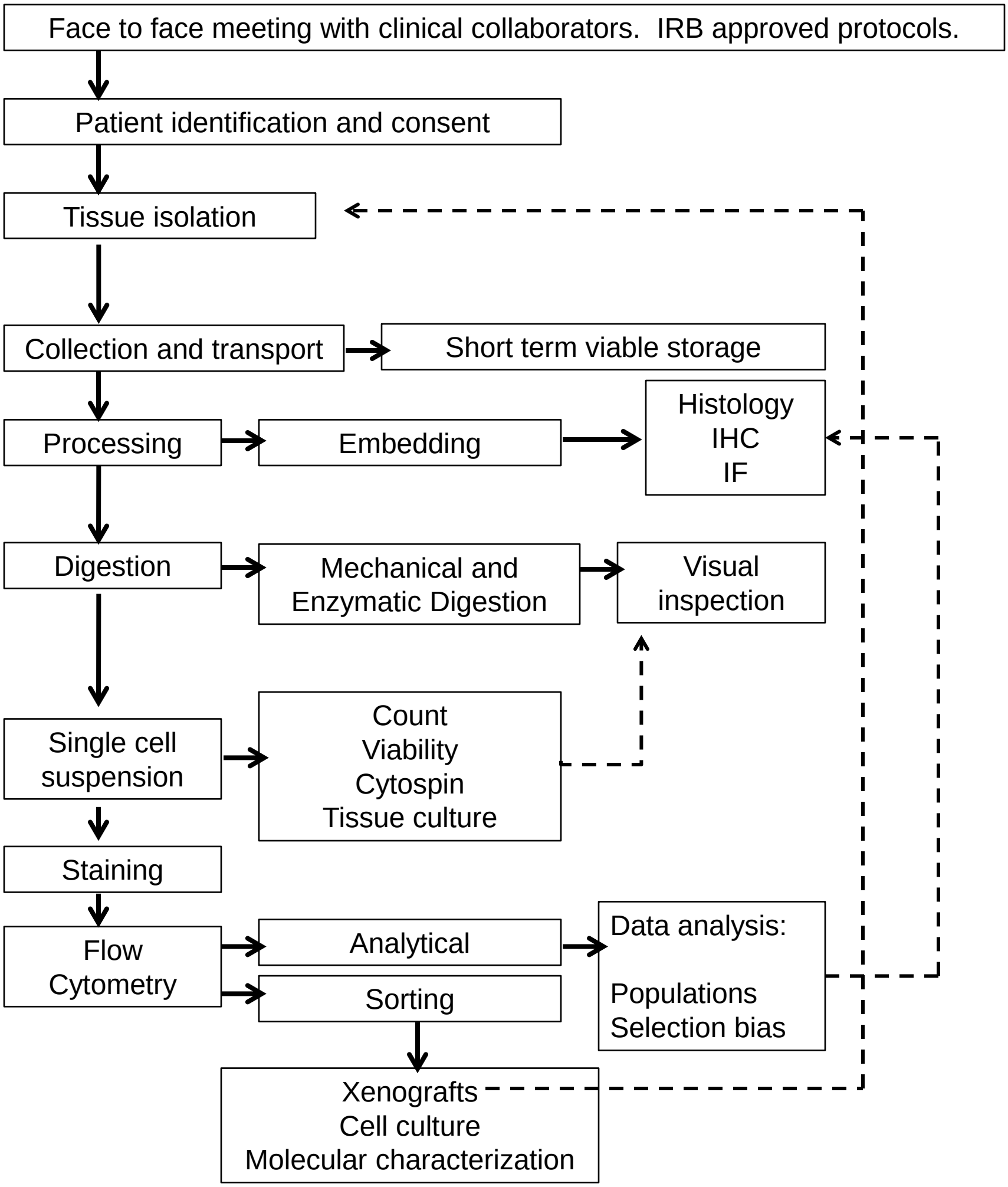


Supplementary Figure 1. Schematic representation of tissue processing, staining, analysis workflow. IHC = immunohistochemistry, IF = immunofluorescence.

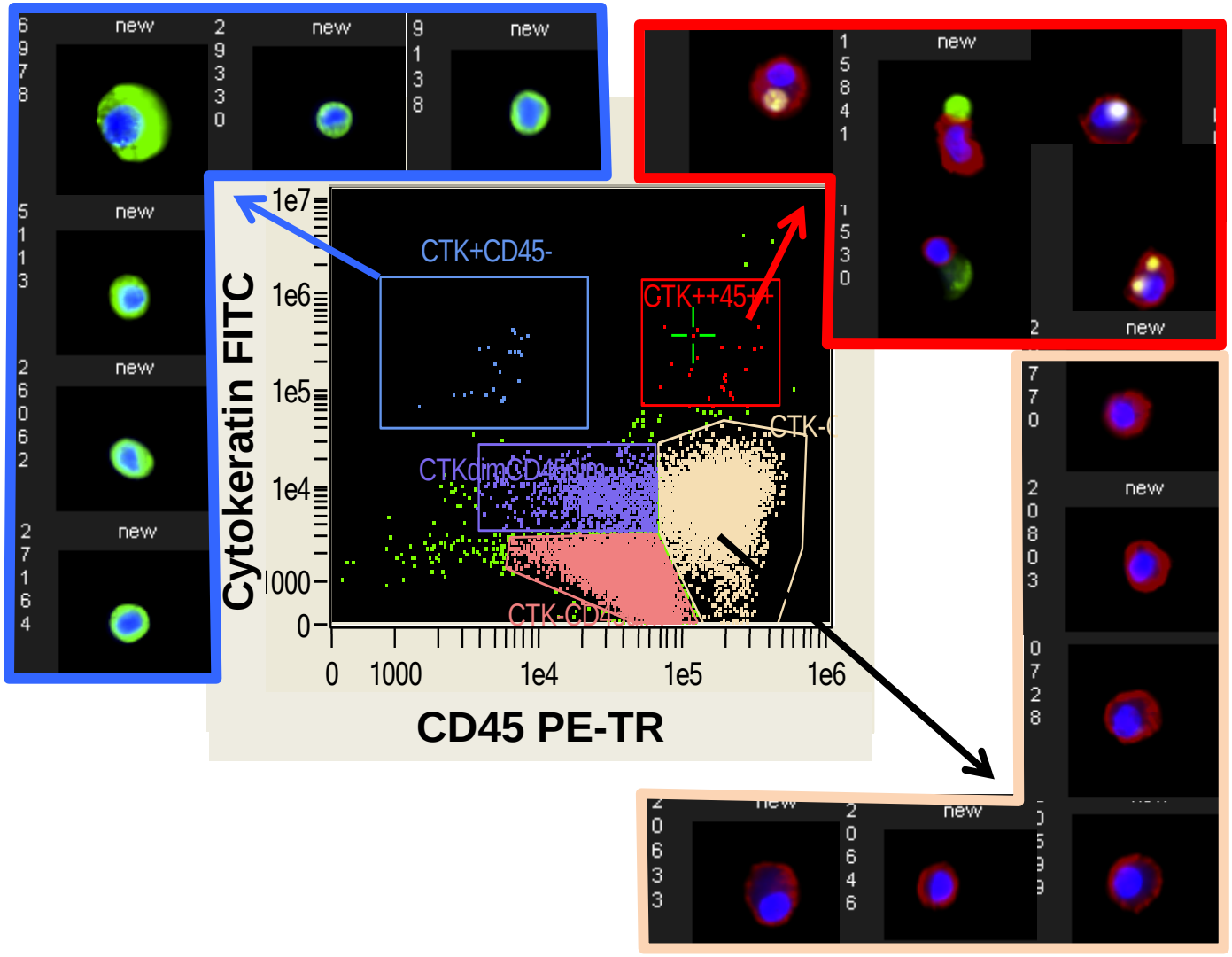
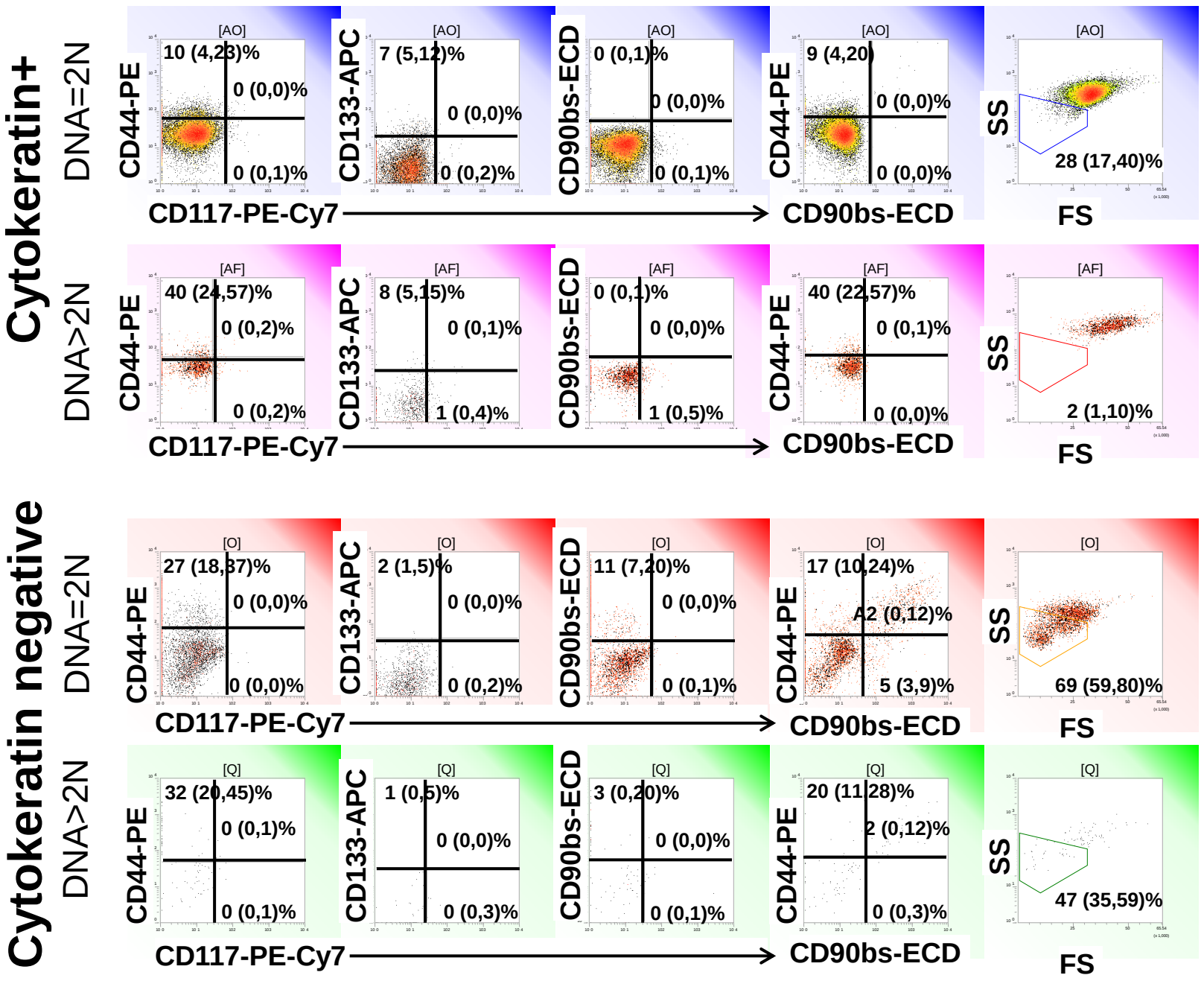


