

# Chapter 10

## Phenotypic Analysis of Bioactive Cells for Application in Regenerative Medicine

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### Abstract

The following chapter outlines methodologies to phenotypically characterize primary cells for the use in tissue-engineered and regenerative medicine applications. Methods covered include analyzing cells using immunocytochemistry, fluorescence-activated cell sorting, and confocal microscopy of adherent and suspended cells, as well as combinations of formulated cell–biomaterial constructs.

**Key words** Phenotype, Primary tissue culture, Immunocytochemistry, FACS, Confocal microscopy

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### 1 Introduction

Analyzing cells using immuno-phenotyping (antibody staining) provides a highly sensitive method to characterize regenerative cell-based tissue-engineered products. Three main approaches to evaluate phenotype include immunocytochemistry, fluorescence-activated cell sorting (FACS), and confocal microscopy. Each approach provides valuable information depending on the process step and the formulation (cells only versus cell(s)–biomaterial construct). Using phenotypic analysis throughout the cell isolation and expansion procedure enables process checks and monitors cell stability. This chapter gives step-by-step methods that can help determine isolated cell phenotype from the initial processing through formulated prototype. Examples cited will reference Tengion's Neo-Bladder Augment™ (NBA), Neo-Urinary Conduit™ (NUC), and Neo-Kidney Augment™ (NKA) products.

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## 2 Materials

### 2.1 Reagents

1. D-PBS without magnesium chloride and calcium chloride.
2. Goat Serum (G9023 Sigma), Horse Serum (H0146 Sigma).
3. Triton-X 100.
4. 40% (w/v) paraformaldehyde.
5. Hoechst nuclear dye.
6. Biologically inert antibodies: Mouse IgG, Rabbit IgG, Goat IgG. Store for 2–3 weeks at 4°C. For longer term storage, keep at –20°C.
7. Cell type-specific primary antibodies (Table 1).
8. Isotype-matched secondary antibodies (Table 1). Anti-mouse/-rabbit/-goat conjugated to either Alexa Fluor 488/Alexa Fluor 594/or Alexa Fluor 647 (Invitrogen). Store at 4°C or according to manufacturer's recommendations.

### 2.2 Solutions

1. Fixation solution for adherent cells: Dilute stock 4% (w/v) paraformaldehyde in D-PBS. Dilute 40% (w/v) stock solution tenfold with D-PBS, solution can be stored at room temperature for 1 month.
2. Fixation solution for suspended cells: 2% (w/v) paraformaldehyde in D-PBS. Dilute 40% (w/v) stock solution tenfold with D-PBS.
3. Surface blocking solution: 2% FBS, 0.09% sodium azide in D-PBS.
4. Surface staining solution: 0.2% BSA, 0.09% sodium azide in D-PBS.
5. Permeabilization and blocking solution for intracellular staining: 0.2% Triton X-100, 10% goat or horse serum in D-PBS.
6. Alcohol-based permeabilization solution: 90% ice-cold methanol. Store at –20°C.
7. Intracellular staining buffer: 0.2% Triton X-100, 2% goat/horse serum in D-PBS.
8. Vectashield Mounting Medium with DAPI (H-1200 Vector Labs).

### 2.3 Equipment

1. Millicell EZ 8-chambered slide (PEZGS0816 Millipore).
2. Fluorescent microscope (Leica DMIL5000B or equivalent) or confocal microscope (BD Pathway 855 or equivalent).
3. 96-well black/clear tissue culture-treated imaging plate, flat bottom.
4. Centrifuge capable of 500×g centrifugal force.

**Table 1**  
**Example antibodies used in FACS analysis and immunocytochemistry (NBA™, NUC™, and NKA™)**

Tissue	Step	Target	Antibody	Manufacturer
Kidney	Primary	Epithelial <sup>a</sup>	CK8/18/19 (mouse IgG <sub>1</sub> )	Abcam (ab41825)
		Proximal tubular <sup>b</sup>	Aquaporin 1 (mouse IgG <sub>2b</sub> )	Abcam (ab9566)
		Distal tubular <sup>b</sup>	Ecadherin (mouse IgG <sub>2a</sub> )	BD (610182)
		Proximal tubular <sup>b</sup>	Lotus tetragonolobus agglutinin (LTA)	Vector (B1325)
		Collecting duct <sup>b</sup>	Dolichos biflorus agglutinin	Vector (B1035)
		Endothelial <sup>b</sup>	PE-CAM (mouse IgG1)	BD (555025)
Bladder and AdSMC		Epithelial <sup>a</sup>	CK(AE1/AE3) (mouse IgG1)	DAKO (M35150)
		Smooth muscle <sup>a</sup>	Smooth muscle actin (mouse IgG2a)	DAKO (M0851)
		Smooth muscle <sup>a</sup>	Caldesmon (mouse IgG1)	DAKO (M3557)
		Smooth muscle <sup>a</sup>	Calponin (mouse IgG1)	DAKO (M3556)
	Isotype control		Mouse IgG1	DAKO (X0931)
		Mouse IgG2a	DAKO (X0932)	
		Mouse IgG2b	Dako (X0944)	
		Rabbit IgG	Invitrogen (02-1202)	
	Secondary		Goat anti-mouse IgG1 (A488/A647)	Invitrogen (A211121/A21240)
		Goat anti-mouse IgG2a (A488/A647)	Invitrogen (A211131/A21241)	
		Goat anti-mouse IgG2b (A488/A647)	Invitrogen (A211141/A21242)	
		Streptavidin (A488/A647)	Invitrogen (S32354/S32357)	

<sup>a</sup>Intracellular expression

<sup>b</sup>Surface expression

5. 15 ml conical polypropylene centrifuge tubes.
6. 1.5 ml polypropylene microcentrifuge tubes.
7. 12 × 75 mm round-bottom polystyrene centrifuge tubes.
8. Flow cytometer (BD FACS Aria or equivalent).
9. FlowJo (Treestar ) flow cytometry analysis software.
10. Cyto centrifuge (7620 Cytopro Wescor) capable of 300 × *g* for spinning cells onto polylysine-coated microscope slides (SS-218 dual-chamber slides and SS-213 dual-sample chambers Cytopro Wescor).
11. Liquid Blocker PAP pen (EMS).
12. Cover glass 24 × 55 mm.

