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14. ABSTRACT We developed a microfluidic device to detect surface protein expression in individual cancer cells in small cell populations without prior labeling. We used LM2 cells (kind gift of Juan Massague, Memorial Sloan-Kettering Cancer Center, NY), derived from murine lung metastases of MDA-MB-231 cells that have a unique potential to re-metastasize to lung. We selected LM2 cells for 100% sustained expression of IL-13RA2 and cells without expression. We coated the channel with an antibody to IL-13RA2 or with BSA and directed cells over the patch by a microsyringe pump at flow rates of nanoliters per second. The transient interactions with surface ligands resulted in retardation of rolling rates in cells expressing the cognate surface protein over the antibody-coated patches and rapid, unimpeded flow on BSA or in the absence of expression of the antigen with 100% specificity. We detected and tracked cells with microscope objectives and computer tracking programs. We have achieved our objectives for the first year of the project in developing this method and are proceeding to achieve high sensitivity with single and tandem patches and maintaining 100% specificity.					
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INTRODUCTION:

Solid tumors are highly heterogeneous with respect to cell type. A small fraction of cells with the capacity to metastasize will dictate the ultimate prognosis of a tumor. Metastatic potential is dictated to a great extent by the expression pattern of cell surface proteins. Population averaged gene expression profiling by microarrays does not capture the signature of this minority subpopulation and therefore lacks the prognostic specificity to impact clinical practice. Needle aspirates of tumors reveal only a malignant phenotype in the few cells that are obtained and there are currently no good methods for characterizing cells when only a few are available. To address this need, we undertook to develop a microfluidic device to detect the surface protein expression profile of individual cancer cells without prior labeling. The device will be a microfluidic channel with multiple tandem antibody-coated patches over which cancer cells are directed by a microsyringe pump at flow rates of nanoliters per second. The transient interactions with surface ligands result in retardation of cell velocities over patches with cognate ligands of expressed surface proteins and normal cell velocities over patches coated with ligands that don't recognize surface proteins expressed by the cell. This will permit assigning an individual expression pattern for the proteins recognized by the immobilized antibodies for each cell. We hypothesize that the use of this device will make it possible to identify rare cells in a tumor with specific capacities to metastasize to unique organs based on their cell surface expression profiles with sensitivities and specificities of greater than 99%.

BODY:

We decided to characterize surface proteins associated with metastatic potential of breast cancer cells to the lung. We are using LM2 cells (kind gift of Juan Massague, Memorial Sloan-Kettering Cancer Center, NY) (1), derived from murine lung metastases of MDA-MB-231 cells that have a unique potential to re-metastasize to lung. We have verified that of the genes with differential expression patterns between LM2 and parental cells on gene arrays, four coded for surface proteins whose protein expression also differed between the two populations. We determined that the surface expression of VCAM, IL13RA2 and EMP1 was upregulated and that of CXCR4 was downregulated in LM2 cells as compared to MDA-MB-231 cells. We sorted the cells into three fractions for no, medium and high expression of these proteins at UMDNJ- Newark in the laboratory of Dr. Robert Wieder, initiating PI. Figure 1 demonstrates that the majority of LM2 cells express high levels of IL-13RA2 and that a sorted population, of which 100% of cells express this surface protein, maintain expression after several weeks of passage. Cells sorted for lack of expression of CXCR4 or for expression of VCAM and EMP1 did not maintain the level of expression of the sorted cells after two weeks in culture and hence, those antigens could not be used for development of the parameters of microfluidic retardation. Thus, all of the studies described were performed with the cells with sustained expression of IL13R and with non-expressing control LM2 P1, MDA-MB-231 and MCF-7 control cells.

