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High-dimensionality Flow Cytometry for Immune Function Analysis of Dissected Implant Tissues

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TITLE:

High-dimensionality Flow Cytometry for Immune Function Analysis of Dissected Implant Tissues

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Immunology, biomaterials, flow cytometry, bioengineering, immunoengineering, medical devices, biocompatibility

SUMMARY:

Isolation of cells from dissected implants and their characterization by flow cytometry can significantly contribute to understanding the pattern of immune response against implants. This paper describes a precise method for the isolation of cells from dissected implants and their staining for flow cytometric analysis.

ABSTRACT:

The success of implanting laboratory-grown tissue or a medical device in an individual is subject to the immune response of the recipient host. Considering an implant as a foreign body, a hostile and dysregulated immune response may result in the rejection of the implant, while a regulated response and regaining of homeostasis can lead to its acceptance. Analyzing the microenvironments of implants dissected out under in vivo or ex vivo settings can help in understanding the pattern of immune response, which can ultimately help in developing new generations of biomaterials. Flow cytometry is a well-known technique for characterizing immune cells and their subsets based on their cell surface markers. This review describes a protocol based on manual dicing, enzymatic digestion, and filtration through a cell strainer for the isolation of uniform cell suspensions from dissected implant tissue. Further, a multicolor flow cytometry staining protocol has been explained, along with steps for initial cytometer settings to characterize and quantify these isolated cells by flow cytometry.

INTRODUCTION:

Advances in the field of medicine have led to the frequent use of implanted materials for supporting the function or re-growth of damaged tissue[1,2]. These include devices such as pacemakers, reconstructive cosmetic implants, and orthopedic plates used for bone fracture fixation[3,4]. However, the materials used to make these implants and the locations in which they are implanted play important roles in determining the success of these implants[5-7]. As foreign bodies, these implants can generate an immune response from the host that can either lead to rejection or tolerance[8]. This factor has driven biomaterial research to generate materials that can attract the desired immune response after implantation[9-12].

The immune response is an essential requirement in the field of regenerative medicine, where a tissue or an organ is grown around a biomaterial skeleton (scaffold) in a laboratory for the replacement of a damaged tissue or organ[13-16]. In regenerative medicine, the goal is to replace missing or damaged tissue through the use of cells, signals, and scaffolds, each of which can be greatly modulated by immune responses[17]. Furthermore, even when a lack of immune response is desired, it is very rarely an absence of immune activity rather than the presence of a regulatory profile that is desired[18]. Techniques such as flow cytometry can play a significant role in characterizing the pattern of immune response to various biomaterials used for coating implant devices or for developing scaffolds for tissue engineering[19].

This information, in turn, will ultimately help in developing biomaterials for implants that can be well-tolerated by the immune system or in developing scaffolds that can play a constructive role in tissue engineering. Proper preparation of samples for analysis by flow cytometry is an important step for avoiding inaccurate results in immune characterization via fluorescence activated cell sorting[20,21]. Therefore, this review presents a detailed methodology that can be utilized for the isolation of cells from scaffold tissue, staining the cell suspension, and analysis by flow cytometry.

PROTOCOL:

NOTE: **Figure 1** gives an overview of the flow cytometry protocol.

1) Reagent preparation

1.1) Prepare media for diluting enzymes and for tissue culture.

1.1.1) Add 5 mL of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer solution into 500 mL of RPMI medium and shake well. Store the medium at 4 °C until further use.

1.2) Calculate the volume of the enzyme solution.

NOTE: The volume of the enzyme solution is the volume of the medium containing the enzymes (collagenase and DNase I) that will be needed to digest diced tissue in 6-well plates; it depends on the number of samples.

1.2.1) Use the following equation to calculate the required volume:

(Number of samples to be digested) × 5 mL of serum-free RPMI with 10 mM HEPES buffer

NOTE: For example, for 0.1 to 0.3 g of dissected spleen, use 5 mL of medium to prepare the enzyme solution. The enzymatic digestion protocol described in this manuscript is primarily for soft tissues (ranging from 0.1 to 1 g) dissected from mice which include the brain, kidney, skin, liver, lungs, spleen, muscles, as well as capsules around subcutaneous implants made of synthetic materials or extracellular matrix proteins. Digesting hard cartilaginous tissues might require different conditions and volumes of digestive enzymes, which can only be ascertained by optimization.