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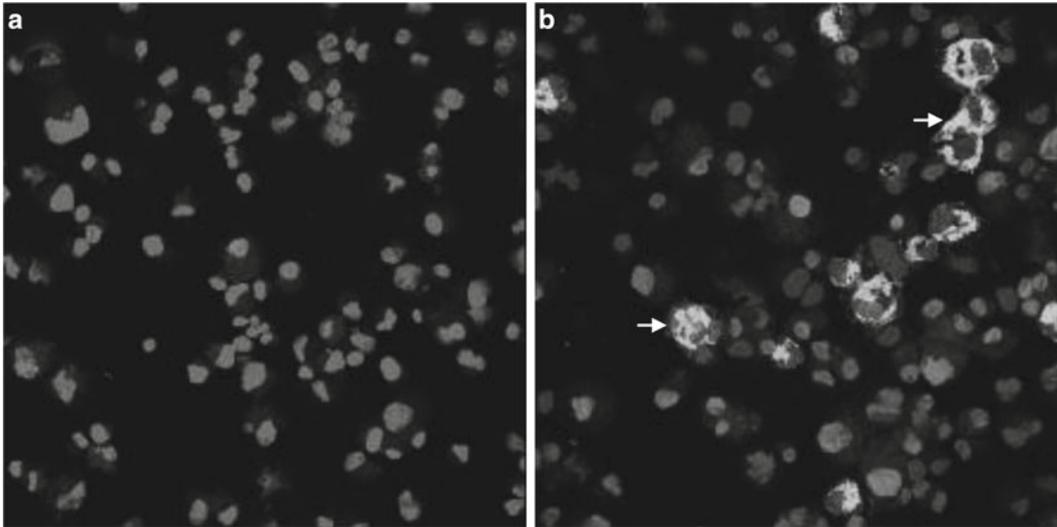
### 3 Methods

#### 3.1 *Initial Identification of Target Cell Types from Primary Tissue Source*

The analysis of cells post-isolation from mammalian primary tissues using enzymatic digestion or tissue disassociation techniques allows for the identification of starting material for certain regenerative medicine/tissue-engineered products. Since single-cell suspensions are difficult to achieve with enzymatic digestion (for example, kidney may generate kidney tubules and glomeruli), flow cytometric analysis may not be the right choice for initial screening. The following steps can be applied to any tissue where the target cells need to be quantitated and identified at the initial phase of the process even if there are aggregates and clusters post-isolation.

Immunocytochemistry can be a useful tool for evaluating and analyzing cytocentrifuged cell aggregates and clusters from the initial cell isolation from multiple species. Although direct staining is usually faster and less complicated, utilizing an indirect single-color method for immunocytochemistry that targets either surface or intracellular antigens removes limitations of species-specific antibody availability.

1. Obtain cell suspension.
2. Fix cells in a final concentration of 2% paraformaldehyde in D-PBS for 20 min at room temperature.
3. Wash cells twice by centrifugation at  $500\times g$  and then resuspend in D-PBS (1 ml per  $1\times 10^6$  cells).
4. Determine cell concentration with a hemocytometer (see Note 1).
5. Adjust concentration to  $1\times 10^5$  cells/ml. For intracellular targets proceed to **step 6**; for surface targets proceed to **step 8**.
6. For intracellular antigens, wash once more and resuspend  $1\times 10^6$  cells in 0.5 ml blocking buffer.
7. Incubate for 30 min at room temperature to permeabilize cell membrane and block nonspecific binding.
8. For surface antigens, wash once and resuspend in surface blocking solution for 5–15 min at room temperature.
9. After blocking, add 200  $\mu$ l of cell suspension into the single Cytopro chamber with glass slide containing a fast white Cytopad.
10. Centrifuge for 4 min at  $300\times g$ .
11. After centrifugation, remove slide and mark the perimeter of the cell spot with a liquid blocker PAP pen.
12. Rehydrate cells on the slide by adding 200  $\mu$ l of D-PBS for 5 min at room temperature.



**Fig. 1** Fluorescent image of cytocentrifuged kidney cells at 200 $\times$  magnification. (a) Negative control with nuclear dye. (b) DBA-positive cells (*arrows*) with nuclear dye

13. Remove D-PBS by aspiration, and add desired primary antibody, diluted in surface or intracellular staining buffer (0.2% Trion X-100 with 2% serum of the species from which the secondary antibodies were generated from) as well as mouse isotype control and stain for 90 min to overnight at 4 $^{\circ}$ C.
14. After staining with primary antibody, carefully wash cells twice using aspiration with D-PBS.
15. Following washing, add isotype-matched secondary antibody for 30 min at room temperature in the dark.
16. Wash cells twice with D-PBS and then add a drop of mounting medium to each spot with a coverslip.
17. Analyze slides using fluorescent microscope using image analysis software (Fig. 1) (see Note 2).

Isolated aggregates and clusters such as glomeruli and intact tubules from post-enzymatically digested kidney can be identified using immunofluorescence and confocal microscopy (1). Phenotypic analysis of these structures can confirm the specificity of antibodies that may be used later in the expansion process. As cells expand, antibody specificity may change due to protein regulation and functional differences caused by culture conditions (2). Having a compartmental specific antibody or genetic tracer that confirms the identity of cells prior to culture is helpful in

monitoring any changes that may be associated with the expansion process (3, 4).