Figure S2. Cytokeratin+/CD45+ events are tumor-laden macrophages. Metastatic pleural effusion cells were stained for CD45, fixed, saponin permeabilized and stained for intracellular cytokeratin. Fixed cells were incubated with the nuclear stain DRAQ5 prior to acquisition on an Amnis ImageStream imaging cytometer (Amnis, Seatle WA). Imagery was false-colored blue for DRAQ5, cytokeratin, green, and CD45, red. A complex staining pattern was observed consistent with conventional flow cytometry. Cytokeratin+/CD45- events imaged as small and large tumor cells typical of a metastatic effusion. The cytokeratin-/CD45+ population was morphologically consistent with lymphocytes and monocyte/macrophages. Cytokeratin+/CD45+ events were either macrophages with cytokeratin+ cytoplasmic inclusions (yellow), or tight conjugates of mononuclear cells and anucleate tumor cells.

Adjacent lung 94



Supplementary Figure 3. Identification of classifiers and outcomes in normal lung. The top panels show identification of cytokeratin+ (E2) and cytokeratin negative (E1) cells among nonhematopoietic (D) cells. These were further subdivided in diploid and aneuploid populations, creating 4 classes on which to measure outcomes (stem/progenitor markers, light scatter). The region percents listed are mean values, parentheses indicate lower and upper 95% confidence intervals.

MIFlowCyt Components

Flow cytometric determination of stem/progenitor content in epithelial tissues: An example from non-small lung cancer and normal lung.

1. Experiment Overview

The experiment overview shall contain the following information:

1.1. Purpose

A brief description of the goal of the experiment. This should include the rationale and hypothesis.

Single cell analysis and cell sorting has enabled the study of development, growth, differentiation, repair and maintenance of "liquid" tissues and their cancers. The application of these methods to solid tissues is equally promising, but several unique technical challenges must be addressed. This report illustrates the application of multiparameter flow cytometry to the identification of candidate stem/progenitor populations in non-small cell lung cancer and paired normal lung tissue.

1.2. Keywords

The keywords should include terms from an appropriate vocabulary (e.g., MeSH) to describe the experiment.

Non-small cell lung cancer, lung stem cells, flow cytometry data analysis, solid tissue, collagenase, CD133, CD44, CD90, CD117

1.3. Experiment Variables

Variables are attribute(s) that differ between samples within an experiment due to pre-existing differences in sample states or due to experimental manipulation of the samples. If applicable, a brief description of the conditional and/or manipulated variables in the experiment shall be provided (e.g., smoker vs. nonsmoker, IL-2 treatment vs. no treatment, knockout versus wild-type, varying number of transplanted cells, varying treatment dosage, etc.). The number of instances per experimental group should also be stated.

Non-small cell lung cancer tumors and adjacent normal lung parenchyma

1.4. Organization

1.4.1. Name

Donnenberg Research Laboratory
University of Pittsburgh
Hillman Cancer Center

1.4.2. Address

2.35 Hillman Cancer Center Research Pavilion
5117 Centre Avenue
Pittsburgh, PA 15213

1.5. Primary Contact
The following shall be specified for the experimental primary contact:

1.5.1. Name
Dr. Vera Donnenberg

1.5.2. Email Address
donnenbergvs@upmc.edu

1.6. Date
The date or time period during which the investigation was performed (i.e., from collecting and treating samples to performing data analysis) shall be stated.
June 30, 2009 – August 31, 2011

1.7. Conclusions
A brief summary of the interpretation of the results or outcome of the experiment shall be provided if available.
We discuss issues unique to performing flow cytometry on disaggregated solid tissues, using lung tumors and normal lung as examples. The manuscript has several unique features: in the methods section each topic begins with a discussion/commentary followed by the methods specific to our example; we have also included a complete and detailed SOP for preparation of single cell suspension from solid tissue for inclusion as an online supplement.

1.8. Quality Control Measures
8pk beads are used for cytometer validation and determining daily target channels. Calibrite beads and IgG capture beads are used as single fluorochrome staining for compensation controls and antibody integrity controls.

1.9. Other Relevant Experiment Information
Additional information about the experiment should be provided if relevant. This may include funding announcements, related publications (which should be referenced by PMID), URIs, databases, etc.
This work was funded by grants BC032981 and BC044784 from the Department of Defense, and supported by Production Assistance for Cellular Therapy (PACT) under contract #N01-HB-37165, the Commonwealth of Pennsylvania, the Hillman Foundation and the Glimmer of Hope Foundation. The UPCI Cytometry Facility is supported by CCSG P30CA047904.

2. Flow Sample/Specimen Details
The flow sample details shall include a description of each sample material used in the experiment (2.1), (2.2), how they were treated (2.3) and what reagents were used (2.4) to fluorescently label the material. Relations between samples, aliquots, different

treatments, and replicates shall be unambiguously described.

2.1. Sample/Specimen Material Description

Sample materials shall be described according to 2.1.1, 2.1.2, or 2.1.3, based on the type of the sample material. Each sample shall be distinguished from other samples within the same experiment.

Non-small cell lung cancer samples and paired adjacent normal lung tissue were obtained from 17 patients at the time of surgical resection of the tumor. Specimens were collected under protocols approved by the University of Pittsburgh Internal Review Board (UPCI 99-053, 020391, 0503126, 07090247).

2.1.1. Biological Samples

The following information about Biological Samples shall be provided:

2.1.1.1. Biological Sample Description

A description of the biological sample shall be provided, including the sample type, if relevant (e.g., C57BL/6 spleen, purified CD4+ lymphocytes, BALB/c thymocyte DNA, control patient PBMCs, protein lysate from lung cancer biopsy, peripheral blood from patient with Type I diabetes, liver biopsy, etc.).

Non small cell lung cancer tumor and adjacent normal parenchyma excised during clinically indicated surgery.

2.1.1.2. Biological Sample Source Description

The source of the biological sample shall be described (e.g., wildtype mouse, C57BL/6 spleen, C57BL/6 splenocytes). If the source is a cell line the description shall include its name, ATCC [17] (or equivalent) number, and cell type.

Seventeen paired tumor/normal lung samples were collected at the time of surgical excision and processed immediately. Collagenase and DNA treated single cell suspensions form NSCLC and normal adult lung.

2.1.1.3. Biological Sample Source Organism Description

2.1.1.3.1. Taxonomy

The source organism shall be specified by genus and species (e.g., *Mus musculus*). The terms should come from an appropriate standard such as the NCBI taxonomy database [12]. Taxonomy information should also contain the type of subspecies and organism strain, if applicable. A standard taxonomy may be extended or a proprietary taxonomy may be used, especially if detailed identification is relevant and beyond the scope of standard taxonomies.

Human

2.1.1.3.2. Age
The age shall be provided if applicable for the particular organism (e.g., 6 weeks). It may also include the developmental state (e.g., Theiler stage 23).
Age range from 42 – 81 years.

