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Characterization of Single Cells from Dissociated Solid Tumors

Aaron J. Middlebrook,[1] Shahryar Niknam,[1] Joyce Ruitenberg,[1] Maria Suni,[1] Albert J. Mach,[1] Friedrich Hahn,[2] Eileen Snowden,[2] Warren Porter,[2] Rainer Blaesius,[2] Smita Ghanekar[1]

[1] BD Biosciences, 2350 Qume Drive, San Jose, CA 95131; [2] BD Technologies, 21 Davis Drive, Research Triangle Park, NC 27709

Abstract

The heterogeneous nature of solid tumors, coupled with the relatively small sample size of available biopsies, has led to an emerging need to glean as much information as possible from these valuable specimens. Current approaches to solid tumor analysis fail to completely reveal the diverse range of cellular compartments that comprise the tumor microenvironment. A comprehensive approach to tumor interrogation requires efficient tissue dissociation to facilitate analysis at the single-cell level. In contrast to current methods, single-cell analysis of tumor derived cell suspensions by flow cytometry has the potential to provide a more complete understanding of the many subpopulations within the tumor microenvironment and the cell-to-cell interactions that govern this space. Here we demonstrate an efficient workflow that enables comprehensive single-cell analysis of solid tumors from breast cancers. Using tumors from clinical samples and mouse models, we evaluated different dissociation and processing techniques for their effects on cellular viability and surface marker expression. Solid tumors were dissociated into single-cell suspensions using a combination of mechanical dissociation and enzymatic digestion. Phenotypic distribution and morphology of cells within the tumor microenvironment were evaluated using flow cytometry. As this approach evolves, and a knowledge base of relevant surface markers is established, this technology has the potential to significantly impact how tumor biopsies are processed to get multiparametric information at a single-cell level.

Methods

Surgical tumor biopsies from breast cancer patients were purchased (Conversant Biologics) and shipped for overnight delivery to BD Biosciences (San Jose, CA). The tumors were dissociated into single-cell suspensions using two methods. The "BD Workflow" (Figure 1) included mechanical dissociation using scalpels in a glass petri dish followed by a 30-minute enzymatic digestion using BD's Dissociation Reagent. An comparator workflow (Method 2) involved an existing and popular commercial method that differed in both the mechanical dissociation and the enzymatic digestion. Following dissociation, the samples were filtered using a 70-micron strainer, washed, and counted via trypan blue exclusion.

Aliquots of single-cell suspensions were stained with live/dead fixable amine viability dye (BD Biosciences, Cat. No. 564406), Hoechst 33342 nuclear stain (BD Biosciences, Cat. No. 561908) and fluorescent-conjugated monoclonal antibodies (BD Biosciences) against a variety of surface markers. Flow cytometry was performed on a BD LSRFortessa™ cytometer using BD FACSDiva™ software.

Figure 1: BD Workflow for Solid Tumor Dissociation

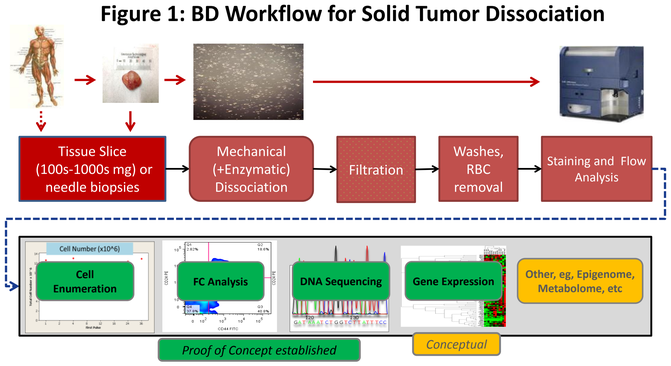
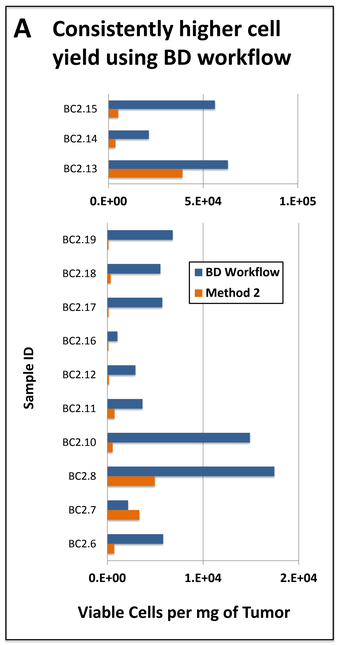