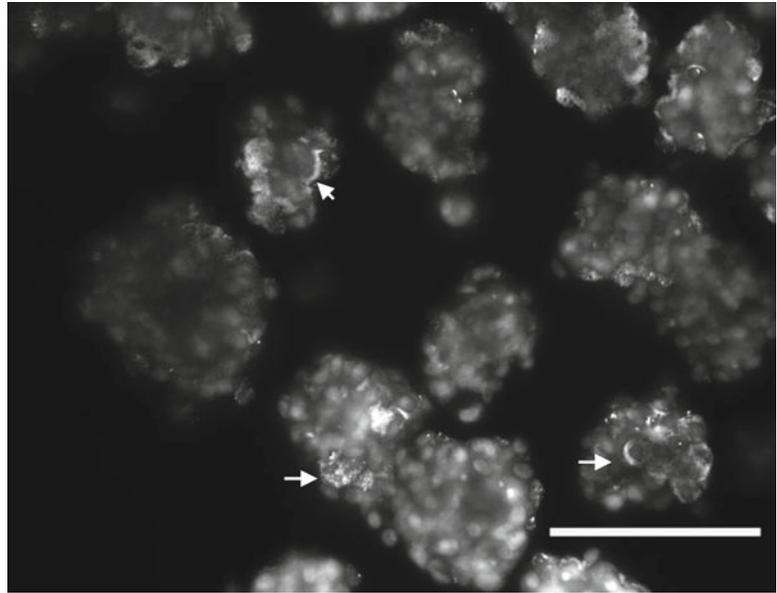
1. Obtain a sample of live cells from isolated tissue suspended in D-PBS.
2. For single-color cell surface phenotypic markers, use  $0.5 \times 10^6$  live cells suspended in 0.5 ml of D-PBS in a 1.5 ml microcentrifuge tube. Divide the number of tubes per antibodies and include isotype-matched controls for each antibody (see Note 3).
3. Block cells for nonspecific binding with 1 ml surface blocking solution for 10 min at  $4^\circ\text{C}$ .
4. After blocking, wash by centrifugation at  $500 \times g$  for 3 min.
5. Resuspend cells with 0.5 ml surface staining buffer containing primary antibody at a concentration of  $(1 \mu\text{g}/\text{ml}/1 \times 10^6 \text{ cells})$  (see Note 4).
6. Incubate for 30 min at  $4^\circ\text{C}$ .
7. After 30 min, wash cells twice with D-PBS by centrifugation at  $500 \times g$  for 3 min.
8. Add secondary antibody diluted in surface staining solution at a concentration of  $1 \mu\text{g}/\text{ml}/1 \times 10^6 \text{ cells}$  and incubate for 30 min at  $4^\circ\text{C}$ .
9. Following secondary incubation, wash cells twice with D-PBS by centrifugation at  $500 \times g$  for 3 min.
10. Fix cells for 10 min with fixation solution containing Hoechst nuclear dye at a concentration of  $1 \mu\text{g}/\text{ml}$ . Transfer 100  $\mu\text{l}$  from each stained sample to a corresponding well of the 96-well imaging plate and select the correct dye and light path prior to imaging.
11. Use image analysis software and the confocal microscope to view stained structures in 3D (Fig. 2) (see Note 5).

### **3.2 Phenotypic Analysis of Expanded Cells Using Immunofluorescent Microscopy**

Expansion of cells from initial cell isolates or primary tissue may be required for immunofluorescence and FACS analysis. Phenotypic analysis using immunofluorescent microscopy can be performed using techniques on adherent cells in many formats. Test plates can range from 6- to 96-wells and should be chosen based on the adherence and growth characteristics that best mimic the expansion culture. The format can also be dictated by the number of cells available and antibodies to be targeted including technical replicates. This can be minimized by multicolor staining referred to later in the chapter (see Note 6).

#### **3.2.1 Characterization of Surface/Membrane Antigens**

1. Obtain adhered cultured cells in a plated format.
2. Wash cultured cells by removing medium and adding appropriate volume of D-PBS to each well and or dish: 3 ml/well for 6-well dish, 2 ml/well for 12-well dish, and 250  $\mu\text{l}$ /well of chamber slide.

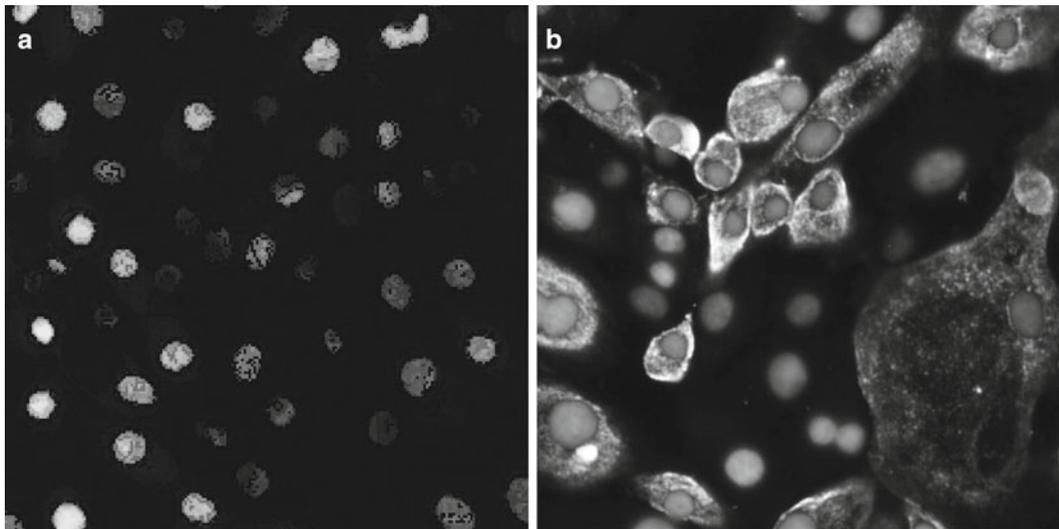


**Fig. 2** Confocal collapsed Z-stack image at 100 $\times$  magnification of immunostained kidney tubular clusters. Bright areas within clusters are positive for anti-mouse pan cadherin antibody (*arrows*). Nuclear staining are dim spots within clusters. Scale bar equals 100  $\mu$ m