1.2.2) Calculate the amount of collagenase and DNAase I needed for the enzyme solution.

NOTE: In this protocol, 0.25 mg/mL of collagenase and 0.2 mg/mL of DNase I were used. The working concentrations required for collagenase and DNase can vary for different types of tissues[19].

1.3) Prepare a 1:1000 viability dye solution by adding 1 µL of the viability dye (see **Table of Materials**) to 999 µL of phosphate-buffered saline (PBS) and vortex the solution. Store the solution in the dark at 4 °C until further use.

1.4) Prepare the staining buffer by adding 1 g of bovine serum albumin (BSA) to 100 mL of PBS, and vortex the solution until BSA is completely dissolved. Store the solution at 4 °C until further use.

2) Setting up enzymatic digestion plates

2.1) Set up a 6-well plate with 70 µm cell strainers in each well. Add 3 mL of the prepared medium from step 1.1.1 into each well, and incubate on ice.

2.2) Store the remaining media (2 mL/well) in an incubator or water bath at 37 °C for suspending the calculated amount of collagenase and DNase I (step 1.2).

3) Isolation of cells

3.1) Place the dissected implants/tissue into plates, and dice finely using scissors. Alternatively, utilize a mechanical disruption method using a tissue dissociator or hand-held

homogenizer. Owing to the high number of leukocytes, dissect out the spleen to utilize as a staining control in each run.

NOTE: As some materials induce higher levels of fibrosis, this protocol is applicable for both highly fibrotic and minimally fibrotic materials. However, each processing method should be evaluated for both cell yield and viability after digestion prior to staining. Avoid peripheral blood contamination during dissection.

3.2) Add collagenase and DNase I (volume calculated in step 1.2) to the remaining RPMI (2 mL) that has been warmed to 37 °C. Add 2 mL of the complete enzyme solution to each well.

3.3) Place the plates in an incubated shaker for 45 min at 37 °C and 100 rpm.

NOTE: Be cautious when stacking plates as this can inhibit proper heat transfer.

3.4) Meanwhile, prepare a suspension-dispensing tip by chopping 3–4 mm from the end of a 1000 µL tip with scissors. After incubation, pipette the suspension in the wells up and down to mix it thoroughly using the chopped tip. Place the cell strainer on the top of a 50 mL conical tube and pass the digested solution through the cell strainer.

NOTE: The 70 µm cell strainer is sufficient to separate cells from implanted biomaterial, such as alginate. If greater purity is required, use processes such as density gradient separation to obtain an enriched population of leukocytes.

3.5) Rinse the wells with 1x PBS solution, and transfer the rinse volume through the strainer, followed by adding the washes of the strainer with 1x PBS solution until the volume in the tube reaches 50 mL.

NOTE: The 1x PBS must be at room temperature to prevent any increase in the viscosity of the digest and to allow cell pelleting during centrifugation.

3.6) Centrifuge the tube at 300 × *g* for 5 min at room temperature. Following centrifugation, aspirate the supernatant carefully using a serological pipette without disturbing the pellet. Re-suspend the pellet in 1000 µL of 1x PBS, and transfer the suspension to a microcentrifuge tube.

3.7) Mix 10 µL of the cell suspension with 10 µL of trypan blue, and load the suspension on counting chamber slides for counting cells using an automatic cell counter or on a hemocytometer to count via the conventional method under a microscope. Alternatively, use flow cytometry cell counting beads.

4) Staining for flow cytometry

4.1) See **Figure 2** for an example plate layout for a 14-color experiment with 45 samples, fluorescence minus one (FMO) controls, compensation controls, and a fully stained lung sample

control. After estimating the total number of cells, calculate the volume of the cell suspension required for staining 1×10^6 cells for each sample, and 0.5×10^6 cells for each compensation and FMO control. Dispense the required volume of the cell suspension into the wells of a V-bottom 96-well plate, and make up the volume to 100 µL with 1x PBS.