Table 1: Summary of Patient Pathology Reports*

BD ID	BC2.6	BC2.7	BC2.8	BC2.10	BC2.11	BC2.12	BC2.13	BC2.14	BC2.15	BC2.16	BC2.17	BC2.18	BC2.19
ID#	120278980	120289271	120292990	120300315	120312932	120326220	120328671	120333773	120333784	120343444	120343445	120345333	120345244
Collection Date	7/29/2014	8/21/2014	9/3/2014	9/23/2014	11/6/2014	12/9/2014	12/10/2014	1/5/2015	1/7/2015	2/1/2015	2/2/2015	2/16/2015	2/17/2015
Age of Patient	60	94	47	68	76	46	69	65	82	65	73	85	72
Race	OTHER	White	White	African American	White	White	White	White	White	White	African American	White	White
Treatment Status		Active (Tamoxife	Pre Tx	Pre Tx	Prev Diagnosis	Pre Tx	Pre Tx	Pre Tx	Pre Tx	Pre Tx	Pre Tx	Pre Tx	Pre Tx
Stage		Grade 2	Grade 3	Grade 3	Grade 3	Grade 2	Grade 3	Grade 3	Grade 2	Grade 1	Grade 1	Grade 2	Grade 2
Tumor Size		1.77 mm	3 cm	6.5 cm	3.0 cm	9.5 cm	5.0 cm	4.2 cm	6.5 cm	0.8 cm	0.8 cm	2.5 cm	2.4 cm
Cancer Class**		DCI	DCI	DCI	LCI	LCI	DCI	DCI	LCI	DCI	DCI	DCI	DCI & LCI
Sentinel Metastatic Nodes (pos/total)		1/3	0/2	none	1/7	1/2	2/17	2/7	none	0/4	0/4	none	2/16
Estrogen Recptr	++			++	++	++	--	++	++	++	++		
Progesterone Recptr	++			++	++	++	--	++	++	NA	--		
Her-2/Neu	++			+/-	--	NA	--	--	--	++	NA		

*Pathology reports are provided 7–10 days after sample is processed courtesy of Conversant Biologics.

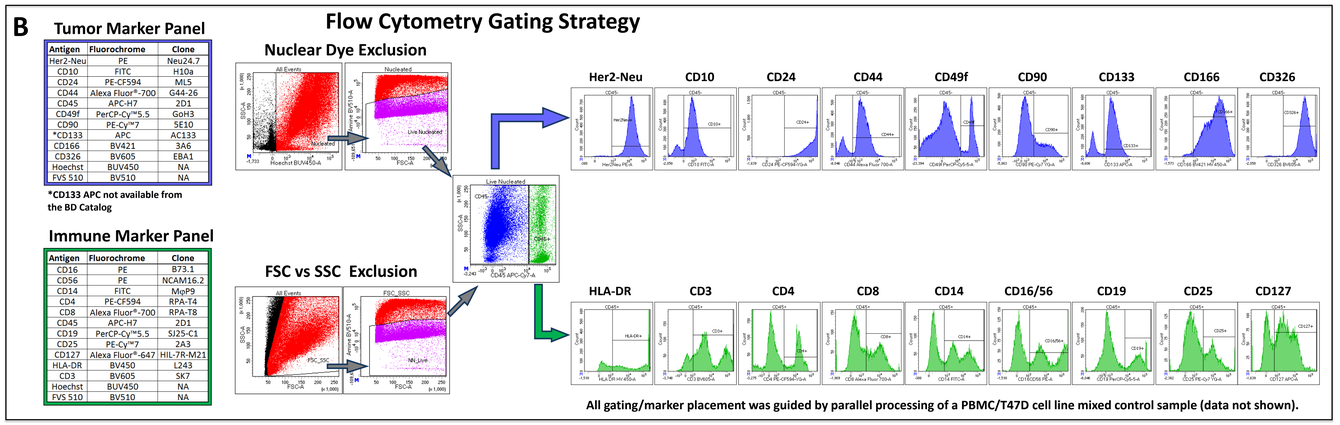
** Ductal carcinoma in situ (DCI); Lobular carcinoma in situ (LCI)