3. After washing with D-PBS, add 0.5 ml/well of 2% paraformaldehyde to fix cells. Incubate at 4°C for 20 min (see Note 7).
4. Remove fixative and gently wash cells twice with D-PBS.
5. Block any nonspecific binding sites with surface blocking solution for 30 min at room temperature.
6. After 30 min, remove blocking solution.
7. Add primary antibody of interest as well as isotype-matched control at a previously titered concentration in a volume of 0.5 ml surface staining solution per well.
8. Incubate overnight at 4°C.
9. Wash cells twice with D-PBS.
10. After washing, add secondary antibody at a previously titered concentration (starting at 1  $\mu$ g/ml) in a volume of 0.5 ml surface staining solution per well for 30 min at room temperature protected from light.
11. Wash cells twice with D-PBS.
12. Add 0.5 ml/well of Hoechst nuclear dye 1  $\mu$ g/ml for 10 min at room temperature protected from light.
13. Wash cells twice with D-PBS.
14. Analyze using inverted fluorescent microscope with image analysis software.

3.2.2 *Characterization of Intracellular/Cytoplasmic Antigens*

1. Obtain adherent cultured cells in a plated format (6-, 12-, 24-, or 48-well tissue culture dish).
2. Wash cultured cells by removing medium and adding appropriate volume of D-PBS to each well and or dish (3 ml/well for 6-well dish, 2 ml/well for 12-well dish, 250  $\mu$ l/well of chamber slide).
3. After washing with D-PBS, add 2% paraformaldehyde to wells to fix cells. Incubate at 4°C for 20 min.
4. Remove fixative and wash cells twice with D-PBS.
5. Permeabilize and block cells by adding permeabilization and blocking solution (0.2% Triton X-100 with 2% serum of the species from which the secondary antibodies were generated from) for 30 min at room temperature.
6. Once cells are blocked, add desired primary antibody diluted in intracellular staining buffer, as well as isotype-matched control, and stain overnight at 4°C.
7. Wash cells twice with D-PBS.
8. Following washing, add secondary antibody (diluted in intracellular staining buffer) for 30 min at room temperature protected from light.
9. Wash cells twice with D-PBS.
10. Add 0.5 ml/well of Hoechst nuclear dye (1  $\mu$ g/ml) for 10 min at room temperature protected from light.
11. Wash cells twice with D-PBS.
12. Analyze using inverted fluorescent microscope with image analysis software (Fig. 3).



**Fig. 3** Immunofluorescence image of expanded cultured kidney cells 200 $\times$  magnification; (a) negative control with nuclear dye. (b) Cell membrane positive for a proximal tubule marker aquaporin 1

### **3.3 FACS Characterization of Primary Cultured Cells Using Single- Color Staining**

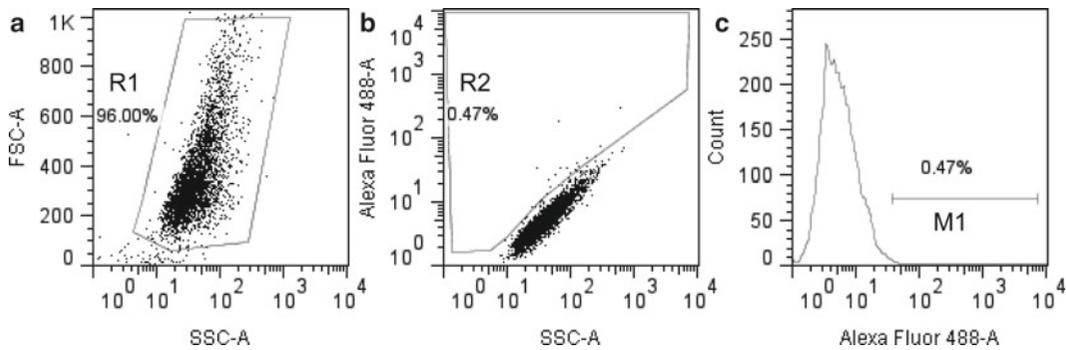
#### *3.3.1 Characterization of Surface/Membrane Antigens*

FACS analysis is a very effective method for generating quantitative results from a given population of expanded, subcultured cells. Direct versus indirect staining depends on the availability and reactivity of the specific antibodies for the species of interest (see Note 8). The following section details an indirect method.

1. Obtain sample of live cells from isolated tissue washed in D-PBS.
2. For surface targets, use live cells in suspension at a starting concentration of  $1 \times 10^6$  cells/ml in a  $12 \times 75$  mm tube. Include isotype-matched controls for each antibody (see Note 9).
3. Block cells for nonspecific binding with 1 ml surface blocking solution for 10 min at  $4^\circ\text{C}$ .
4. After blocking any nonspecific binding, wash by centrifugation at  $500 \times g$  for 3 min.
5. Resuspend cells with 1 ml surface staining buffer containing primary antibody of interest at a concentration of  $1 \mu\text{g}/\text{ml}/1 \times 10^6$  cells (see Note 4).
6. Incubate for 30 min at  $4^\circ\text{C}$ .
7. After 30 min, wash cells twice with D-PBS by centrifugation at  $500 \times g$  for 3 min.
8. Add secondary antibody diluted in surface staining solution at a concentration of  $1 \mu\text{g}/\text{ml}/1 \times 10^6$  cells and incubate for 30 min at  $4^\circ\text{C}$ .
9. Following secondary incubation, wash cells twice with D-PBS by centrifugation ( $500 \times g$  for 3 min) and then fix cells with 2% paraformaldehyde in D-PBS prior to analysis on the flow cytometer.