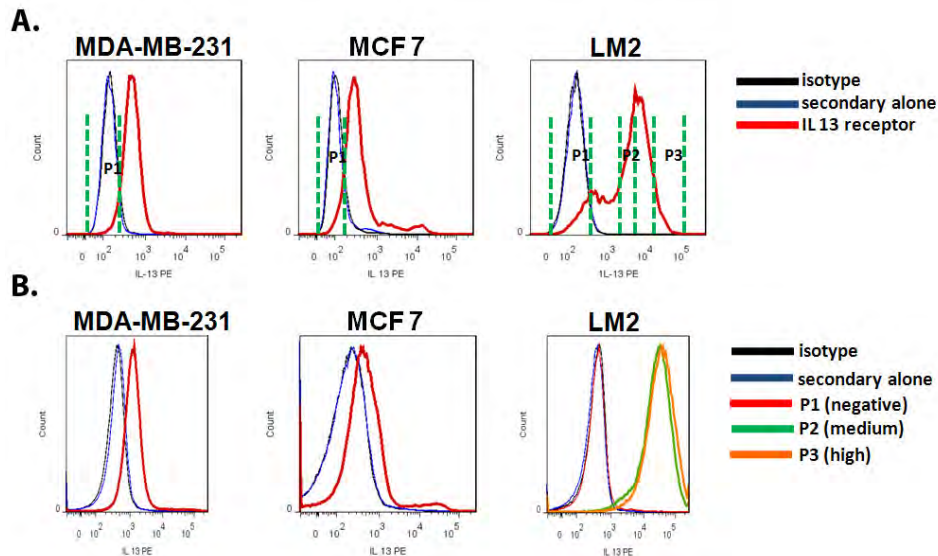


Figure 1. A. Sterile sorting of LM2 cells (P1, P2, P3), MDA-MB-231 and MCF-7 cells and maintenance of high levels of IL13 receptor (IL13R) expression in culture. A. LM2 cells expressed a significant fraction of cells with high expression of IL13R of >2 logs over other cells. Cells were sorted for low, medium and high expression and passaged for 2 weeks. B. Analysis of sorted and passaged cells demonstrated that P2 and P3 cells maintained a homogeneous, approximately 200x higher expression of IL13R than P1 or the other two cell lines. Scatchard analysis is being conducted to correlate these measurements with receptor numbers (Performed at UMDNJ, Newark).

We constructed a flow channel coated with IL13R antibody and BSA and conducted a series of experiments with different shear rates due to variable channel dimension, flow rates and viscosity from variable glycerol concentrations. The device (Figure 2) consisted of apposable glass plates with an inlet port for buffer, an inlet port for cells 5 mm downstream, and an outlet port for collection of buffer and cells. Tandem patches for BSA and IL-13RA2 antibody immobilization were separated by spacers of variable widths. Channel height was established by variable sized gaskets. We developed and characterized quantitative antibody adhesion to a microfluidic channel. More than 50% of adhered antibody remained on the patch after extensive washing with buffer pumped through the channel.

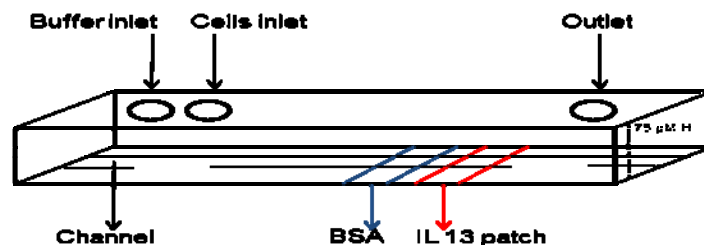


Figure 2. Shown is a sketch of the flow device used. Patches of immobilized ligands are 0.5 cm in length, but recent variations that provided reproducible flow rates include 100-200 micron patches with 100 micron spacers. Chamber heights varied from 25-100 μ m depending on gaskets used to separate plates and provided variations in shear rate.

Cells are suspended in standard media at approximately 100,000 cells/ml. This is sufficiently dilute to permit sufficient distance between single file cells to prevent interference with each other's flow. Volumes of 3.5 ml drawn up into a syringe are loaded onto the Harvard Apparatus Pump II syringe pump controller and pumped through Teflon tubing into the cell inlet port of the microfluidic channel in single file. Equal flow rate of buffer solution containing DMEM + 2.0 mM EDTA is pumped into the Buffer inlet. Flow rates are modulated in the 5-15 microliters/min

range, determined in calibration experiments to establish a consensus shear rate, as before (2). Individual cells with varying phenotypes tracked by imaging will be collected from the outflow channel into different tubes for further analysis, initially manually. The current detection and tracking of cells with microscope objectives and computer tracking programs and measurement of the velocity of individual cells as they traverse individually coated patches is sketched below (Figure 3).