2.1.1.3.3. Gender
The gender shall be provided if applicable for the particular organism.
Females and males were recruited.

2.1.1.3.4. Phenotype
Appropriate phenotype characteristics such as disease state shall be described (e.g., increased tumorigenesis).
Non small cell lung cancer and adjacent normal adult lung.

2.1.1.3.5. Genotype
Appropriate genotype characteristics shall be described (e.g., p53-/-). N/A

2.1.1.3.6. Treatment
All source organism treatments shall be described (e.g., treatment with cyclooxygenase-2 (COX-2) inhibitor).

Tissues were mechanically and enzymatically dissociated into single cell suspension and stained with a panel of antibodies used for negative gating (CD45, CD14, CD33, glycophorin A), identification of epithelial cells (intracellular cytokeratin), and detection of stem/progenitor markers (CD44, CD90, CD117, CD133). DAPI was added to measure DNA content. Formalin fixed paraffin embedded tissue samples were stained with key markers (cytokeratin, CD117, DAPI) for immunofluorescent tissue localization of populations detected by flow cytometry.

2.1.1.3.7. Other Relevant Biological Sample Source Organism Information
Additional information about the source organism should be provided if relevant. This may include anatomic location of the source, visit time/date, or additional details and comments.

2.1.1.4. Other Relevant Biological Sample Information
Additional information about the biological sample should be provided if relevant. This may include relevant information such as *in vivo* or *in vitro* treatment, stimulation, preparation /

enrichment protocol, culture/growth protocol, collection time/date, whether the sample was frozen/banked/fixed, etc.

2.1.2. Environmental Samples
N/A.

2.1.3. Other Samples
SPHERO Rainbow Calibration Particles-8 peaks (Spherotech, RCP-30-5A)
BD Calibrite Beads-Unlabeled, FITC, PE (BD Biosciences, 349502)
BD Calibrite Beads-APC (BD Biosciences, 340487)
BD CompBeads Anti-mouse Ig, κ / negative control (FBS) Compensation Particle Set

2.1.3.1. Other Sample Description
8 peak Rainbow Calibration Particles contain different fluorescence intensities similar sized particles. Anti-mouse IgG BD CompBeads are polystyrene microparticles that may be labeled with a fluorochrome-conjugated mouse antibody, while Calibrite Beads are hard-dyed with a particular fluorochrome.

2.2. Sample Characteristics
Key information about the possible and expected sample characteristics should be noted as they provide the rationale for the experimental design, including the choice of appropriate reagents. Sample characteristics description should include the expected and possible types of cells or other particles in the sample material.

2.3. Sample Treatment(s) Description
The description shall include details about treatment agents, which play the role of experiment variables, or shall specify that samples were untreated. For example, a sample treated with an agent might be compared to an untreated sample; when reproducing and interpreting such an experiment, having access to details on treatment agents and conditions is essential in order to query and retrieve samples for further analysis. The treatment description should also contain other relevant treatment details such as, but not limited to, time, temperature, and concentration.

Tissues were mechanically and enzymatically dissociated into single cell suspension and stained with a panel of antibodies used for negative gating (CD45, CD14, CD33, glycophorin A), identification of epithelial cells (intracellular cytokeratin), and detection of stem/progenitor markers (CD44, CD90, CD117, CD133). DAPI was added to measure DNA content. Formalin fixed paraffin embedded tissue samples were stained with key markers (cytokeratin, CD117, DAPI) for immunofluorescent tissue localization of populations detected by flow cytometry. Detailes procedures are presented in the Methods section. Further we have also included a complete and detailed SOP for preparation of single cell suspension from solid tissue for inclusion as an online supplement.

2.4. Fluorescence Reagent(s) Description

The expected and possible types of cells or other particles in the sample material, and their expected and possible measurable characteristics should be noted as these represent the key information for choosing appropriate reagents with respect to the experimental goal. The following information shall be provided about each fluorescence reagent used. Information about the characteristic(s) being measured, as well as details about what plays the role of analyte, analyte detector, and analyte reporter should be descriptive enough to allow for accurate interpretation of the experiment data. Table 1 lists some examples of reagent descriptions.

Laser	Fluorochrome	Abs/dye	Clone	Manufacturer	Catalog #
488nm	FITC	Anti-pan Cytokeratin	J1B3	Beckman Coulter	IM2356
	PE	CD44	J.173	Beckman Coulter	A32537
	Biotinylated	CD90	5E10	BD Biosciences	555594
	ECD	Streptavidin		Beckman Coulter	IM3326
	PE-Cy5	CD14	RMO52	Beckman Coulter	IM2640U
	PE-Cy5	CD33	D3HL60.251	Beckman Coulter	IM2647U
	PE-Cy5	Glycophorin A	GA-R2(HIR2)	BD Biosciences	559944
	PE-Cy7	CD117	104D2D1	Beckman Coulter	IM3698
638nm	APC	CD133	293C3	Miltenyi Biotech	130-090-854
	APC-Cy7	CD45	2D1	BD Biosciences	348805
405nm	FL9 Log	DAPI		Molecular Probes	D1306
	FL10 Lin	DAPI		Molecular Probes	D1306

2.4.1. Characteristic(s) Being Measured

The relative amount of molecules, properties, or processes being evaluated (e.g., CD25, apoptosis, membrane permeability, cell viability, oxidative burst). The characteristic(s) being measured shall be provided

whenever there is ambiguity about the analyte being measured, such as when sample processing affects detection of the analyte (e.g., propidium iodide example in Table 1). The optical detector (e.g., FL1) or parameter (e.g., FL1-H, see 3.3.6) used primarily for this measurement shall be indicated.
Flow cytometry: Single cell suspensions were stained with a panel of antibodies used for negative gating (CD45, CD14, CD33, glycophorin A), identification of epithelial cells (intracellular cytokeratin), and detection of stem/progenitor markers (CD44, CD90, CD117, CD133). DAPI was added to measure DNA content.

Immunofluorescence and histology: Formalin fixed paraffin embedded tissue samples were stained with key markers (cytokeratin, CD117, DAPI) for immunofluorescent tissue localization of populations detected by flow cytometry.

2.4.2. Analyte
What plays the role of the analyte? Which substance or chemical constituent is the subject of interest of the analytical procedure, what target is specifically bound by the analyte detector (e.g., CD25)?
Pan-cytokeratin, CD44, CD90, CD117 and CD133

2.4.3. Analyte Detector
What plays the role of analyte detector? Which component of the fluorescence reagent specifically binds to the analyte to make it detectable (e.g., anti-CD25 antibody)?

2.4.4. Analyte Reporter (Fluorochrome)
What plays the role of analyte reporter? Which component of the fluorescence reagent reports the presents of the analyte to the flow cytometer? What substance (label) is used to generate the measured signal (e.g., FITC)? FITC, PE, PE-Texas Red, PE-Cy7, APC, DAPI

2.4.5. Clone Name or Number
If the probe is a monoclonal antibody, the clone name or number shall be provided. See table 1. Prior to intracellular cytokeratin staining, cells were stained for surface markers (2 uL each added to the cell pellet, 15-30 minutes on ice; CD44-PE (Beckman-Coulter, Cat No. A32537), CD90-biotin (BD, Cat.No. 555594), Streptavidin-ECD (Beckman Coulter, Fullerton, CA Cat. No. IM3326), CD14-PECy5 (Beckman-Coulter, Cat. No. IM2640U), CD33-PECy5 (Beckman-Coulter, Cat. No. IM2647U), Glycophorin A-PECy5 (BD Biosciences, Cat.No.559944), CD133-APC (Miltenyi Biotech Cat.No. 130-090-854), CD117-PC7 (Beckman Coulter, Cat. No. IM3698), CD45-APCCy7 (BD, Cat. No. 348805)), and fixed with 2% methanol-free formaldehyde (Polysciences, Warrington, PA). Cells were then permeabilized with 0.1% saponin (Beckman Coulter) in phosphate buffered saline with 0.5% human serum albumin (10 minutes at room temperature), cell pellets were incubated with 5 uL of neat mouse serum for 5 minutes, centrifuged and decanted. The cell pellet was disaggregated and incubated with 2 uL of anti-pan cytokeratin-FITC (Beckman Coulter, Cat. No. IM2356)

for 30 minutes. Cells pellets were diluted to a concentration of 10 million cells/400 uL of staining buffer and DAPI (Life Technologies, Grand Island NY, Cat. D1306) was added 10 minutes before sample acquisition, to a final concentration of 7.7ug/mL and 40 µL/10^6 cells

2.4.6. Reagent Manufacturer Name
The reagent manufacturer shall be specified (e.g., MIFlowCyt Reagent Inc.). Tabel 1.

2.4.7. Reagent Catalogue Number
The reagent catalogue number shall be specified.
Table 1.

2.4.8. Other Relevant Reagent Information
When secondary antibodies are used, the primary analyte (the analyte bound by the primary antibody) and the reporter component of the secondary antibody (the reporter that generates the measured signal) shall be clearly specified as described in 2.4.2, 2.4.3, and 2.4.4. If relevant, the primary and secondary antibodies shall be described individually (e.g., in the case that the use of a secondary antibody significantly impacts the interpretation of results because of nonspecific fluorescence, etc.). The description should also contain other relevant details which may include lot number, concentration, label incorporation method, separation technique, antibody staining procedures, and wash steps. Table 1 and section 2.4.5

3. Instrument Details
3.1. Instrument Manufacturer
Beckman Coulter, Inc.
www.beckmancoulter.com

3.2. Instrument Model
Beckman Coulter Gallios Flow Cytometer

3.3. Instrument Configuration and Settings
The cytometer has been configured by the manufacturer and has not been altered from that configuration.