#### *3.3.2 Characterization of Intracellular/Cytoplasmic Antigens*

1. Obtain sample of live cells from isolated tissue washed in D-PBS.
2. Determine the number of tubes to be used, including antibody targets and isotype-matched controls, as well as an unlabeled sample to measure intrinsic autofluorescence using a starting cell concentration of  $1 \times 10^6/\text{ml}/\text{tube}$ .
3. For intracellular targets, cells must be fixed and cell membranes permeabilized. Fix cells at a concentration of  $1 \times 10^6/\text{ml}$  in 2% paraformaldehyde in D-PBS for 20 min at room temperature.
4. After fixation, wash cells thoroughly in D-PBS by centrifugation at  $500 \times g$  for 3 min at room temperature. Prior to last wash, distribute  $1 \times 10^6$  cells into 1.5 ml microcentrifuge tubes for staining.
5. Resuspend cells in a concentration of  $1 \times 10^6$  cells/0.2 ml of permeabilization and blocking solution (10% serum of the species from which the secondary antibodies were generated from in 0.2% Triton X-100 in D-PBS) and incubate for 30 min at room temperature.

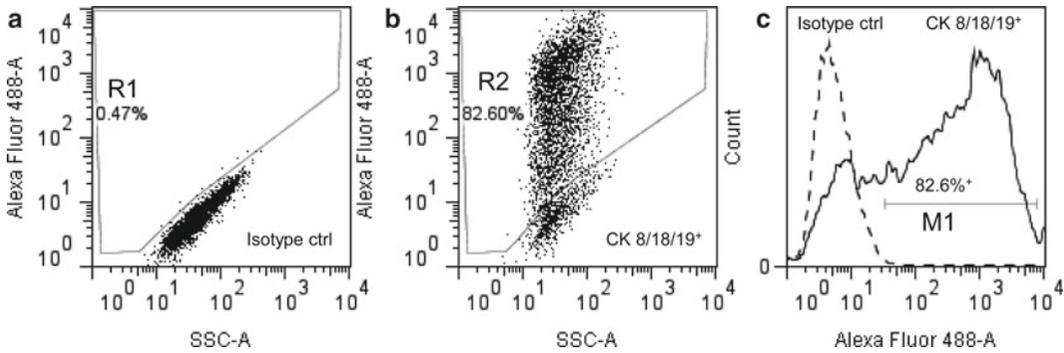


**Fig. 4** Recommended starting dot plots for single-color FACS analysis. (a) Selected cell population of interest (R1). (b) The R1 gate from the first plot displayed in a second dot plot where a second region (R2) is drawn using the isotype-matched control as a negative population. (c) Setting the positive marker (M1) for fluorescence using a histogram

6. After permeabilization, add 0.8 ml of 0.2% Triton X-100 in D-PBS containing primary antibody and isotyped-matched controls to each tube. Final concentration of antibodies should be determined beforehand using optimal titration assays. A general starting point is 1  $\mu\text{g}/1 \times 10^6$  cells/ml of intracellular staining solution.
7. Following blocking, wash by centrifugation at  $500 \times g$  for 3 min.
8. Incubate for 90 min to overnight at  $4^\circ\text{C}$ .
9. After 90 min, wash cells twice with D-PBS by centrifugation at  $500 \times g$  for 3 min.
10. Add secondary antibody diluted in intracellular staining buffer at a concentration of 1  $\mu\text{g}/\text{ml}/1 \times 10^6$  cells and incubate for 30 min at  $4^\circ\text{C}$ .
11. Following secondary incubation, wash cells twice with D-PBS by centrifugation at  $500 \times g$  for 3 min and then resuspend cells in 0.5 ml of D-PBS and filter if necessary through a 40  $\mu\text{m}$  filter (BD Falcon) to remove clumps prior to analysis on the flow cytometer.

### 3.3.3 Flow Cytometer Plot Setup for Basic Single-Color Analysis (see Note 10)

1. Make three starting plots: two dot plots and one histogram. The first dot plot measures size and granularity of the entire population; set up as SSC area (log) on X-axis and FSC area (linear) on the Y-axis. Make a capture gate for the region of interest (Fig. 4a) (see Note 11).
2. The second dot plot should be set up to capture the region of interest. Set the parameter for the X-axis to be SSC-area (log) vs. fluorophore-area (log) on Y-axis.
3. The third plot can be a histogram showing fluorophore such as A488-Area (log) vs. counts (Fig. 4c).



**Fig. 5** FACS analysis of cultured kidney cells stained with cytokeratin (CK) 8/18/19: **(a)** Isotype-matched control with gated population (R1) of positive stained cells, **(b)** percentage positive CK8/18/19 (R2) by dot plot, **(c)** overlay histogram of the same data showing percentage positive CK8/18/19 (M1) compared to control

4. Place unlabeled or isotype-matched control tube in loader and start acquisition. Adjust the PMT voltages for the forward side scatter and side scatter detectors to capture the entire cell population in the plot window. Since the initial cell culture population at early passage might have a mixture of cell types with varied sizes and granularities, start off by placing the entire population within the window.
5. Adjust the capture gate (R1) to exclude debris (Fig. 4a).
6. Select the gated population (R1) from the first plot to show in plot two and adjust the fluorophore PMT voltage (such as AlexaFluor 488-Area) by placing the population between the first and second decade of the Y-axis scale.
7. Make a second polygon-shaped gate in plot 2 (R2) (Fig. 4b) that captures the positively stained cells. The gate from the unlabeled and isotype-matched control should be similar if the antibodies have been titered properly (5) and can be adjusted to include 0.5% or less of the control population in order to capture any of the “low bright” positively stained cells of interest. The histogram marker (M1) can also reflect the percentage of positive cells that shifts to the right of the control peak (6) (Fig. 4c).
8. Sample acquisition can begin once the regions of interest have been gated. The total number of cells to be acquired and recorded can range between 5 and 50,000 events and can depend on the frequency of the rarest cell population of interest.
9. Percentage positivity using single-color fluorophores can be determined by subtracting the percentage of cells from the isotype-matched controls from the positive stained cells in the capture gate. Percentage positive equals 82.15% in the example of Fig. 5 (see Note 12).