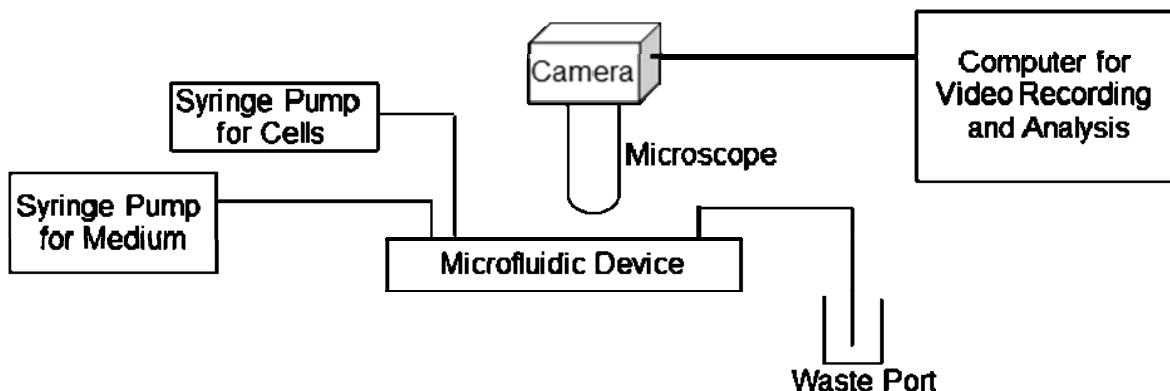


Figure 3. Current setup of the microfluidic device, pumping system and imaging and computational support. The waste port has the capacity to recapture specific cells for further analysis.

One of the major challenges is imaging a large protein-coated region while maintaining a high resolution that contains enough detail to allow tracking of cells over time. We use a latest generation high-resolution camera, an Epix Silicon Video 642 Camera with PIXCI Digital Frame Grabber attached to an inverted microscope with a 2X lens.

Interval timing is set to 2 images per second using a remote control cable. Images are recorded for 12 minutes on internal memory. Format used for recording images is high quality JPEG that allows facile import of images into image analysis software. Each series of images is imported into XCAP v3.2 software (Epix Inc., Buffalo Grove, IL). XCAP software permits tracking of each cell in successive images. An image containing no cells is subtracted from all images to remove all background noise. Cells are identified using 'blob' finding algorithm, which identifies all features with diameters between 5 and 15 pixels. Since cells are projected at 10 pixels each, they are readily identified. 'Tracking' algorithm identifies tracks of the cells and calculates velocity of cells (ΔH) by measuring distance traveled in a half second. The data is imported into Microsoft Excel and percentage retardation will be calculated.

We demonstrated statistically significant shifts in mean flow rates in sorted populations of IL13R (figure 4, D&E) on IL13R antibody. The standard deviations of the flow rate distributions across cognate antibody patches were much higher than those over control patches. They were restored with blocking antibody (Figure 4, F&G). The reason for the wider distribution curve was the significant rate of retardation in about 20% of the cells, which had cell velocities more than two standard deviations lower than the mean velocity of the cells on control patches in the experiment shown in figure 6, where the chamber height was 50 μm and flow velocity was 15 $\mu\text{l}/\text{min}$, giving a shear rate of 100/s with a glycerol concentration of 1.5%. The velocity of the remaining 80% of the cells fell within the distribution curve of the velocity of the control patches, despite having a uniformly high expression of IL13R. This rendered the specificity greater than 99% but the sensitivity, using this measurement, only about 26% (Figure 4).