3.3.1. Flow Cell and Fluidics
The flow cell is a BioSense rectangular cuvette. Sheath consumption during acquisition is 780mL/hour. The 8 peak Rainbow beads were acquired at approximately 10 µL/min to determine daily target channels. Samples and compensation controls were acquired at 30 µL/min.

3.3.2. Light Source(s)
The instrument contains three solid-state, software controlled lasers.

1) 22 mW, blue laser operating at 488 nm, elliptical beam spot 10 µm x 84 µm
2) 25 mW, diode laser operating at 638 nm, elliptical beam spot 9.6 µm x 72 µm
3) 40 mW, violet laser operating at 405 nm, elliptical beam spot 8.9 µm x 70 µm

3.3.2.4. Light Source Polarization

Polarization shall be specified (e.g., linear, >100:1, in the direction of the sample flow). If possible, this should be specified at the intersection of the light source beam and particles; see also 3.3.3.

3.3.2.5. Light Source Beam

Light source beam dimensions and geometry shall be specified at the flow cell or at the intersection of the beam with particles (e.g., elliptical 22 x 66 µm).

3.3.2.6. Other Relevant Light Source Information

Additional relevant light source details should be provided and may include light source manufacturer name (e.g., MIFlowCyt Lasers, Inc.), light source model name (e.g., 302C Krypton-Ion Laser), light source part number (e.g., I-326), noise (e.g., <1%), pointing and power stability (e.g., <1% over 1 hour), etc.

3.3.3. Excitation Optics Configuration

If possible, the light source power (3.3.2.3), polarization (3.3.2.4), and beam profile (3.3.2.5) should be specified at the intersection of light source beam with particles. If this is not feasible for a particular researcher then details about the excitation optics shall be included to allow for an approximation of the light characteristics at the beam/sample intersection point. This description shall detail all components along the excitation optical path (i.e., from light sources to the flow cell). Each component shall be described stating its type (e.g., beam expander), manufacturer (e.g., MIFlowCyt Optics, Inc.), and model number (e.g., BE03M).

3.3.4. Optical Filters

Each optical filter of the instrument shall be described stating the following:

3.3.4.1. Optical Filter Type

The optical filter type shall be specified (e.g., band pass filter, long pass filter, dichroic long pass filters, short pass filter, dichroic short pass filter, polarizer excitation filter, grating, prism).

3.3.4.2. Transmitted Wavelengths

Light wavelengths transmitted by the filter shall be specified (e.g., 488/25 nm, >670 nm, <620 nm).

3.3.4.3. Optical Filter Installation Date

As optical filters of all types are subject to degradation, filter performance needs to be monitored at intervals to verify continued performance at an acceptable level [19]. The optical filter installation date shall be specified (e.g., January 15, 2007).

3.3.4.4. Optical Filter Manufacturer

The optical filter manufacturer shall be specified (e.g., MIFlowCyt Filter Inc.).

3.3.4.5. Optical Filter Model Number

The optical filter model number shall be specified (e.g., model #1234).

3.3.4.6. Other Relevant Optical Filter Information

Other relevant optical filter details should be provided.

3.3.5. Optical Detectors

Each optical detector (e.g., photomultiplier tube) shall be described stating the following:

3.3.5.1. Optical Detector Name

The optical detector name (e.g., FSC, SSC, FL1, FL2, FL3, FL4, etc.) shall be specified.

3.3.5.2. Optical Detector Type

The type of the optical detector shall be provided (e.g., photodiode, photomultiplier tube).

3.3.5.3. Optical Detector Voltage

The optical detector voltage shall be specified (e.g., 300V, 700V).

3.3.5.4. Optical Detector Amplification Type

The optical detector amplification type shall be specified (e.g., linear, log). For log amplification, the number of decades shall be provided (e.g., 4 decades). For a linear amplification, the linear gain shall be provided (e.g., linear gain of 2.0). Additional details including the actual amplifier profile may be provided.

3.3.5.5. Other Relevant Optical Detector Information

Additional relevant optical detector details should be provided and may include the optical detector manufacturer name and model number (e.g., MIFlowCyt Photonics Inc., PMT #R9220), minimal, maximal, and peak wavelength sensitivity (e.g., 185 nm, 900 nm, and 650 nm), the internal gain (e.g., 10_7), or the rise time (e.g., 2.2ns).

3.3.6. Optical Paths

The full optical path shall be given for each measured parameter where applicable (i.e., excluding time). The optical path shall start with specification of the light source(s), which shall be followed by enumeration of all optical components (e.g., optical filters, beam splitters, mirrors, spectrometer, etc.) that contribute to the particular parameter. If nonimaging components are used, this shall be explicitly noted. The components shall be enumerated corresponding to their order along the light path in the instrument. The description shall include how components are used if relevant (e.g., passed vs. reflected light for a dichroic filter). The optical path description shall specify the optical detector used to measure the particular parameter. It shall also state whether the height, width, or area of the detected signal is used, and specify the threshold value if set. In addition, the collection angle shall be specified for the forward scatter detector. See Table 2 for an example of optical path components and Table 3 for an example of optical path details. As for other sections, we are not specifying the format for providing this information.

3.4. Other Relevant Instrument Details

Additional relevant instrument details should be provided and may include machine-specific information such as information about automated agitation, temperature control, controlled volume dispensing, sampling from microtiter plates, auto-boost, auto-flush, etc. Other custom settings shall be provided if relevant and may include setting name, description, and value.

Laser	Detecto r	Detector type	Filter Type	Wavelength	Parameter	Target Channel Range (MFI)	Sample Voltage (V)	Amplification	Thresh
488nm	Wide	Photodiode	BP	488	FS			Linear	
488nm		Photodiode	BP	488	SS			Log	
488nm	FL1	PMT	SP BP	550 525/40	FITC	344-358	531	Log	
	FL2	PMT	SP BP	595 575/30	PE	325-338	472	Log	
	FL3	PMT	SP BP	655 620/30	ECD	213-222	464	Log	

	FL4	PMT	SP BP	730 695/30	PE-Cy5	66-69	554	Log	
	FL5	PMT	LP	755	PE-Cy7	12-14	590	Log	
638nm	FL6	PMT	SP BP	710 660/20	APC	121-134	524	Log	
	FL7	PMT	SP BP	750 725/20	APC-AlexaFluor700	133-149	520	Log	
	FL8	PMT	LP	755	APC-Cy7	113-128	571	Log	
405nm	FL9	PMT	SP BP	480 450/40	DAPI Log	87-99	300-350	Log	10
	FL10	PMT	BP	550/40	DAPI Lin	43-47	300-350	Log	

Target channels determined by the 7th peak of the Spherotech 8 peak beads

4. Data Analysis Details

If data analysis has been performed the following details shall be specified:

. The goal of cytometer standardization is to assure that the instrument is functioning properly and that prospective samples of equivalent fluorescence will be yield the same measured channel value. Beads standards are used to check fluidics, optics, laser output and PMT gain. Before acquiring a sample, standardize the PMT gain to pre-established bead target channels rather than to fixed voltages. A set of broadly fluorescent multi-peak beads serves multiple purposes: The bright peaks can be used to set PMT voltage to target values. The relative spacing of the peaks can be used to determine linearity across a range of fluorescence, and the distance between the negative and dimmest peak can be used to measure cytometer sensitivity (signal to noise). The intensity of DNA staining depends on cell and dye concentration and can be quite variable. Lymphocytes in the tissue sample should be identified and used to set the brightness of the 2N peak in a convenient fluorescence channel. After PMT gain is set, test-run an experimental sample to check for saturated PMTs. Events falling in the last fluorescence channels cannot be spectrally compensated. Especially in tumor cells some markers (e.g. cytokeratin, CD44, CD90) are expressed over a very broad dynamic range. Sometimes PMT gains can be adjusted in concert to accommodate a very bright staining, but more often an adjustment to the staining protocol is required. We dilute

dye-conjugated antibodies in an equal concentration of unconjugated antibody of the same clone to lower staining brightness without increasing the spread of the population. When possible, sample acquisition speed should be regulated by cell concentration, and not by the cytometer flow rate. A faster flow rate yields a wider sample stream and increases measurement error. Spectral compensation should be performed off-line during data analysis using automated compensation software.
In this example acquisition was performed using a 10-color Gallios cytometer (Beckman Coulter, Miami FL). An effort was made to acquire a total of 1.8 million events per sample at rates not exceeding 10,000 events/second. For DAPI staining, PMT was gain optimized for linear (cell cycle) detection of 2N cells (tissue lymphocytes). The cytometer was calibrated to predetermined photomultiplier target channels prior to each use using SpectrAlign beads (DAKO, Cat. No. KO111) and 8-peak Rainbow Calibration Particles (Spherotech, Libertyville, IL, Cat. No. RCP-30-5A).