### **3.4 Multicolor Immunophenotyping**

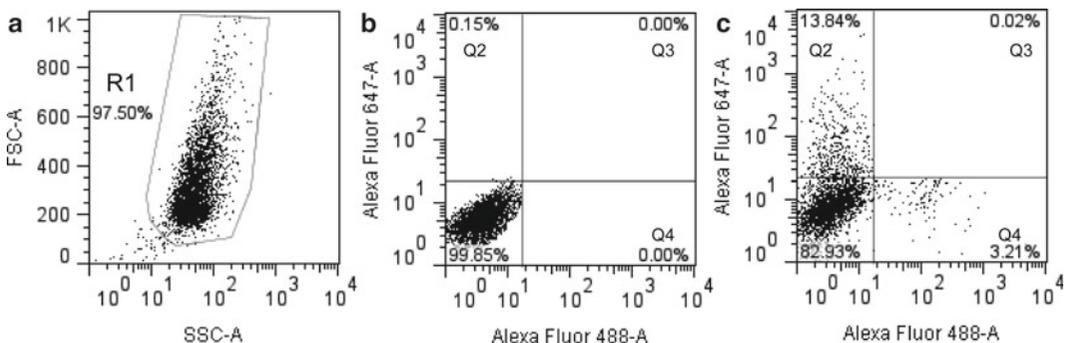
Multicolor immunophenotyping can be used effectively on passaged cells when more information from the expanded cultured cells is needed. The ability to simultaneously stain for multiple surface antigens can enhance cell characterization and distinguish between different cell populations during the expansion process. Most flow cytometers have the ability to distinguish many different fluorochromes. The addition of colors requires more comprehensive antibody panels that deal with compensation issues (7). Most of the larger panels for multicolor staining use directly conjugated antibodies for surface antigens on lymphocytes (8). This section describes a basic method for FACS that can distinguish two-color immunophenotyping targeting two surface antigens.

#### *3.4.1 Multicolor Indirect Immunophenotyping of Surface Antigens Utilizing Different Fluorescent Spectra on a Flow Cytometer*

1. Obtain sample of live cells from isolated tissue (i.e., kidney) washed in D-PBS.
2. Determine the number of tubes to be used including antibody targets and isotype-matched controls. For multicolor experiments greater than three colors, the use of “fluorescence minus one” (FMO) is recommended (see Note 13).
3. Set up 1.5 ml microcentrifuge tubes with a starting concentration of  $1 \times 10^6$  cells per tube.
4. Block cells for nonspecific binding with 1 ml surface blocking solution for 10 min at  $4^\circ\text{C}$ .
5. After blocking, wash by centrifugation at  $500 \times g$  for 3 min.
6. Resuspend cells with 1 ml surface staining buffer containing unconjugated primary monoclonal antibodies of interest with different isotypes at a concentration of  $1 \mu\text{g}/\text{ml}/1 \times 10^6$  cells, for example, mouse IgG1 and mouse IgG<sub>2b</sub>.
7. Incubate for at least 30 min at  $4^\circ\text{C}$ .
8. After primary incubation, wash cells twice with D-PBS by centrifugation at  $500 \times g$  for 3 min.
9. Add secondary antibodies such as Alexa Fluor 488 or Alexa Fluor 647 that can be excited by either the blue or red laser to minimize bleed over from one channel to another and to minimize compensation issues. Dilute antibodies in surface staining buffer to a concentration of  $1 \mu\text{g}/\text{ml}/1 \times 10^6$  cells and incubate for 30 min at  $4^\circ\text{C}$ .
10. After secondary incubation, wash cells twice with D-PBS by centrifugation at  $500 \times g$  for 3 min and then fix cells in 1% paraformaldehyde in D-PBS for 10 min.
11. Filter cells if necessary through a  $40 \mu\text{m}$  filter to remove clumps prior to analysis on the flow cytometer.

3.4.2 *Flow Cytometer Plot Setup and Basic Two-Color Analysis of Culture Cells*

1. Make two starting plots: The first dot plot measures size and granularity of the entire population; set up as SSC area (log) on *X*-axis and FSC area (linear) on the *Y*-axis. Make a capture gate for the population of interest (R1).
2. The second dot plot should be set up to show both fluorophores, one on each axis. Placing a four-quadrant region within the plot will enable the distinction of single- as well as dual-positive cells. Set the parameter for the *X*-axis to be A488-area (log) vs. A647-area (log) on *Y*-axis (Fig. 6).
3. Place the control tube for each fluorophore in the flow cytometer cell loader and start acquisition. Adjust the PMT voltages for the forward side scatter and side scatter detectors to capture the entire cell population in the plot window. Since the initial cell culture population at early passage might have a mixture of cell types with varied sizes and granularities, start off by placing the entire population within the window.
4. Adjust the capture gate (R1) to include nucleated cells, excluding debris (Fig. 6a).
5. Select the gated population from plot one to show in plot two. Adjust both of the fluorophores' PMT voltages, placing the population between the first and second decade of the *X*- and *Y*-axis scale and within the quadrant I region (lower left).
6. Adjust PMT voltages from controls to ensure that the positive controls fall within the upper region on the scale (Fig. 6b).
7. Sample acquisition can begin once the regions of interest have been gated. The total number of cells to be acquired and recorded can range between 5 and 50,000 events and can depend on the frequency of the rarest cell population of interest.



