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Sorting Out the FACS: A Devil in the Details

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The reproduction of results is the cornerstone of science; yet, at times, reproducing the results of others can be a difficult challenge. Our two laboratories, one on the East and the other on the West Coast of the United States, decided to collaborate on a problem of mutual interest—namely, the heterogeneity of the human breast. Despite using seemingly identical methods, reagents, and specimens, our two laboratories quite reproducibly were unable to replicate each other's fluorescence-activated cell sorting (FACS) profiles of primary breast cells. Frustration mounted, given that we had not found the correct answer(s), even after a year. Rather than giving up or each publishing our data without the other laboratory, we decided to work together to solve these differences, even traveling from one laboratory to the other in order to perform experiments side by side on the same human breast tissue sample. This exercise confirmed our suspicions and resolved our problem. Here, we summarize our cautionary tale and provide advice to our colleagues.

The ever-increasing use of flow cytometry and FACS in the past decade has been accompanied by a surge of interest in learning how to incorporate primary normal breast tissues and breast tumors in biomedical research. This interest in primary-tissue-based research stems from increased awareness that cell polarity and shape, mechanical forces, and tissue organization are all potent regulators of cell and tissue phenotype, function, and physiology (for a review, see Nelson and Bissell, 2006). Taking these factors into account adds many more dimensions to an already considerable body of work on cellular heterogeneity of breast tissues and its tumors. The heightened awareness of the critical importance of studying cells close to their context in vivo makes the exercise even more challenging.

Paired with in situ characterizations, FACS has emerged as the technology most suitable for distinguishing diversity among different cell populations in the mammary gland. Flow instruments have evolved from being able to detect only a few parameters to those now capable of measuring up to—and beyond—an astonishing 50 individual markers per cell (Cheung and Utz, 2011). As with any exponential increase in data complexity, the importance of developing robust preparation and analytical protocols that generate reproducible results increases commensurately (Alexander et al., 2009; Herzenberg et al., 2006). Here, we share our surprising and time-consuming experience of trying to achieve similar data sets in the East and West Coast settings while collaborating on a shared grant. The task at hand was to confirm each other's data so that we could move to the next stage of collaboration. Given that CD44 and CD10 are frequently used as markers in most lineage and "cancer stem cell" studies, and because there are substantial disagreements and confusion about the significance of what FACS fractions from mammary gland and breast tumors signify in different laboratories, we paid special attention to these two markers. A set of data that was supposed to be completed in a few months took 2 years to understand and sort out.

Our challenges began when our two laboratories, located in Boston and in Berkeley, began our joint funded project to study the involvement of myoepithelial cells in breast tumor progression. An early aim was to separate and characterize cellular subpopulations derived from breast reduction mammoplasties. Molecular analysis of separated fractions was to be performed in Boston (K.P.'s laboratory, Dana-Farber Cancer Institute, Harvard Medical School), whereas functional analysis of separated cell populations grown in 3D matrices was to take place in Berkeley (M.J.B.'s laboratory, Lawrence Berkeley National Lab, University of California, Berkeley). Both our laboratories have decades of experience and established protocols for isolating cells from primary normal breast tissues as well as the capabilities required for flow sorting primary cells from mice and women.

We settled on isolating cell populations independently in each laboratory in order to avoid potentially adverse effects caused by shipping freshly sorted cells across the country (Figure S1A). We carefully characterized antibodies for CD24, CD10, and CD44 and gauged their specificity. Because we were initially interested in the nonluminal subfractions, we were subtracting (gating out) the $CD24^+$ luminal cells on the cytometer and analyzing CD10 and CD44 expression in the remaining subpopulations. We quickly discovered, however, that reproducing each other's FACS profiles would not be so straightforward. Despite the fact that both groups began with primary breast tissues from reduction mammoplasty and the set of FACS profiles obtained in each laboratory was consistently reproducible, the profiles obtained in Boston and Berkeley were not similar (Figure S1B). The question was why.