4.1. List-mode Data File

The list-mode data files (e.g., FCS files [18]) shall be provided directly or details on how they may be requested shall be stated. Please contact Dr. Vera Donnenberg by E-mail donnenbergvs@upmc.edu

4.2. Compensation Details

4.2.1. Compensation Description

A description of the type of compensation used shall be included (e.g., no compensation, hardware compensation, computed compensation) and the spillover or compensation matrix shall be provided when possible (i.e., it may not be available for old data but shall be provided when available). While the spillover matrix is preferred, the compensation matrix is also acceptable. The type of the matrix (i.e., spillover vs. compensation) shall be explicitly stated.

Offline compensation and analyses were performed using VenturiOne software designed for multiparameter rare event problems (Applied Cytometry, Dinnington, Sheffield, UK). Spectral compensation matrices were calculated for each experiment using single-stained mouse IgG capture beads (Becton Dickinson, Cat. No. 552843) for each tandem antibody and hard stained beads (Calibrite, BD) for single molecule dyes (Becton Dickinson, FITC, PE (Cat. No. 349502), APC (Cat. No. 340487)).

4.2.2. Other Relevant Compensation Information

Additional relevant compensation details shall be provided and may include information such as the FMO control.

4.3. Data Transformation Details

The following shall be described for each data/parameter transformation performed during analysis when the transformation does not qualify as compensation (4.2): none

4.3.1. Purpose of Data Transformation
The purpose of each performed transformation shall be specified (e.g., data visualization, background correction, statistical analysis, quantitative flow cytometry, etc.).

4.3.2. Data Transformation Description
Either the exact mathematical formulas/algorithms of each data transformation shall be supplied using an open and freely available specification, or a description of each transformation shall be provided.

4.3.3. Other Relevant Data Transformation Details
Other relevant information about data transformation should be provided and may include specification of software (e.g., name, version, operating system), analysis date, and graphical visualization of the transformation process, which is especially essential for stepwise transformations such as used in quantitative cytometry, i.e., transformation from measured voltage to count of photons, to count of reporter molecules, to count of detector molecules, to count of analytes.

4.4. Gating (Data Filtering) Details
Gating, or data filtering, is a process in flow cytometry in which a subset (subpopulation) of a larger set (population) is defined phenotypically. Gating significantly impacts all statistical and analytical results and thus it is crucial that all the gates be exactly mathematically described (e.g., using Gating-ML [9]). In case the exact gating/filtering description cannot be produced (e.g., software is incapable of exporting an exact description, unknown gate boundaries, probabilistic filtering algorithms, clustering analysis, etc.) detailed membership information should be provided for each gate/subpopulation. This should consist of a complete list of events within each particular subpopulation.
The following information about gating shall be provided, or it shall be specified that no gate was applied.

Disaggregated tumor and lung preparations contained a high proportion of events that would interfere with analysis, were they not eliminated by logical gating. This is the purpose of this article, where we demonstrate how inclusion of doublets, events with hypodiploid DNA, and cytokeratin+ events also staining for hematopoietic markers reduces the ability to quantify epithelial cells and their precursors. Using the lung cancer/normal lung data set, we present an approach to multiparameter data analysis that consists of artifact removal, identification of classes of cells to be studied further (classifiers) and the measurement of outcome variables on these cell classes. Detailed gating strategies are dpresented in Figures 2, 3, 4 and supplementary Figures 1 and 2.

4.4.1. Gate Description
The subpopulation identified by the gate shall be briefly described (e.g.,

“IL-4 producing helper T cells of the CD3+CD4+ phenotype”). The gating strategy or a reference to where it is described in detail (e.g., a manuscript) should be provided.

4.4.2. Gate Statistics

Percentage of events within the gate shall be provided specifically stating the denominator. The denominator shall be either the total population of events (e.g., percentage of lymphocytes based on the total number of events) or another gate (e.g., percentage of CD4+ lymphocytes based on all lymphocytes). When the denominator is another gate, this shall also be exactly specified in recursive fashion so that the gating strategy can be followed up to the original data set. The denominator may not necessarily be a containing (superset) population for some statistics (e.g., the donor / host blood cell ratio).

The region percents shown are mean values, parentheses indicate lower and upper 95% confidence intervals of all samples analysed.

4.4.3. Gate Boundaries

Either the exact mathematical descriptions of each gate boundary shall be provided using an open and freely available specification, or this information shall be provided in the form of images.

The identification of cytokeratin+ and cytokeratin negative cells among nonhematopoietic cells is shown in Figure 4. These were further subdivided in diploid and aneuploid populations, creating 4 classes on which to measure outcomes (stem/progenitor markers, light scatter).

4.4.4. Other relevant gate information

Other descriptive statistics may be provided including the count of events, arithmetic mean, mode(s), median, coefficient of variation (CV), minimum value, maximum value, standard deviation, etc. A description of the relative intensity of staining of the markers defining the subpopulation identified by the gate may also be provided (e.g., CD3 negative, dim, moderate, or bright) [20]. A qualitative description of the subpopulation (e.g., between first and second log decade) and a reference (e.g., publication, individual, or other) for the definition of the qualitative descriptor may also provide useful information.

References

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Procedure Name: 2011-01-R0 PROCEDURE FOR DISAGGREGATION OF SOLID TISSUES

Date Adopted: January 1, 2012 **Date Revised:**

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Authors: Pfeifer, ME,, Donnenberg, A.D., Donnenberg, V.S.

Supersedes Procedure:

Distribution:

Laboratory Director Donnenberg **Date**_____

Technologist Review

Date	Signature

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2011-01-R0 PROCEDURE FOR DISAGGREGATION OF SOLID TISSUES

Principle

Preparation of single cell suspensions from solid tissue is an excellent source of cells for flow cytometry, culture, molecular studies, and viable cell cryopreservation. Obtaining these cells from solid tissue will allow for examination of millions of cells instead of the limited number in a slide-based assay of a tissue section. This procedure will yield viable cells which can be used immediately or cryopreserved for future assays.

Specimen or Component Requirement

Human tissue is often a byproduct of surgery. To ensure viability and sterility, it is best obtained in the operating room. This requires the cooperation of the responsible pathologist who will assure that sufficient tissue is first procured for diagnostic purposes. This protocol applies to tissue samples in the range of 0.1 to 100 g. It has been tested with normal breast, lung and esophagus tissues, and breast, lung, esophagus and oral tumors (1-4). Tissue will be placed in anticoagulated collection medium and kept on wet ice until delivery to the research laboratory. In the event of time constraint, tissue can be stored overnight at 4°C.

Reagents and Supplies

Supplies	Supplier	Catalog number
Forceps		
Plastic waste container for liquid waste		
Bleach	Stockroom	SU-070
60mm Petri Dishes, BD Falcon	Fisher Scientific	08-772E
Scalpels, BD Bard-Parker protected disposal, blade#10	Fisher Scientific	02-688-78
Dulbecco's Phosphate Buffered Saline, without calcium/magnesium, PBS-A	Sigma-Aldrich	D5652
70µm cell strainer, BD Falcon	Fisher Scientific	08-771-2

50mL polypropylene (pp) conical tube, BD Falcon	Fisher Scientific	14-959-49A
15mL polypropylene (pp) conical tube, BD Falcon	Fisher Scientific	14-959-70C
10mL serological pipet, BD Falcon	Fisher Scientific	13-675-20
2mL serological pipet, BD Falcon	Fisher Scientific	13-675-17
Bovine Serum	Invitrogen	16170-078
Parafilm, 4”	Stockroom	SU-380
Collagenase Type I	Sigma	C-0130
RPMI 1640 w/o L-glutamine, Biowhittaker, #12167F	Stockroom	CB-017
Heparin	Fisher	NC9556044
DNase I, 750KU	Sigma	D-5025-750KU
Hanks’ Balanced Salt Solution 1X, w/ Calcium chloride and magnesium chloride	Gibco	14025
Albumin, Bovine 30% solution	Sigma	A-7284
Corning Bottle Top Filter System, 0.45 µm pore, 500mL bottle and funnel	Fisher Scientific	09-761-7
Thioglycolate		
12x75mm polypropylene tube	Fisher Scientific	14-959-16A+
1-200µL pipet tips, racking system	VWR	53512-260
Vi-CELL AS, S Quad Pack (reagents)	Beckman Coulter	383198
Vi-CELL sample cups	Beckman Coulter	383721
Vi-CELL concentration control	Beckman Coulter	175478

diH_2O		
IO test 3 rbc lysing solution	Beckman Coulter	A07799
0.45µm Syringe Filter	Fisher Scientific	09-719D
60cc syringe, BD Falcon	Fisher Scientific	13-689-8
Histopaque-1077	Sigma	10771-6x100mL

Instrumentation

Item	**Manufacturer**	**Model number**
Balance	Mettler	PJ 3000
BD Falcon Express portable Pipet-Aid	BD Biosciences	357590
Sorvall RT 6000B centrifuge	Dupont	RT6000B
Shaking Water bath, The Belly Dancer hybridization water bath	Stovall Life Science	CMBAA115S
ViCell Cell Counter	Beckman Coulter	ViCell
Vortex	Scientific Industries, Inc.	K-550-G
P20 pipetman	Gilson	
P200 pipetman	Gilson	
Hemacytometer	American Optical Corp.	
Rocker, Orbitron Rotator 1	Boetel Industries, Inc.	260200

Reagent Preparation

Cautionary Notes

- All reagents should be prepared under sterile conditions in a Biological Safety Cabinet.
- Collection medium

1. RPMI without L-glutamine with 10U/mL of heparin.

- Decomplemented Bovine Serum
    1. Thaw 500mL bottle of serum at 4°C protected from light.
    2. Place in a water bath at 56° C for 30 min.
    3. Cool to room temperature and aliquot in 50 mL pp conical tube and cryopreserve at -86°C.
    4. Test sterility with thioglycolate.
- Collagenase Type I, Sigma # C-0130
    1. Resuspend 5 g in 1250 mL of RPMI medium as a source of Ca^{++} and Mg^{++}
    2. Aliquot 9 mL into a 15 mL pp Conical tube
    3. Test sterility with thioglycolate.
    4. Cryopreserve at -86°C.
- DNase Type I, Sigma # D-5025 (10X stock solution)
    1. Use the following equation with the lot specific information provided by the manufacturer.