NOTE: As an example, if 1000 µL of cell suspension obtained at step 3.13 contain 40×10^6 cells, then for 1×10^6 cells, $1000/40 \times 10^6 = 25$ µL of cell suspension will be required.

4.2) Centrifuge the plate at 300 × *g* for 5 min at 4 °C, aspirate the supernatant, and then resuspend the cells in the sample wells and in a compensation control well to determine viability, with 100 µL of a 1:1000 solution of the viability dye (see **Table of Materials**).

4.3) For cells in the other compensation wells as well as FMO and unstained wells, resuspend them with 100 µL of 1x PBS.

4.4) Incubate the cells in the dark for 30 min at 4 °C.

4.5) In the meantime, prepare a surface antibody cocktail for staining the sample and FMO controls: 50 µL per sample or FMO. See **Table 1** for an example of the antibody cocktail.

4.6) After incubation, add 100 µl of 1x PBS to each well, spin down at 300 × *g* for 5 min at 4 °C.

4.7) Aspirate the supernatant and resuspend in 200 µL of staining buffer (1x PBS + 1% BSA); centrifuge at 300 × *g* for 5 min at 4 °C.

4.8) Aspirate the supernatant, and resuspend the cells in the compensation, FMO, and unstained wells with 50 µL of 1x PBS. Add 50 µL of the antibody cocktail to the respective sample wells and FMO wells. Add the respective antibodies to the different compensation wells.

4.9) Incubate the plate for 45 min in the dark at 4 °C. After incubation, add 150 µL of staining buffer in each well, and centrifuge the cell suspension at 300 × *g* for 5 min at 4 °C.

4.10) Aspirate the supernatant, resuspend the cells with 200 µL of staining buffer, and centrifuge the cell suspension at 300 × *g* for 5 min at 4 °C.

4.11) If analyzing samples without fixation, resuspend them in 200 µL of staining buffer, and proceed to section 6 on cytometer setup and sample analysis. If fixing the cells, aspirate the supernatant after washing, and add 100 µL of a fixative such as 4% paraformaldehyde. If staining for intracellular markers, proceed to section 5 on intracellular staining, and fix and permeabilize the cells (see **Table of Materials**). Incubate cells for 20 min in the dark at 4 °C.

NOTE: As fixation can affect the fluorescence intensity of some fluorophores, evaluate each panel both with and without fixation.

4.12) After incubation, add 100 µL of 1x PBS, followed by centrifugation at 300 × *g* for 5 min at 4 °C. Aspirate the supernatant, resuspend in 1x PBS, and centrifuge at 300 × *g* for 5 min at 4 °C.

4.13) Re-suspend the cells in 200 µL of staining buffer, and store at 4 °C prior to flow cytometric analysis.

5) Intracellular staining

5.1) Continuing from step 4.11 for fixation and permeabilization for intracellular markers, add 100 µL of the appropriate buffer. Centrifuge the cells at 350 × *g* for 5 min at 4 °C. Aspirate the supernatant, and re-suspend the pellets in 200 µL of the fixative-permeabilizing agent solution; centrifuge the cells at 350 × *g* for 5 min at 4 °C. Aspirate the supernatant, and resuspend the pellets with intracellular antibodies diluted in the appropriate buffer.

NOTE: Antibody types and dilutions will depend on the target cells. For example, one common intracellular marker is forkhead box P3 for regulatory T cells, which is frequently used at a 1:100 dilution.

5.2) Incubate the cell suspension in the dark for 45 min at 4 °C. After incubation, add 150 µL of the appropriate buffer, and centrifuge the suspension at 350 × *g* for 5 min at 4 °C.

5.3) Aspirate the supernatant, and re-suspend the cells in 200 µL of staining buffer followed by centrifugation at 300 × *g* for 5 min at 4 °C. Aspirate and re-suspend the cells in 200 µL of staining buffer for flow cytometric analysis.

6) Cytometer and compensation setup

6.1) Immediately before running the samples, prepare compensation beads (see **Table of Materials**) if utilizing bead-based compensation as opposed to cell-based compensation.

6.1.1) Label separate microcentrifuge tubes for each fluorochrome-conjugated antibody, and add 100 µL of 1x PBS followed by one full drop of anti-mouse negative control compensation beads and one drop of positive control compensation beads to each tube. Add 1 µL of the appropriate antibody to each separate tube. Vortex and incubate for 5 min before acquisition.