**Fig. 6** Multicolored analysis of cultured human kidney cells stained with an endothelial marker CD31-A488 and a proximal tubular marker LTA- A647; (a) selected population. (b) Quadrant adjustment based on placing the isotype-matched control in lower left quadrant 1. (c) Bivariate 2-color analysis showing 13.84% positive for LTA and 3.21% for CD31

8. Percentage positivity using multicolor fluorophores can be determined by the percentage of cells located in each quadrant. Cells located in Q2 (upper left) for this example would be positive for LTA-A647, Q3 (upper right) would be positive for both CD31 A488 and LTA-A647, and Q4 (lower right) would be positive for CD31-A488 only (Fig. 6c).

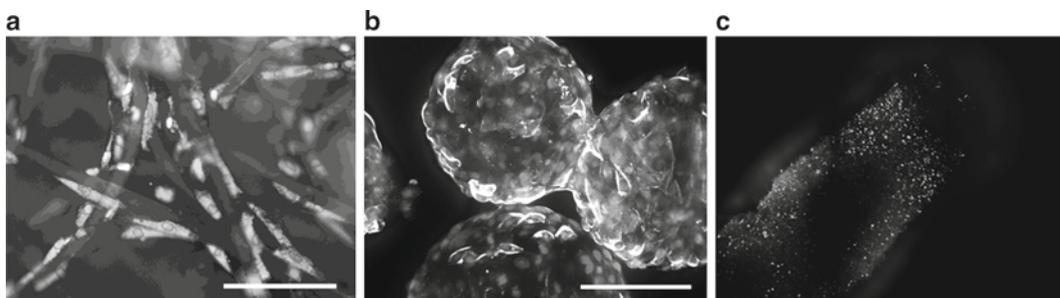
### 3.5 Three-Dimensional Phenotypic Analysis of Cell/Biomaterial Combinations Using Confocal Microscopy

#### 3.5.1 Immunophenotyping Cell/Biomaterial Combinations

The ability to analyze cells seeded onto or embedded within biomaterials can be helpful in characterizing combination products for the use in regenerative medicine (9). The following section describes immunophenotyping methodology using a cell–biomaterial combination analyzed by spinning disk confocal microscopy. Examples of cell–biomaterial combinations include adipose-derived smooth muscle cells (Ad-SMC) seeded onto PGA/PLGA scaffolds, kidney tubular epithelial cells seeded on Collagen Type 1 beads and embedded in gelatin-based hydrogels (Figure 7).

Different biomaterials may require separate procedures for targeting membrane or cytoplasmic antigens. Considering the biomaterials' natural autofluorescence will help in deciding which fluorophores to use to maximize the signal-to-noise ratios. Other considerations may include fixation with paraformaldehyde which may increase cross-linking, depth of seeded cells within the biomaterial, and cell morphology. This section provides a method for single-color immunophenotyping of cells on paraformaldehyde-fixable biomaterials using an indirect method for a cytoplasmic antigen target. Multiple colors can be added using the same principles if autofluorescence, isotype, and compensation can be accounted for.

1. Obtain sample of a cell-seeded biomaterial (see Note 14).
2. Fix cells seeded onto biomaterial with 2% paraformaldehyde in D-PBS for 20 min at room temperature.



**Fig. 7** Immunophenotyping cell/biomaterial combinations: (a) Smooth muscle actin-positive cells stained on PGA/PLGA scaffold, 200 $\times$  collapsed z-stack image with 100  $\mu$ m scale bar. (b) Cultured kidney epithelial cells stained with cytokeratin 8/18/19 on type 1 collagen beads, 200 $\times$  collapsed Z-stack image with 100  $\mu$ m scale bar. (c) Cytokeratin 8/18/19-positive epithelial cells embedded in gelatin-based hydrogel, 100 $\times$  collapsed Z-stack 3  $\times$  3 montage image

3. After fixation, wash twice with D-PBS and for cell membrane targets, block nonspecific binding using surface blocking solution for 30 min at room temperature.
4. Add primary antibody in 0.5 ml surface staining buffer at a starting concentration of 3  $\mu\text{g}/\text{ml}$ .
5. For intracellular targets, the cells need to be permeabilized. Add 1 ml of permeabilization and blocking solution (10% serum of the species from which the secondary antibodies were generated from) and incubate for 30 min at room temperature.
6. After permeabilization, aspirate permeabilization and blocking solution, and add 0.5 ml of intracellular staining solution containing primary antibody and isotype-matched controls to each piece of cell–biomaterial combination. Final concentration of antibodies should be determined beforehand using optimal titration assays. A general starting point is 3  $\mu\text{g}/\text{ml}$  of intracellular staining solution.
7. Incubate for 90 min to overnight at 4°C.
8. Following primary antibody incubation, wash cell–biomaterial combination twice with D-PBS by aspiration.
9. Add secondary antibody diluted in intracellular staining buffer at a concentration of 1  $\mu\text{g}/\text{ml}$  and incubate for 30 min at 4°C.
10. After secondary incubation, wash cells twice with D-PBS by aspiration, then add 0.5 ml of D-PBS containing 1  $\mu\text{g}/\text{ml}$  of Hoechst nuclear dye, and incubate for 10 min.
11. Following incubation, wash twice using aspiration and then transfer cell–biomaterial combination to an appropriate imaging plate to match the size of biomaterial (see Note 15).
12. Image cell-seeded biomaterial using confocal microscope with image analysis software such as Attovision™ (BD) for the Pathway 855 microscope (BD). Depending on the material, manually setting the depth at which the signal is brightest in the Z-plane is recommended; this will vary with material composition. Generally, for the Pathway 855, 70 microns is the maximum depth for PGA/PLGA materials. 3D analysis can be done using post-acquisition collapsed Z-Stack processing.