       Weight DNase (mg) x activity of stock (U/mg) = total units

       Total units / 3500 (U/mL) = total reconstitution volume
    2. Reconstitute with 1x HBSS with 0.5% albumin
    3. Filter sterilize with 0.45 µm bottle top filter system.
    4. Test sterility with thioglycolate.
    5. Store in 1mL aliquots at -86°C

Procedure

Cautionary Notes

- All techniques should be conducted under sterile conditions in a Biological Safety Cabinet.
- All instruments, consumables, etc. should be disinfected with 70% ethanol prior to placement in the biological safety cabinet.
- All specimens should be considered potentially infectious and therefore handled with universal precautions.

Detailed Methods

Tissue Dissociation and Enzymatic Digestion

1. Using sterile forceps, remove the tissue from the collection container and place into a 60mm pre-weighed Petri dish.

2. Weigh the Petri dish containing the tissue. Subtract the initial weight of the dish from the current weight of the dish containing the tissue to obtain the tissue weight.
3. Using 2 scalpels, finely dissociate the tissue by using a cross-cutting motion. The tissue should be minced until any remaining tissue pieces are smaller than roughly 3 mm.
4. Wash the tissue in the Petri dish with PBS-A in order to remove cells that have dissociated from the tissue during mincing. Pass through a 70 µm cell strainer into a 50mL polypropylene (pp) conical tube.
5. Wash the Petri dish again with PBS-A in order to remove tissue fragments. Pass through a 70µm cell strainer into a 50 mL pp conical tube.
6. Centrifuge the liquid that passed through the cell strainer at 400 g for 7 min at 4°C. Decant supernatant and dislodge pellet. Resuspend in 2 mL of bovine serum and place on wet ice until use.
7. Transfer all tissue that did not pass through the cell strainer into a new 50 mL pp conical tube containing 9 mL collagenase type I (0.4%, Sigma C-0130 in RPMI) and 1 mL DNase I (3500KU/mL stock solution).
8. Place parafilm around the lid of the conical tube containing the tissue/collagenase/DNase. Place the conical tube in a shaking waterbath for 30 min at 37° C. Do not submerge the lid of the conical tube in the water.
9. Pass the tissue/collagenase/DNase mixture through a new 70 µm cell strainer into the 50 mL pp conical tube containing the single cells in bovine serum.
10. Wash the digested tissue and the conical tube used during digestion with PBS-A in order to remove any cells that may have been digested. Pass through the 70µm cell strainer into the 50 mL pp conical tube.
11. Fill the 50 mL pp conical tube containing the digested cells to 50 mL with PBS-A. Centrifuge at 400g for 7 min at 4°C. Decant supernatant and dislodge pellet. Resuspend in 2 mL of bovine serum and place on wet ice until use.
12. Any remaining tissue that did not pass through the 70 µm cell strainer should be collected into a 50 mL pp conical tube. Repeat steps 7 through 11 in order to digest for an additional 30 min.
13. After the single cells have been pooled in the 50 mL pp conical tube with 2 mL of bovine serum, any remaining undigested tissue may be discarded in the biohazard waste box.

CELL COUNTING AND VIABILITY

Note: The following steps are specific to the Beckman-Coulter Vi-CELL analyzer, which is used to determine a preliminary cell count and viability determination. (Alternative cell counting protocols with a hemacytometer may be used).

Optional procedures for red blood cell lysis and separation by Ficoll/Hypaque density separation are also provided. RBC lysis, when required, is performed prior to counting. Ficoll/Hypaque separation may be performed when viability of digested cells is below 50%, in an attempt to remove dead cells (this is not always effective).

14. Turn on counting machine (there is a switch in the back) it should only take a few seconds and you will notice a green light on the front.
15. Open "Vi-CELL" software on the attached laptop. You will notice a white LED light at the sample port.
16. Double-click the icon labeled "Daily Concentration Control" (it is a yellow Erlenmeyer flask)
17. A box should appear to "Log in sample" and the fields should be filled in automatically
    a. Sample ID = Daily Concentration Control [xxxxxx]
    b. Cell Type = Conc. Control
    c. Dilution Factor = 1.0
    d. Date
    e. Time
    f. Save Images box should be checked
    g. Click "OK"
18. Vortex the concentration control and add 1.0 mL to a sample cup. (be precise)
19. Click "Start"
    a. The system should take a few minutes to mix and count the control.
    b. When it is finished, it will display the results on the plot. It should be within the acceptable range shown by two red lines (these are +/- 10% of the lot-specific concentration value.)
    c. If the concentration value is within the acceptable range, proceed to sample analysis.
20. Click "New Sample"
    a. A box should appear to "Log in sample"
    b. Sample ID: (example: LungTSC_12_30_2011)
    c. Cell Type: (we have been using the "default" setting)
    d. Dilution Factor: The range for the Vi-CELL is $1x10^4$ – $1x10^7$ cells/mL
    e. Date, Time should update automatically
    f. Click "OK"
    g. Click "Start"
    h. The system should take a few minutes to mix and count. If you want to view the images as it is counting, click "camera image"
    i. The machine will flush after each sample.
21. Close the software when finished.
22. Turn off the machine (switch located in the back)
23. Cell counts and viabilities will be provided with associated standard errors.

RBC LYSIS

24. If a visible "red" cell pellet remains, red blood cell lysis may be performed.
25. Dilute Beckman Coulter's 10X Lysing Solution with sterile diH_2O. For example, add 5 mL of the lysing solution to 45 mL of water.
26. Filter sterilize the solution into a new 50 mL pp conical tube, using a 0.45 µL syringe filter with a 60 cc syringe.
27. Centrifuge the sample at 400 g for 7 min at 4° C. Decant supernatant and dislodge pellet.
28. Add the 1X lysing solution to the pelleted cells. Place at room temperature for 10 min on a rocker.
29. Centrifuge at 400 g for 7 min at 4° C. Decant supernatant and dislodge pellet.
30. Wash twice with PBS-A.
31. Continue on to cell counting.

CELL SEPARATION BY DENSITY GRADIENT

Note: This protocol is a modification of the original Ficoll/Hypaque separation procedure described by Böyum (5).

32. If cell viability is low, a Histopaque separation may be performed to remove dead cells.
33. Histopaque should be filter sterilized with a 0.45µL syringe filter.
34. All reagents should be at room temperature for the cell separation.
35. Dilute the sample to 35 mL with RPMI containing 20% bovine serum.
36. Overlay the 35 mL of diluted sample onto 15mL of Histopaque in a 50 mL pp conical tube. Ensure that mixing of the liquids does not occur.
37. Centrifuge the sample at 400g for 30 min at room temperature with the centrifuge brake off.
38. After centrifugation, aspirate the upper layer and discard.
39. Collect the white "buffy coat" layer directly above the Histopaque layer into a new 50 mL pp conical tube. This layer contains the viable cells.
40. Wash three times with PBS-A to thoroughly remove the Histopaque. The first wash will be at room temperature. The last two washes should be at 4° C.
41. Resuspend in 2 mL bovine serum and continue to cell counting.

Acceptable Endpoints

Endpoints are specific to individual research protocols. Generally, viability >50% is required.

Result Reporting

Tissue input (weight in grams), total cells recovered and cell viability are always recorded. Photomicrographs of cytocentrifuge preparations and flow cytometry results are recorded when available.

Quality Control

The Vi-CELL concentration control standard is run according the manufacturer's instructions.

Revision	Change	Rationale	CFR/FACT/CAP/PADH Standards	Start Date	End Date
0	Creation	New procedure.			
1					
2					
3					
4					

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Supplementary Methods with Commentary

Donnenberg *et al*, Flow cytometric determination of stem/progenitor content in epithelial tissues: An example from non-small lung cancer and normal lung, Cytometry A. 2013

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Tissue procurement and transport. Human tissue is isolated in the operating or procedure room, as a part of a medically indicated procedure or an approved research protocol. Often it is surgical waste. Once the tissue is transported to the laboratory it will require a great deal of time and resources to process and use in an experimental setting. Since specimen quality is critical, work with the surgeon and pathologist to ensure that your requirements are known (e.g. sterility, type and size of sample, need for viability, transport vessel and medium). If it is a surgical sample, the pathologist or pathology technician is responsible for obtaining a sufficient diagnostic sample before waste material is allocated for research. This activity is optimally performed on the "back table" of the OR where the surgeon can provide input and the pathologist can provide orientation marks. The excess tissue can be divided immediately, placed into prelabeled collection vessels and rushed back to the research laboratory immersed in anticoagulated collection medium on wet ice. Small tissue fragments can sometimes be stored overnight under these conditions without appreciably affecting viability, but this varies with the tissue source. It is important to prepare a form for all needed data that will be difficult or impossible to retrieve later. This includes a diagram indicating the precise anatomical location from which the sample was taken.