NOTE: As some dyes, such as BUV737, cannot be bound to beads, use a cell-based control.

6.2) Calibrate the flow cytometer before each experiment by running the cytometer setup with the tracking beads, and maintain the flow cytometer settings for each experiment.

6.3) Before analyzing the sample, adjust the flow cytometer by running an unstained sample to adjust the cell population on a side scatter (SSC) versus forward scatter (FSC) plot so that the cell population falls in the center of the plot and is not off-scale.

6.4) Run the stained sample briefly to adjust the voltages for FSC and SSC and for each channel to make sure that no events fall outside the logarithmic scale of each channel. Record 5,000 events for each compensation control, followed by gating of positive and negative populations. Calculate the compensation matrix and then run the samples, gathering at least 1,000 events for the populations of interest.

NOTE: Compensation on larger-color panels should be verified, as automated compensation can be prone to over- or under-compensation. Each parameter should be graphed against every other parameter to monitor for signs of over- or under-compensation, and each single-color control should be evaluated. Complex compensation of larger-color experiments should be carried out with the assistance of a collaborator or a core facility with extensive flow cytometry experience. Newer types of cytometers—spectral cytometers—utilize the full spectrum of a fluorophore through a process known as spectral unmixing, which can yield a cleaner distinction of fluorescent overlap as compared to standard compensation alone[22].

REPRESENTATIVE RESULTS:

The process of development of flow cytometry panels for immune analysis often relies on the comparison of results to existing data and the literature in the field. Knowledge of how populations may present in flow cytometry is critical for proper interpretation of data. Regardless, populations and cell types can appear differently in different tissues, so some variability is to be expected. In the context of well-defined control tissues, such staining optimization can be evaluated against known tissue that has well-researched cell types. **Figure 3** shows the results of a 14-color FACS on control mouse tissue. In this case, the spleen was used to identify all markers that were stained for, and to show that the staining was technically functional. From this point onwards, it became easier to test in unknown conditions, be confident of the fluorophore selection, and optimize the protocol for different tissue sources. **Figure 3** also shows populations of different cell types isolated from a murine spleen[19].

Here, several obvious populations, such as Ly6G+ neutrophils, and different expression levels of Ly6C on different monocyte classes could be observed. $CD11c^{hi}MHCII^{+}$ dendritic cells were readily apparent when gated against CD11b to rule out macrophages and other myeloid lineage cells such as neutrophils and monocytes. A subset of CD206+CD86+ dendritic cells could be found by focusing on this CD11c+ population. CD86 and CD206 were included to phenotype myeloid cells as being in a more M1-like (CD86hi) versus in a more M2-like (CD206hi) polarization state. F4/80 showed a gradient of expression, that can be commonly observed in various macrophage populations. Siglec-F was present in two populations, F4/80+ and F4/80-, most likely corresponding to a macrophage subset and eosinophils, respectively. Although these designations of cell types can be made, it is important to note that cells expressing different markers should be viewed in a functional manner as opposed to a more binary classification. Acknowledgement that what is considered a macrophage may differ in the spleen and the foreign body capsule around an implant, is important and avoids the potential misinterpretation of results.

FIGURES AND TABLES LEGENDS

Figure 1: Overview of flow cytometry staining protocol. Preparation includes dissection of the tissue of interest followed by 1) manual dicing, 2) enzymatic digestion, 3) cell straining and washing, and 4) staining with fluorescently tagged antibodies followed by flow cytometric analysis.

Figure 2: Example of a plate layout for flow cytometry staining. This layout includes the samples, fluorescence minus one (FMO) controls, compensation controls, and a control tissue sample (spleen).

Figure 3: Representative results from 14-color FACS staining on control mouse tissue. Myeloid cell phenotyping of murine spleen cells with examples of hand-gating and automated t-stochastic neighbor-embedding (t-SNE) clustering algorithm for data display. This figure has been reproduced from Sadtler and Elisseeff[19]. Abbreviations: FACS = fluorescence-activated cell sorting; SSC = side scatter; CD = cluster of differentiation; PE = phycoerythrin; CCR = C-C chemokine receptor type; MHC = major histocompatibility complex; PerCP = peridinin chlorophyll protein complex.