Results: Figure 2

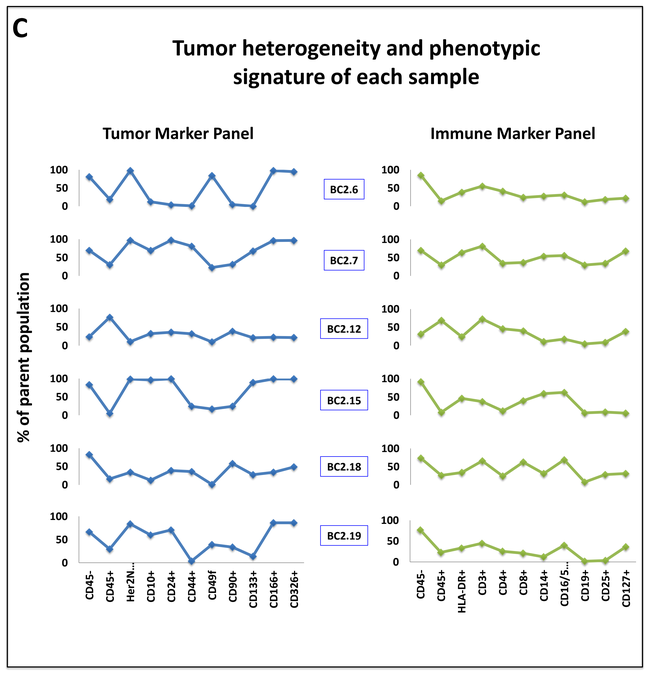
(A) Following tumor dissociation, the viability and total cell recovery were measured using a VI-CELL™ automated cell counter. The number of viable cells per mg of tumor tissue processed (yield) was then calculated using viability and the total number of cells recovered.

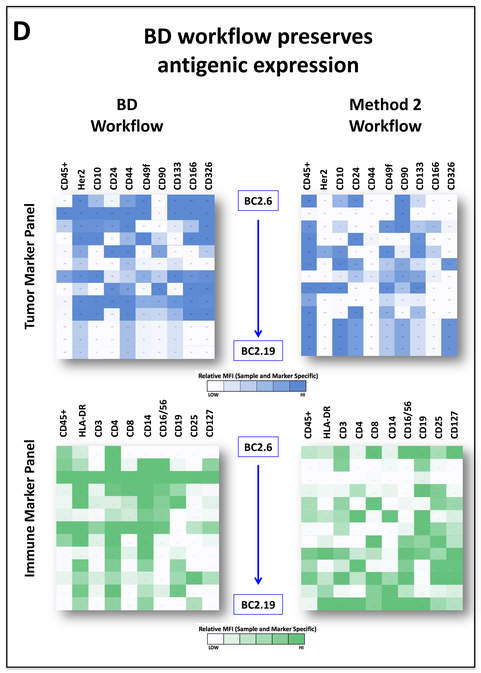
(B) Aliquots of cell-suspensions from each of the two workflows were stained with a tumor marker panel (blue box below) and an immune marker panel (green box below) and acquired as described in methods. The flow data were analyzed using either a nuclear stain (Hoechst 33342) as an inclusion/exclusion parameter or using a standard FSC vs. SSC debris exclusion strategy. Data in this figure is representative of BD Workflow. The hierarchical gating progresses from left to right across the figure. The blue histograms (top row) show profiles of Her2-Neu, CD10, CD24, CD44, CD49f, CD90, CD133, CD166, and CD326 (Epcam) within the CD45- subpopulation. The green histograms (bottom row) show profiles of HLA-DR, CD3, CD4, CD8, CD14, CD16/CD56, CD19, CD25 and CD127 within the CD45+ subpopulation.



Results: Figure 3

(C) Expression levels of each of the different markers measured plotted as % of parent population. Plots demonstrate the inherent heterogeneity of human clinical samples. (D) Median Fluorescence Intensity (MFI) was compared across tumor processing methods (BD workflow vs Method 2) and across analysis methods (Nuclear stain exclusion vs FSC/SSC exclusion; data not shown) to determine any effects. The MFI within each marker and within each sample were ranked relative to each other and assigned one of 4 shades of green or blue (darkest, medium dark, light, and lightest). A shade change represents a 10% change such that dark green or blue is approximately 40% higher in MFI than the lightest green or blue.





Conclusions

- **Solid tumor dissociation and subsequent analyses by flow cytometry** of the resulting cell suspension is a novel sample evaluation platform with a wide array of potential applications.
- The BD workflow for solid tumor dissociation demonstrates **significant increases in overall viable cell recovery per mg of tumor tissue processed** compared to a commercially available alternative.
- **The use of a nuclear stain (such as Hoechst 33342) can improve the overall quality of data by including only nucleated cells and excluding debri, tissue fragments**; however, mere use of FSC vs SSC as a means of excluding debris does not significantly impact the results within the context of cellular phenotype other than adding subjectivity to draw a gate.
- Surface marker expression levels as measured by median fluorescence intensity **(MFI), can be significantly impacted by the dissociation method used**.
- **Flow cytometry enables the quantification of tumor cell and immune infiltrates heterogeneity** and can be used to establish a phenotypic signature of a given tumor sample. This signature has immediate implications for the tumor sample and can further be used to identify and isolate subpopulations of interest.