In the example presented here, non-small cell lung cancer samples and paired adjacent normal lung tissue were obtained from 17 patients at the time of surgical resection of the tumor.

Specimens were collected under protocols approved by the University of Pittsburgh Internal Review Board (UPCI 99-053, 020391, 0503126, 07090247). Our research laboratory worked closely with the surgeon and sent a dedicated tissue collector to the OR in time to obtain the tissue as soon as it was divided. The tissues were immediately immersed in sterile heparinized tissue culture medium (sodium heparin, 10 U/mL) and transported to the laboratory on an ice pack in a cooler.

Tissue processing. After the tissue is accessioned, it is weighed, photographed, a physical description is recorded, and a sample is taken for formalin fixation and paraffin embedding. It is important to standardize fixation time and conditions if antigens will later be detected on tissue sections. The remaining tissue is weighed again and mechanically and enzymatically disaggregated into a single cell suspension. Tumors vary greatly in the degree of fibrosis and necrosis and will require more or less aggressive treatment. Collagenase is the most commonly used enzyme, followed by dispase and trypsin. The first two require calcium and magnesium, whereas trypsin works optimally in the absence of divalent cations and is often combined with a chelating agent such as EDTA. Mechanical disaggregation can be accomplished with paired scalpels, scissors, screens, or devices such as the Miltenyi GentleMACS or the Becton Dickinson Medimachine. Enzymatic digestion is performed in an incubator (small samples) or a shaking water bath such as the Stovall Belly Dancer. The Miltenyi MACSmix is battery operated, fits in a standard incubator, and is very useful for small tissue fragments. The process often requires centrifugation, harvest of single cells and redigestion of tissue fragments. Addition of DNAse is often critical because dying cells release DNA which traps viable cells, greatly reducing cell yield. Released DNA can be visualized as macroscopic mucous-like clumps and strands. The sample should be visually inspected at all phases of tissue digestion. In the final stage, cell suspensions are passed through a 70 to 200 micron filter to remove aggregates. Cell suspensions are then counted and viability is determined by a dye exclusion assay, such as trypan blue. Automated hematology counters overestimate the count of such samples and should be avoided. A simple

hemocytometer, or an image-based cell counter such as the Beckman Coulter Vi-CELL, works best. At this point, the cells can be examined microscopically using a cytocentrifuge preparation and histological stains. This provides a permanent record of the results of sample dissociation. Samples may also be removed for tissue culture. A schematic diagram of tissue processing workflow is provided in supplementary Figure 1, and the expected cell recovery of several tissue types prepared by mechanical dissociation (scalpels and screens) and collagenase digestion is shown in Table S1.

Tissue	**Number**	**Cells/Gram**	**Viability**
Lung, Normal	42	32.9 ± 4.6 x 10^5	62.8 ± 15%
Lung, NSC Carcinoma	41	226.0 ± 60.3 x 10^5	72.8 ± 3.2%
Breast, Normal	21	8.9 ± 1.7 x 10^5	71.3 ± 0.6%
Breast, Adenocarcinoma	23	72.0 ± 28.6 x 10^5	66.2 ± 6.3%
Adipose, Whole	10	3.0 ± 0.9 x 10^5	57.6 ± 6.8%
Adipose, Lipoaspirate	35	3.0± 0.5 x 10^5	60.1 ± 2.0%
Adipose, LipiVage	14	5.8 ± 0.7 x 10^5	74.7 ± 3.6%

Table S1. Cell recovery from mechanically and enzymatically digested tissues. Tumors give much better cell yield than normal tissues. LipiVage is a proprietary adipose harvest system (Genesis Biosystems).

In the present example single cell suspensions were prepared from malignant lesions and tumor-free adjacent lung tissue as previously described (1). Briefly, tumors and lung tissue were minced with paired scalpels and digested with type I collagenase (0.4% in RPMI 1640 medium, Cat. No. C-0130, Sigma Chemicals, St. Louis MO) and DNase (350 KU/mL, Sigma Chemicals, St. Louis MO, Cat. No. D-5025) and disaggregated through 100 mesh stainless steel screens. Undigested tissue clumps were subjected to repeated rounds of digestion. Viable cells were separated from erythrocytes and debris on a Ficoll-Hypaque gradient (Histopaque 1077, Sigma

Chemicals). Erythrocytes were lysed using an ammonium chloride lysing solution without fixative (Beckman-Coulter, Cat No. IM3630d). The complete laboratory procedure for tissue disaggregation is provided in this online supplement.

Histology and Immunohistostaining. Preparing conventional hematoxylin/eosin and immunofluorescent or immunohistochemical stained slides is essential to the understanding of flow cytometric data from disaggregated solid tissue. Fixation is performed according to standard pathology procedures, but it is important that maximum sample size during fixation (~ $1cm^3$) and fixation time (24 hours) be standardized. Improper fixation results in antigen degradation. Ink marks made on the tissue during collection will aid in orientation of the sample when it is embedded and sectioned. When appropriate, full thickness sections and tumor sections with normal tissue margins will help in the understanding of the anatomical niche in which populations of interest reside. Tissue microarrays can be used for larger validation studies, but the small area of individual sections often precludes anatomic orientation. Antigen retrieval and antibody staining in fixed tissues are empirical processes that require trial and error. The choice of immunohistochemical versus immunofluorescent staining depends upon the application: immunohistochemical staining provides a permanent record but is not suited to antigen colocalization; immunofluorescent preparations cannot be stored long term, but provide higher resolution of stained features and can resolve multiple markers localized to the same structures.

In this example, samples were fixed for 24 hours in neutral buffered formalin (Sigma Cat. No. F5554). Paraffin sections (5-6μm) were prepared from embedded tissues. Tissue sections were heated (60°C, 20 min), deparaffinized (3 washes in xylenes), rehydrated by successive washes in absolute ethanol, 90% ethanol, 75% ethanol and deionized water and rinsed twice in Dako wash buffer (Dako). Antigen retrieval was performed at 125°C for 20 min in pH 9.0-EDTA buffer (Dako). After 2 washes in Dako wash buffer, the tissue sections were incubated for 1 hour in a

blocking solution (PBS, 5% goat serum, 0.05% Tween 20) to reduce nonspecific antibody binding.

In addition to CD117 and cytokeratin staining described in the main manuscript, we offer staining conditions for several other markers that tie in with the present flow cytometry panel. Immunofluorescent staining was performed using the following primary antibodies: mouse anti-human CD44 (1:25 (7.6 µg/mL final concentration), Dako, Cat. No. M7082, clone DF1485), CD90 (1:10 (7.6 µg/mL), BD Biosciences Cat. No. 550402, clone 5E10), pan-cytokeratin (1:100 (1.64µg/mL), Dako, Cat. No. M3515, clone AE1-AE3) and Ki67 (ready-to-use, Dako, Cat. No. N1633, clone MIB-1) or rabbit anti-human CD44 (1:100, Epitomics, Cat. No. 1998-1, clone EPR1013Y), CD90 (1:100, Epitomics, Cat. No. 2695-1, clone EPR3133), and CD117 (1:400 (35.7µg/mL), Dako Cat. No. A4502, polyclonal). Primary mouse and rabbit antibodies were substituted by Dako Universal Negative Control for Mouse Antibodies (ready to use, Dako Cat. No. N1698) or Dako Universal Negative Control for Rabbit Antibodies (ready to use, Dako Cat.No.N1699) respectively. All primary antibodies and controls were incubated for overnight at 4°C. Tissue sections were washed twice using DAKO Wash Buffer prior to applying biotinylated secondary goat anti-mouse (1:500 (1.58 µg/mL), Dako Cat. No.E0433) or goat anti-rabbit antibody (1:500 (1.52µg/mL), Dako Cat.No. E0432) for 1 hour at room temperature. Tissue sections were washed twice with Dako wash buffer and incubated with streptavidin-Cy3 (1:500 (2µg/mL), Sigma, Cat. No. 6402) for 30 minutes at room temperature. Slides were washed again and tissue sections were incubated with Alexa 488-conjugated anti-pan-cytokeratin (1:200 (2.5µg/mL), clone AE1-AE-3, eBiosciences Cat.No. 53-9003-80) or FITC-conjugated anti-αSMA (1:100, Sigma, Cat. No. F3777, clone 1A4) antibodies for 1 hour at room temperature. Alternatively, tissue sections (rabbit-antibody prestained slides only) were incubated with mouse anti-human pan-cytokeratin (1:100 (1.64µg/mL), Dako, Cat. No. M3515, clone AE1-AE3) or Ki67 (ready-to-use, Dako, Cat. No. N1633, clone MIB-1) for 1 hour at room temperature, washed twice for 5 minutes and incubated with cross-adsorbed Alexa 488-

conjugated goat anti-mouse secondary antibody (1:200 (10µg/mL), Invitrogen, Cat. No. A11029) for 1 hour at room temperature. Stained tissue sections were washed again twice in Dako wash buffer and nuclear staining was attained through 10 minute incubation with DAPI (7.15µM Invitrogen, Cat. No. D1306). Slides were washed twice in PBS-A and mounted in Prolong Gold anti-fade reagent (Invitrogen, Cat. No. P36934). Immunofluorescent staining was observed and photographed using an epi-fluorescence microscope (Nikon Eclipse TE 2000-U).

