers Expression—Molecular Profiles of Cancer Cells Disseminated by Lymphatic and Hematogenous Routes in Breast Cancer.* Cancers, 2013. ***5***(4): p. 1485-1503.

[2] Brewster, A.M.; Hortobagyi, G.N.; Broglio, K.R.; Kau, S.W.; Santa-Maria, C.A.; Arun, B.; Buzdar, A.U.; Booser, D.J.; Valero, V.; Bondy, M.; *et al*. *Residual risk of breast cancer recurrence 5 years after adjuvant therapy.* J. Natl. Cancer Inst*.,* 2008. **100**: p. 1179–1183.

[3] Hayes, D.F.; Cristofanilli, M.; Budd, G.T.; Ellis, M.J.; Stopeck, A.; Miller, M.C.; Matera, J.; Allard, W.J.; Doyle, G.V.; Terstappen, L.W. *Circulating tumor cells at each follow-up time point during therapy of metastatic breast cancer patients predict progression-free and overall survival.* Clin. Cancer Res., 2006. **12**: p. 4218–4224.

[4] Pierga, J.Y.; Hajage, D.; Bachelot, T.; Delaloge, S.; Brain, E.; Campone, M.; Dieras, V.; Rolland, E.; Mignot, L.; Mathiot, C.; *et al*. *High independent prognostic and predictive value of circulating tumor cells compared with serum tumor markers in a large prospective trial in first-line chemotherapy for metastatic breast cancer patients.* Ann. Oncol., 2012. **23**: p. 618–624.

[5] Rack, B.; Schindlbeck, C.; Andergassen, A.; Schneeweiss, A.; Zwingers, T.; Lichtenegger, W.; Beckmann, M.; Sommer, H.; Pantel, K.; Janni, W. *Use of circulating tumor cells (CTC) in peripheral blood of breast cancer patients before and after adjuvant chemotherapy to predict risk of relapse: The SUCCESS trial*. J. Clin. Oncol., 2010: **28**: Abstract No. 1003.

[6] Bempt, I.V.; van Loo, P.; Drijkoningen, M.; Neven, P.; Smeets, A.; Christiaens, M.R.; Paridaens, R.; de Wolf-Peeters, C. Polysomy 17 in breast cancer: *Clinicopathologic significance and impact on HER-2 testing*. J. Clin. Oncol., 2008. ***26***: p. 4869–4874.

[7] Fehm, T.; Muller, V.; Aktas, B.; Janni, W.; Schneeweiss, A.; Stickeler, E.; Lattrich, C.; Lohberg, C.R.; Solomayer, E.; Rack, B.; *et al*. *HER2 status of circulating tumor cells in patients with metastatic breast cancer: A prospective, multicenter trial*. Breast Cancer Res. Treat*.,* 2010. **124**: p. 403–412.

[8] Gee, M. S., Upadhyay, R., Bergquist, H., Alencar, H., Reynolds, F., Maricevich, M., ... & Mahmood, U. *Human breast cancer tumor models: molecular imaging of drug susceptibility and dosing during HER2/neu-targeted therapy*. Radiology, 2008. **248**(3): p. 925.

[9] Riahi, R., Gogoi, P., Sepehri, S., Zhou, Y., Handique, K., Godsey, J., & Wang, Y. *A novel microchannel-based device to capture and analyze circulating tumor cells (CTCs) of breast cancer*. International journal of oncology, 2014. **44**(6): p.1870-1878.

[10] Carter, A. D., Bonyadi, R. O. X. A. N. N. A., & Gifford, M. L. *The use of fluorescence-activated cell sorting in studying plant development and environmental responses.* Int. J. Dev. Biol, 2013. **57**: p. 545-552.

[11] Gossett, D. R., Weaver, W. M., Mach, A. J., Hur, S. C., Tse, H. T. K., Lee, W., & Di Carlo, D. *Label-free cell separation and sorting in microfluidic systems*. Analytical and bioanalytical chemistry, 2010. **397**(8): p. 3249-3267.

[12] Mayer, G., Ahmed, M. S. L., Dolf, A., Endl, E., Knolle, P. A., & Famulok, M. *Fluorescence-activated cell sorting for aptamer SELEX with cell mixtures*. nature protocols, 2010. **5**(12): p. 1993-2004.

[13] Iglesias-Ussel, M., Marchionni, L., & Romerio, F. *Isolation of microarray-quality RNA from primary human cells after intracellular immunostaining and fluorescence-activated cell sorting.* Journal of immunological methods, 2013. **391**(1): p. 22-30.

[14] Hayashi, T., Shibata, N., Okumura, R., Kudome, T., Nishimura, O., Tarui, H., & Agata, K. *Single-cell gene profiling of planarian stem cells using fluorescent activated cell sorting and its "index sorting" function for stem cell research.* Development, growth & differentiation, 2010. **52**(1): p. 131-144.