Table 1: Example of surface antibody cocktail. An example antibody cocktail for myeloid phenotyping of mouse tissue.

DISCUSSION:

This review describes a detailed methodology for isolating cells from biomaterial implants to obtain a uniform cell suspension. In addition, a detailed protocol has been provided for staining the cell suspension for multicolor flow cytometry, along with the steps for configuring a flow cytometer for optimal results. Cell isolation methods can involve multiple steps, often utilizing manual tissue dissection followed by enzymatic digestion with proteolytic enzymes to dissociate the extracellular matrix in the tissue and disrupt the cell-cell junctions to liberate individual cells from the tissue. After digestion, further processing, such as cell straining, is needed to remove remaining debris and ensure a single-cell suspension. Some samples that have high levels of debris or other cell types (such as tumors) may require more thorough clean-up through the use of density separation media[23]. Without proper sample clean-up, data may be skewed due to debris or other cell populations obscuring the populations of interest. Excess debris can also lead to full or partial clogging of the cytometer fluidics.

While the characterization of immune cells can also be performed by other methods, such as light microscopy, by performing a differential cell count on a cell cytospin preparation, flow cytometry provides a more accurate characterization of the cells based on cell surface markers. Additionally, flow cytometry data are more precise as they can characterize and quantify millions

of cells in suspension and can provide accurate estimates of distinct cell subsets much faster than manual differential counting based on only 400 cells[24]. The results shown in this paper rely on an iterative panel design with antibody selection done by the utilization of multiple online tools to compare the excitation and emission spectra and theoretical overlap depending on the cytometer configuration. When designing larger color experiments, it is best to start from a clean slate, as opposed to the addition to a smaller color panel, so as to fully consider antigen abundance, fluorophore brightness, and spectral overlap.

Studies analyzing distinct subsets of specific immune cells require sufficient number of overall cells to perform flow cytometry, as having a smaller number of cells can pose a significant challenge in their isolation by cell sorting or obtaining accurate estimates. This challenge can be overcome by using magnetic beads for isolating and enriching specific immune cells, such as neutrophils, in shorter periods and with limited washing[25]. Isolated cells from tissues of organs such as lungs can contain significant amounts of mucus, which can make the cell suspension "sticky" and result in an increased number of doublet events during flow cytometry[26,27]. Adding chelating agents such as 2 mM ethylenediamine tetraacetic acid to the staining buffer can prevent the aggregation of cells in such samples.

Additionally, suspending cells in an increased volume of buffer can also limit doublet creation by reducing cell-cell interactions. Staining procedures should be thoroughly optimized for each different tissue, staining protocol, flow cytometer, and antibody panel. Some fluorophores can be more sensitive to fixatives, some cell types are more sensitive to different cell isolation and processing methods, and many cell types behave differently in different tissues and different locations. With proper preparation and diligent analysis, flow cytometry can yield a detailed cellular and protein-level analysis to characterize the scaffold and biomaterial immune microenvironment.

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DISCLOSURE:

The authors have nothing to disclose.