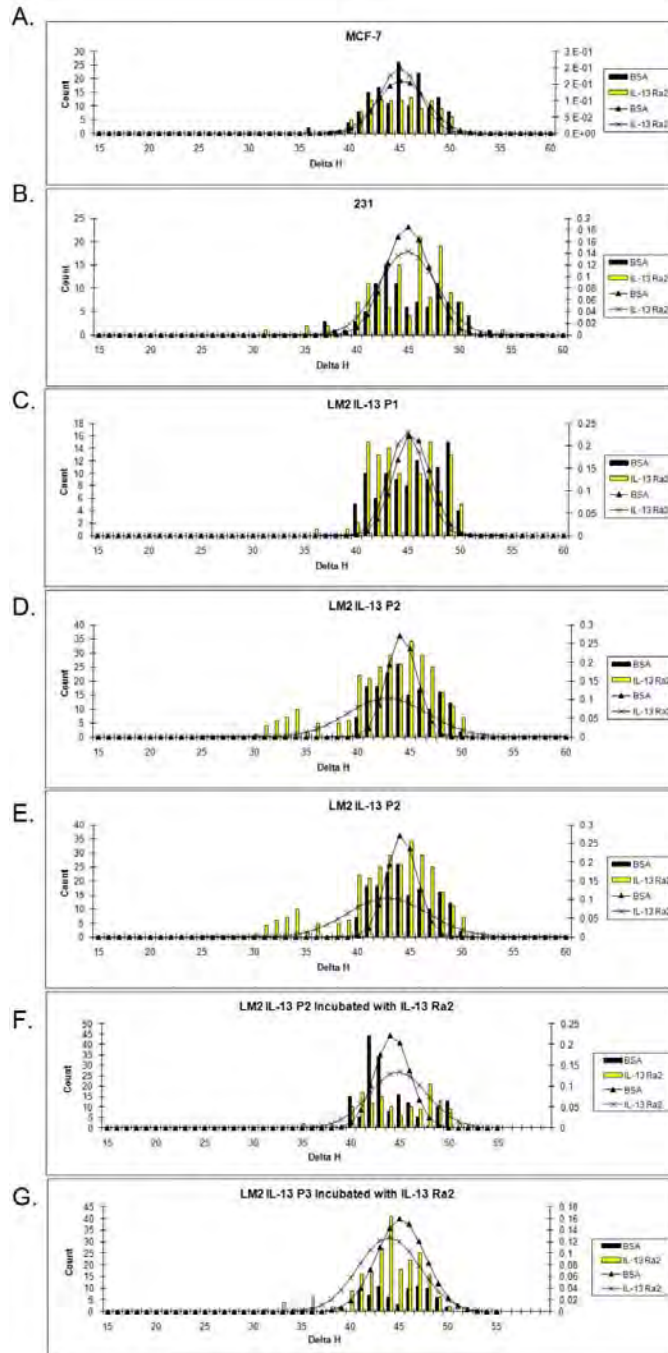


Figure 4. Sample flow retardation experiment with immobilized anti-IL13 receptor antibody. Below is the sketch of the flow device used. Table1 delineates the results demonstrating the percentage differences in the mean flow rates correlating with the presence of IL13R expression in sorted cells in this experiment. More significantly, the raw data depicted in figures 4 A-G demonstrate flow rates, depicted as differences in the number of pixels between cells (ΔH) in photographic frames taken at 0.5 second intervals. Only a subset of LM2 P3 and P3 cells had ΔH values below 35 (D & E), rendering specificity at greater than 99%, Sensitivity was around 26%. Blocking antibodies to IL13R eliminated flow retardation of LM2 P2 and P3 cells (F&G).

Table 1. Flow retardation experiment data from figure 4 conducted at conditions where chamber height was 50 μm and flow velocity was 15 $\mu\text{l}/\text{min}$ (shear rate of 100/s) with a glycerol concentration of 1.5%.