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## 4 Notes

1. Determining cell count using a standard hemocytometer on a cluster of cells can be difficult and not always as accurate as counting a single-cell suspension. For this reason, an approximation is reasonable when performing a cytocentrifugation to ensure that there is separation of clusters and aggregates on the slides prior to staining.

2. Image analysis can be performed on a variety of fluorescent microscopes. Both inverted and upright microscopes are suitable for viewing immunostained slides. The most common filters used are the ones that will excite fluorescein or rhodamine as well as DAPI. Quantification can be completed by using image analysis software that recognizes individual cells and uses segmentation masks to differentiate a positive signal over background.
3. Prior to confocal microscopy, the immunostaining procedure can be completed first in 1.5 ml microcentrifuge tubes. After the last wash, the cells can be concentrated and resuspended in 100  $\mu$ l and transferred into the 96-well thin plastic imaging plate.
4. Antibody titration may be needed to maximize signal-to-noise ratios. A general rule of thumb is to use a known positive control cell and use a matrix to test a twofold dilution series for both primary and secondary antibodies. If there is not a positive control cell available, a general starting concentration of 1–3  $\mu$ g/ml may suffice. Determining the best signal-to-noise ratios may increase sensitivity and decrease false positives.
5. Image acquisition using a confocal microscope needs to be performed by someone trained in imaging to ensure that the most optimal image parameters are set correctly. Parameters such as image processing, flat field correction, background subtraction, and segmentation are some of the things to consider. For the Pathway 855<sup>TM</sup>, AttoVision<sup>TM</sup> is the preferred software for image acquisition.
6. Deciding on which plating format to use to maximize the number of antibodies to screen (including controls) can depend on target cell number frequency. For rare cells with low frequencies, a larger plating format may be required to capture enough cells for statistical significance such as a 6-well dish or a 100 mm plate. For larger cell target frequencies greater than 10%, a smaller plating format could be used such as a 12- or a 24-well plate. This will enable the researcher to use a minimum volume of antibody solution to stain the cells. For multiplexing and high-throughput studies, it is possible with some cell types to use a 96- or 384-well format.
7. Fixation solutions can vary depending on the localization of epitopes. For most applications, a low percentage of 1–4% paraformaldehyde will suffice and maintain expression consistency. Zinc salt-based fixation for analysis of intracellular and surface epitopes has been recently described (10) to allow for long-term storage without changing labeling. Detergent-based versus ethanol-based permeabilization depends on whether the surface epitopes need to be stained prior to fixation. Using paraformaldehyde or zinc-based fixative that preserves surface epitopes for initial staining followed by a detergent-based permeabilization solution allows for multiple analyses of surface and intracellular antigen targets.

8. Direct versus indirect staining mainly depends on the availability of species-specific conjugated antibodies. Direct staining removes steps due to the primary antibody being conjugated to a fluorophore. Whenever possible, try to select conjugated antibodies. In many instances, when either multiplexing or dealing with species other than human, unconjugated primary antibodies may be the only choice. Recognition of both primary and secondary isotype has to be considered and more planning may be required to ensure specificity or nonspecific binding of target antigen. Sources for more information on cytometry can be found on the Internet at Purdue University Cytometry Laboratory Web site ([www.cyto.purdue.edu](http://www.cyto.purdue.edu)) as well as the International Society for Analytical Cytometry ([www.isac-net.org](http://www.isac-net.org)).
9. Staining for surface epitopes on live cells can be accomplished using the 12 × 75 mm polystyrene round bottom tubes (BD Falcon). For intracellular targets post-fixation and permeabilization, there can be a significant loss of cells when using the 12 × 75 mm tubes. The 1.5 ml microcentrifuge tubes work well when centrifuging at 300–500 × *g* for 3 min; the losses are minimal. Upon the last wash in the process, the cells can then be transferred to the 12 × 75 mm tubes for acquisition.
10. Prior to acquiring cells on the flow cytometer, follow manufacturer's recommendations on instrument start-up procedures and instrument QC.
11. When analyzing whole-organ primary cultures, cell size (FSC) and granularity (SSC) are important parameters in determining the heterogeneity of the population. Placing either of these parameters on the *X*- or *Y*-axis is a personal preference. Starting with broad markers that characterize epithelial, mesenchymal, and endothelial cells is a good way to divide the population into categories. Once those percentages are determined, more selective antibodies can be used to further specify the population.
12. The release criteria for cell-based regenerative medicine/tissue-engineered products may vary from product to product and is represented by the phenotypic characterization of cells that best defines the population of interest prior to being released for product use. There can be more than one marker to base the criteria on and usually these markers have to meet a certain percentage of positive cells in order for the product to meet acceptance for release.
13. FMO control refers to controls that measure the fluorescence in multicolor experiments of all reagents, except the one of interest. They are necessary in determining which cells do not express the given target antigen. FMOs are usually run on peripheral blood mononuclear cell (PBMC) analysis using directly conjugated antibodies. For experiments that require a two-color analysis, using isotype-matched controls and a

separate wavelength where fluorescence spillover from one channel into the other channel does not occur is acceptable.

14. Cell–biomaterial combinations can be stained in many formats depending on the size of the sample. A larger scaffold may fit into a 6-well dish, whereas a smaller cell-seeded bead sample can fit into one well of a 96-well imaging plate. Picking a format that optimizes the staining surface and limits the reagents may be the best choice.
15. Choosing imaging plates for confocal microscopy can depend on a few factors such as size of sample, cost, and background fluorescence. Imaging plates are available in different materials such as glass (<200  $\mu\text{m}$ ), thick plastic (>500  $\mu\text{m}$ ), and thin plastic (<250  $\mu\text{m}$ ). Each has its pros and cons but a general rule is to choose one that is flat and allows the use of high-resolution objectives.

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