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Figure 1

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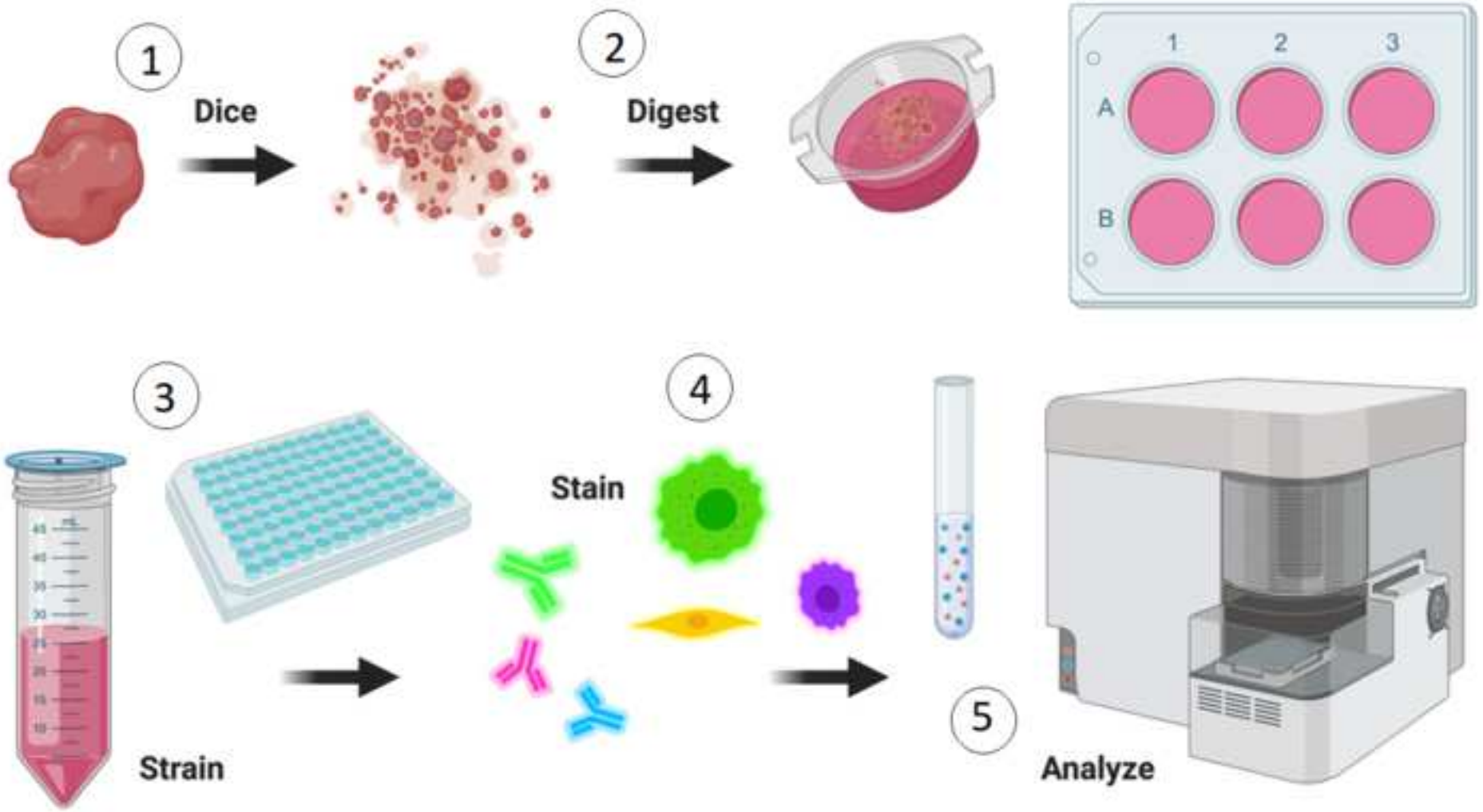

Figure 2

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	1	2	3	4	5	6	7	8	9	10	11	12
A	SAMPLE-1	SAMPLE-9	SAMPLE-17	SAMPLE-25	SAMPLE-33	SAMPLE-41		FMO-1	FMO-9		COMP-1	COMP-9
B	SAMPLE-2	SAMPLE-10	SAMPLE-18	SAMPLE-26	SAMPLE-34	SAMPLE-42		FMO-2	FMO-10		COMP-2	COMP-10
C	SAMPLE-3	SAMPLE-11	SAMPLE-19	SAMPLE-27	SAMPLE-35	SAMPLE-43		FMO-3	FMO-11		COMP-3	COMP-11
D	SAMPLE-4	SAMPLE-12	SAMPLE-20	SAMPLE-28	SAMPLE-36	SAMPLE-44		FMO-4	FMO-12		COMP-4	COMP-12
E	SAMPLE-5	SAMPLE-13	SAMPLE-21	SAMPLE-29	SAMPLE-37	SAMPLE-45		FMO-5	FMO-13		COMP-5	COMP-13
F	SAMPLE-6	SAMPLE-14	SAMPLE-22	SAMPLE-30	SAMPLE-38			FMO-6	FMO-14		COMP-6	COMP-14
G	SAMPLE-7	SAMPLE-15	SAMPLE-23	SAMPLE-31	SAMPLE-39			FMO-7			COMP-7	UNSTAIN
H	SAMPLE-8	SAMPLE-16	SAMPLE-24	SAMPLE-32	SAMPLE-40			FMO-8	SPLEEN CTRL		COMP-8	