A simple explanation could have been that the FACS instruments used at each institution differed: a FACSAria was used in Boston, whereas a FACSVantage was used in Berkeley. However, we quickly

ruled out instrumentation as a source of the problem by testing on each instrument calibration beads and cell line standards (mixtures of five breast cell lines with distinguishable CD24, CD10, and CD44 levels; Figure S1C and data not shown). Other possibilities we considered were the specific sources of tissues (e.g., East versus West Coast subjects and the method of procurement), media composition, source of serum and additives, tissue processing, and methods of staining cell populations. We compared protocols and ensured that we were using identical enzymes, antibodies, and reagents by either making purchases from identical lots or shipping aliquots of different reagents to each other. We did discover slight differences in some parameters and could quickly fix these. Still, tissues processed in Boston always produced the Boston profile and tissues processed in Berkeley produced the Berkeley profile. We were losing time and resources, and we were perplexed.

The back and forth exchange lasted about a year until we realized that, when tissues were digested into organoids at one location and shipped to the other location to be separated and analyzed, the resultant FACS profiles matched the original institution where the collagenase digestion of the tissue was performed. This was an important clue.

With our attention focused on how we were processing tissues, the two first coauthors met in Berkeley to work side by side so we could observe every step of each other's methods. With a fresh specimen in hand, we diced and split the tissue fragments into two equal parts and prepared each half independently—Boston versus Berkeley. The results were surprising but clear: the two profiles prepared from the same reduction mammoplasty were different and bore the signature of the respective laboratory (Figure S1D)! However, this time-consuming and expensive exercise gave us the clue we had been waiting for: our methods for incubating the collagenase digests were distinctly different. In the Boston method, tissue was being stirred comparatively more vigorously in a flask with a stir bar at a speed that achieved constant agitation (300–500 revolutions per min [RPM]) until the digest was observed to be complete, which typically took 6–8 hr. In the Berkeley method, tissues were digested in 50 ml tubes using half the concentration of collagenase used in Boston (1 versus 2 mg/ml) while rocking relatively gently on a rotating platform (80 RPM) and for a much longer time (18–24 hr). We found that, in addition to the distinct FACS profiles obtained by each method of digestion, there was a dramatic difference in the efficiency of organoid recovery—roughly 5× more organoids were recovered from the slower and longer digest. We now had a binary problem to solve: was it the collagenase concentration or the agitation speed and length of digestion (or both). To test this, we divided another fresh tissue sample and digested it with the two different enzyme concentrations but with a slow/gentle agitation method for both. This resulted in a similar (large) yield of organoids and identical FACS profiles (matching those from Berkeley; e.g., Figure S1B, right), thus identifying the quality of agitation as the culprit.

We have reproduced these results several times in each of our laboratories and are confident that the speed and length of agitation during the collagenase digestion has a dramatic effect both on organoid yield and CD44 antigen presentation in the $CD10^+$ cell fraction. Why this is so awaits further experiments. One possibility is that, in both of our methods, organoids are further enzymatically treated with trypsin in order to yield single-cell suspensions. CD44 is notoriously sensitive to trypsin cleavage (Camp et al., 1991; Takeda et al., 2006), and different splice variants demonstrate distinct sensitivities (Biddle et al., 2013). However, neither trypsin nor collagenase was the direct cause of the observed differences here. Instead, it was the vigorous agitation during collagenase treatment itself that lead to reduced CD44 antigen detection on the $CD10^+$ cell population (CD44 levels were preserved on $CD10^-$ cells independently of the agitation procedure used). Possible explanations we have considered include mechanical destruction of CD44 or relevant epitopes, CD44 downregulation or epitopes becoming cryptic during the faster, more vigorous digest method, or release of metalloproteinases or other peptidases that cleave CD44 variants on the $CD10^+$ population. The method of agitation affects observed CD44, and this sensitivity has obvious implications for those optimizing their own digestion protocols as well as for those depending on CD44 antibodies to characterize different cell subpopulations, such as the widely used $CD44^+CD24^-$ protocols for isolating cancer stem cells. It is important to emphasize that we did not detect similar sensitivities for other markers we use commonly, including CD10, CD24, CD227, CD49f, CD90, CD31, CD34, or CD45. It is educational that CD44 staining used by countless laboratories in FACS analysis can so easily be altered by an apparently minor difference in methodology. The irreproducibility of CD44 in FACS analysis has become a legend. We expect sharing these experiences demonstrates that much can be learned through open collaboration and persistence.

SUPPLEMENTAL INFORMATION

Supplemental Information contains Supplemental Experimental Procedures and one figure and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.02.021.

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