Flow cytometric staining. Efficient staining depends chiefly on using the optimal monoclonal antibody concentration. Calculations based on volume of antibody per million cells are misleading and result in wasted reagent. Using too high a concentration of antibody results in a decreased separation between negative and positive populations because of increased nonspecific binding to the negative population. Using too low a concentration results in a dimmer positive population with a greater spread (*i.e.* greater coefficient of variation). In both cases, the signal to noise ratio is reduced. Optimization of antibody concentration is an empirical process which must take into account the dilution of each antibody with other antibodies in the preparation. Rules of thumb for staining include staining in the smallest practical volume, preparing a number of cells appropriate to the frequency of the rarest population of interest, keeping reagents and cells cold and protected from light, preincubating with decomplemented mouse serum to block nonspecific antibody binding. Fluorochromes should be chosen based on reagent availability, knowledge of sample autofluorescence (usually highest in the FITC to PE-Texas red emission range), and antigen density (highest quantum efficiency for low density markers, avoid saturation for high density markers). Another important consideration comes in the use of tandem dyes that excite twice when interrogated by spatially separated lasers. For example, PE-Cy5 will be excited by both blue and red lasers; the blue laser excites PE which frets to Cy5, and the red laser excites Cy5 directly. When PE-Cy5 is used in the same panel as the red-excited APC, caution must be used, because the Cy5 fluorescence will have to be compensated out of the APC channel. In the best scenario, PE-Cy5

and APC are conjugated to mutually exclusive markers (e.g. CD19 and CD3), or PE-Cy5 is used in a "dump" channel. Less desirable, but sometimes workable, PE-Cy5 is used for a dim marker relative to the marker labeled with APC. Coexpression of a bright marker in PE-Cy5 with a dim marker in APC should be avoided.

Single stained controls should be run with every sample. Hard-stained beads can be used for single molecule dyes (FITC, PE, APC, PerCP). For tandem dyes, which vary in emission spectrum from product to product and are very sensitive to degradation, we stain Ig capture beads with each individual tandem dye conjugated antibody. Capture beads bind antibodies irrespective of antigen density on cells, providing a bright compensation standard. Run all FMO controls (staining combinations eliminating antibodies one at a time) initially during assay development until staining patterns are known. Continue to use FMO controls that are critical to region placement. Fix cells with methanol-free EM grade formaldehyde, hold stained cells in the dark, and run on the flow cytometer as soon as possible because samples never improve with age. Gentle cell permeabilization after extracellular staining and fixation is necessary for intracellular staining. Consider permeabilization after surface staining even when intracellular antigens are not measured. This permits DNA intercalating dyes such as DAPI and 7-AAD to be used to measure DNA content: very useful for excluding events with degraded DNA or no DNA. DNA staining also permits superior doublet discrimination.

We minimized non-specific binding of fluorochrome-conjugated antibodies by preincubating pelleted cell suspensions for 5 minutes with neat decomplemented (56°C, 30 minutes) mouse serum (5 μL) (2). Prior to intracellular cytokeratin staining, cells were stained for surface markers (2 μL each added to the cell pellet, 15-30 minutes on ice; CD44-PE (Beckman-Coulter, Cat No. A32537), CD90-biotin (BD, Cat.No. 555594), Streptavidin-ECD (Beckman Coulter, Fullerton, CA Cat. No. IM3326), CD14-PECy5 (Beckman-Coulter, Cat. No. IM2640U), CD33-PECy5 (Beckman-Coulter, Cat. No. IM2647U), Glycophorin A-PECy5 (BD Biosciences,

Cat.No.559944), CD133-APC (Miltenyi Biotech Cat.No. 130-090-854), CD117-PC7 (Beckman Coulter, Cat. No. IM3698), CD45-APCCy7 (BD, Cat. No. 348805)), and fixed with 2% methanol-free formaldehyde (Polysciences, Warrington, PA). Cells were then permeabilized with 0.1% saponin (Beckman Coulter) in phosphate buffered saline with 0.5% human serum albumin (10 minutes at room temperature); cell pellets were incubated with 5 μL of neat mouse serum for 5 minutes, centrifuged and decanted. The cell pellet was disaggregated and incubated with 2 μL of anti-pan cytokeratin-FITC (Beckman Coulter, Cat. No. IM2356) for 30 minutes. Cell pellets were diluted to a concentration of 10 million cells/400 μL of staining buffer and DAPI (Life Technologies, Grand Island NY, Cat. D1306) was added 10 minutes before sample acquisition, to a final concentration of 7.7μg/mL and 40 μL/10^6 cells (2).

Sample Acquisition. The goal of cytometer standardization is to assure that the instrument is functioning properly and that prospective samples of equivalent fluorescence will be yield the same measured channel value. Bead standards are used to check fluidics, optics, laser output and PMT gain. Before acquiring a sample, standardize the PMT gain to pre-established bead target channels rather than to fixed voltages. A set of broadly fluorescent multi-peak beads, such as Spherotech 6-peak Ultra Rainbow Calibration Particles, serves multiple purposes: The bright peaks can be used to set PMT voltage to target values. The relative spacing of the peaks can be used to determine linearity across a range of fluorescence, and the distance between the negative and dimmest peak can be used to measure cytometer sensitivity (signal to noise). The intensity of DNA staining depends on cell and dye concentration and can be quite variable. Lymphocytes in the tissue sample should be identified and used to set the brightness of the 2N peak in a convenient fluorescence channel. After PMT gain is set, test-run an experimental sample to check for saturated PMTs. Events falling in the last fluorescence channels cannot be spectrally compensated. Especially in tumor cells some markers (e.g. cytokeratin, CD44, CD90) are expressed over a very broad dynamic range. Sometimes PMT gains can be adjusted in

concert to accommodate a very bright staining, but more often an adjustment to the staining protocol is required. We dilute dye-conjugated antibodies in an equal concentration of unconjugated antibody of the same clone to lower staining brightness without increasing the spread of the population. When possible, sample acquisition speed should be regulated by cell concentration, and not by the cytometer flow rate. A faster flow rate yields a wider sample stream and increases measurement error. Spectral compensation should be performed off-line during data analysis using automated compensation software.

In the accompanying manuscript acquisition was performed using a 10-color Gallios cytometer (Beckman Coulter, Miami FL). Because some of the populations of interest are present as rare events (3,4), an effort was made to acquire a total of 1.8 million events per sample at rates not exceeding 10,000 events/second. For DAPI staining, PMT was gain optimized for linear (cell cycle) detection of 2N cells (tissue lymphocytes). The cytometer was calibrated to predetermined photomultiplier target channels prior to each use using SpectrAlign beads (DAKO, Cat. No. KO111) and 8-peak Rainbow Calibration Particles (Spherotech, Libertyville, IL, Cat. No. RCP-30-5A). Offline compensation and analyses were performed using VenturiOne software designed for multiparameter rare event problems (Applied Cytometry, Dinnington, Sheffield, UK). Spectral compensation matrices were calculated for each experiment using single-stained mouse IgG capture beads (Becton Dickinson, Cat. No. 552843) for each tandem antibody and hard stained beads (Calibrite, BD) for single molecule dyes (Becton Dickinson, FITC, PE (Cat. No. 349502), APC (Cat. No. 340487)).

Multi-dimensional flow cytometry data analysis.

Artifacts of tissue digestion. Enzymatic digestion and mechanical disaggregation is necessary for preparing solid tissues for analytic flow cytometry or cell sorting. Unfortunately, this results in a variety of artifacts that must be addressed prior to analysis. Overdigestion can kill cells, damage antigenic epitopes, and release DNA that traps viable cells. Underdigestion results in

clusters of adherent cells. Nonadherent and loosely adherent cells (*e.g*. tumor infiltrating immune cells) are preferentially recovered, whereas fragile cells are underrepresented. Further, subcellular debris can bind antibody and be misclassified as cells. Apoptotic and necrotic cells have aberrant and unpredictable antigen expression. Some of these problems can be mitigated by careful tissue preparation and through post-digestion cleanup with a density gradient. Even under the best of circumstances, a significant proportion of acquired events are not single cells in good health at the time of staining and fixation. We have designed specific analytical methods to deal with these sources of artifact, as detailed in the main manuscript.

Choosing classifiers and outcomes. Following artifact removal, an analytical strategy is needed to avoid the "all possible permutations" problem inherent in multivariate data. Analysis can be focused by considering certain parameters as "classifiers" and others as "outcomes", although this distinction is somewhat fluid. Often the choice is suggested by biology or hypothesis. In the accompanying manuscript we chose to divide our valid events into four classes based on cytokeratin expression and DNA content. "Outcome" (stem/progenitor markers and light scatter) were measured on the four classes.

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