Figure 3

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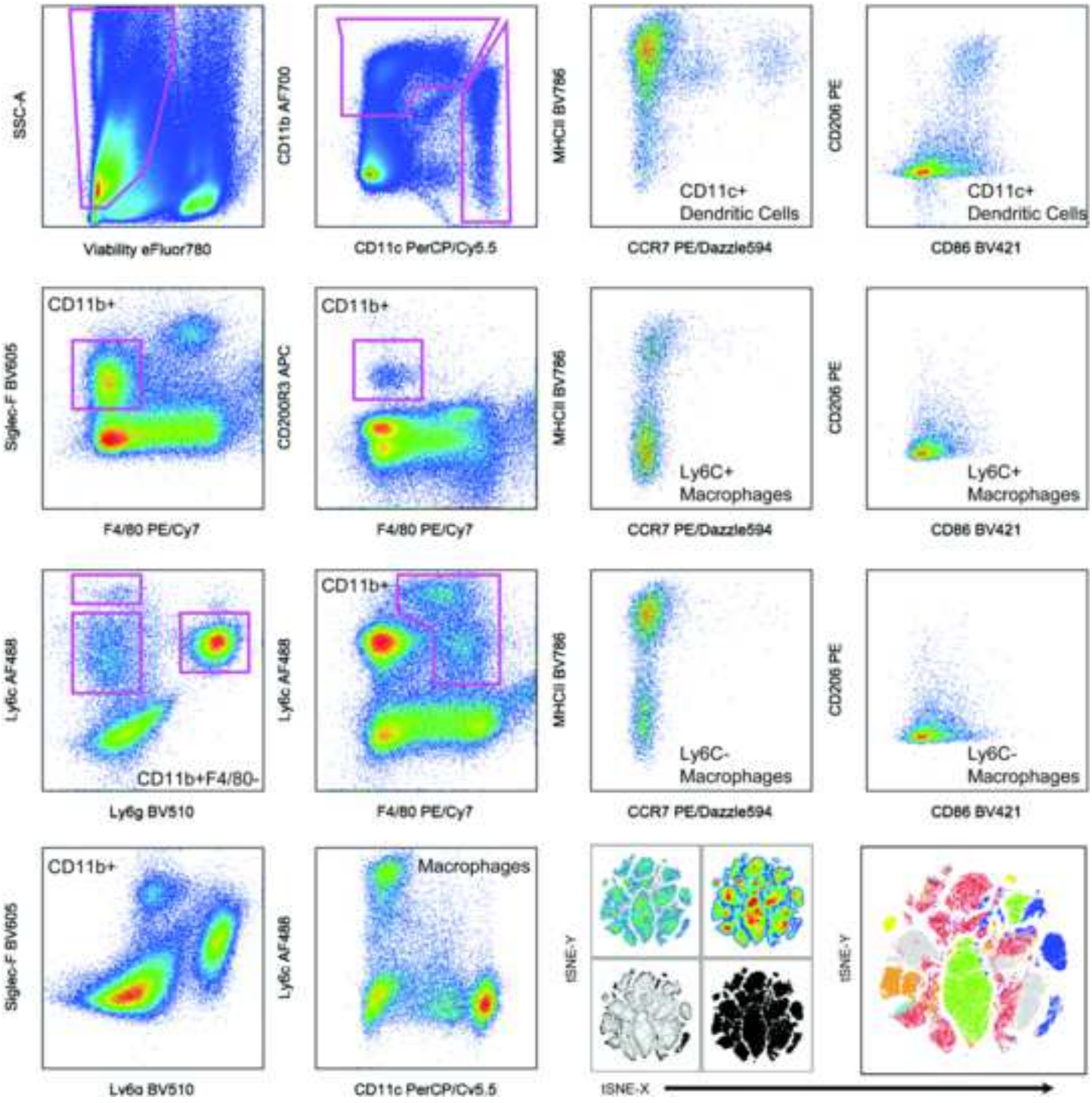