Cell Line	Device Coating	Average	St. Dev.	% CV	% Decrease	P value
MCF-7	BSA	48.359	3.821	8%	0.5%	0.15423
	IL-13	48.140	3.081	6%		
231	BSA	47.912	5.489	11%	1.5%	0.34076
	IL-13	47.204	8.839	19%		
LM2 IL-13 P1	BSA	48.968	5.174	11%	1.6%	0.14377
	IL-13	48.161	3.786	8%		
LM2 IL-13 P2	BSA	47.271	4.740	10%	2.8%	0.09254
	IL-13	45.925	6.555	14%		
LM2 IL-13 P3	BSA	48.322	5.073	10%	11.4%	0.00009
	IL-13	42.828	3.596	8%		

The sensitivity improved somewhat to ~34% and specificity remained greater than 99% with chamber height of 50 μm , flow velocity of 9 $\mu\text{l}/\text{min}$ (shear rate of 60/s) at a glycerol concentration of 0.75%. Decreasing the height of the chamber to 25 μm did not have a significant impact on further increasing sensitivity of specific interactions and retardation, even at slightly lower flow velocities. This was likely due to significantly increased shear rates negating any effect of increased cell-substratum contact compared to that in chambers of greater channel height.

By reanalyzing the data for rates in a 0.5 mm patch, we considered specific interactions as any that were more than two standard deviations below the average rates for all cells over control patches (Table 2). This provided a 100% specificity and a similar sensitivity at the 2 standard deviation cutoff, as did data when all runs across a patch were averaged for each cell. However, specificity remained 100% at lower differences than 2 standard deviations, while the sensitivity continuously increases to a maximum of 55%. If the cutoff was raised to around 1 standard deviation, the specificity dropped to 80% but the sensitivity rose to 71%. These analyses demonstrate that the unconsidered impulse to simply average rates over a patch will not necessarily differentiate between cells with specific and nonspecific binding with complete sensitivity and specificity.

Table 2. Reanalysis of data from the experiment shown demonstrating reciprocal relationship between sensitivity and specificity during analysis of the data.

Cutoff	Sensitivity	Specificity
40	32%	100%
41	35%	100%
42	45%	100%
43	55%	100%
44	55%	94%
45	71%	80%
46	81%	65%
47	94%	51%
48	97%	49%
49	97%	43%
50	100%	37%

We demonstrated differential flow rates of LM2 cells sorted for no expression, intermediate and high expression of the IL13 receptor over patches with adhered IL13R antibody, compared with appropriate controls. Similar experiments are ongoing for the other three antigens in single antibody-coated channels and proceeding to multiple antibody coated channels. Physical parameters like shear rates, shear stress, flow velocity, solvent viscosity, detergent concentration, divalent cations and antibody coating density are being modulated to affect maximum separation. This tool, in its final iteration, will permit validation of metastasis markers in clinical trials of cells obtained by needle aspirate without prior processing or labeling.

KEY RESEARCH ACCOMPLISHMENTS:

- Developed a biologically relevant model for studying flow retardation devices through the use of LM2 breast cancer cells with exclusive lung metastasizing capacity that had reliable and sustained cell surface expression of IL-13RA2.
- Developed a microfluidic device with
 - controlled fluid flow
 - cell introduction and collection capability
 - chamber dimension variability,
 - ligand patch distribution and dimension variability
 - antibody coating reliability
 - imaging capability needed for specific areas
 - data collection capability
 - data analysis capability
- Achieved 100% specificity in identifying cells expressing surface protein on breast cancer cells only present on cells with exclusive lung metastatic potential
- Began to address increasing sensitivity without significant loss of specificity
- Began development of multipatch device

REPORTABLE OUTCOMES:

The data has been presented at the 2011 Annual American Association of Cancer Research Meeting (3) and will be presented at the 2011 DOD Era of Hope meeting.

CONCLUSION:

We developed a microfluidic device with the capability to identify cells that express a surface protein present only on a breast cancer cell line capable of metastasizing exclusively to the lungs without the need tag the cells. The device detects the presence of the cell with a specific surface protein by slowing its rate of passage as it is pumped over a patch in the device coated with an antibody recognizing this protein. The rated of detection is 100% specific. These efforts will result in development of a device that will identify biologically significant cells in a sample derived by needle aspirates without having to subject them to labeling procedures that would result in their loss.

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APPENDICES:

None

SUPPORTING DATA:

All data appear in the body.