Table 1

Click here to access/download;Table;Table 1 Antibody cocktail.xlsx

Reagent/Antibody	µL per sample
CD86 BUV395	0.25
CD45 BUV737	0.5
CD8a BV421	0.25
Ly6g BV510	0.125
Siglec F BV605	0.25
MHCII BV786	0.25
Ly6c AF488	0.125
CD11c PerCP/Cy5.5	0.2
CD206 PE	0.2
CD197 PE/Dazzle594	0.125
F4/80 PE/Cy7	0.25
CD200R3 APC	0.25
CD11b AF700	0.25
Fc Block	1
BD Brilliant Stain Buffer Plus	10
1x PBS	35.975
Total Volume:	**50 µL**

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
50 mL conical tubes	Fisher Scientific	14-432-22	
6 Well Plate	Fisher Scientific	07-000-646	
BD Brilliant Stain Buffer Plus	BD Biosciences	566385	
BD Cytofix	BD Biosciences	554655	For only fixing cells
Bovine serum albumin	Millipore Sigma	A7906	For preparing FACS staining buffer
CD11b AF700	Biolegend	101222	Clone: M1/70
CD11c PerCP/Cy5.5	Biolegend	117325	Clone: N418
CD197 PE/Dazzle594	Biolegend	120121	Clone: 4B12
CD200R3 APC	Biolegend	142207	Clone: Ba13
CD206 PE	Biolegend	141705	Clone: C068C2
CD45 BUV737	BD Biosciences	612778	Clone: 104/A20
CD86 BUV395	BD Biosciences	564199	Clone: GL1
CD8a BV421	Biolegend	100737	Clone: 53-6.7
Comp Bead anti-mouse	BD Biosciences	552843	For compensation control
DNase I	Millipore Sigma	11284932001	Bovine pancreatic deoxyribonuclease I (DNase I)
F4/80 PE/Cy7	Biolegend	123113	Clone: BM8
Fc Block	Biolegend	101301	Clone: 93
Fixation/Permeabilization Solution Kit	BD Biosciences	554714	For fixing and permeabilization of cells.
HEPES buffer	Thermo Fisher	15630080	Buffer to supplement cell media
Liberase	Millipore Sigma	5401127001	Blend of purified Collagenase I and Collagenase II
LIVE/DEAD Fixable Blue Dead Cell Stain Kit	Thermo Fisher	L23105	Viability dye
Ly6c AF488	Biolegend	128015	Clone: HK1.4
Ly6g BV510	Biolegend	127633	Clone: 1A8
MHCII BV786	BD Biosciences	742894	Clone: M5/114.15.2
Phosphate buffer saline	Thermo Fisher	D8537	

RPMI	Thermo Fisher	11875176	Cell culture media
Siglec F BV605	BD Biosciences	740388	Clone: E50-2440
V-bottom 96-well plate			

RESPONSE TO REVIEWERS: JoVE61767R1

Lokwani & Sadtler

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling and grammar.

Response: We would like to thank the editor for pointing our attention, we have thoroughly proof read the manuscript again and tried to address previous shortcomings.

2. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets or dashes.

Response: We have addressed this point now

3. Please include a one line space between each protocol step and then highlight up to 3 pages of protocol text for inclusion in the protocol section of the video.

Response: We have addressed this point now

4. As you mention using implants/tissue, please specify the source and include an ethics statement before the numbered protocol steps, indicating that the protocol follows the guidelines of your institution's human/animal research ethics committee. If patients/healthy human subjects are involved, please specify selection criteria. As you state in the representative results that you have used a murine spleen, please specify age, strain, sex etc of the animals, and include the ethics statement regarding approval from your institution's animal research ethics committee.

As these representative results are published elsewhere and not completed as original research but reviewed, the ethics statement is in the cited article as opposed to this review article that reviews previously completed research.

5. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: Liberase TM (Sigma, Cat # 5401127001), DNase I (Sigma, Cat # 11284932001), gentleMACS (Miltneyi Biotec), Eppendorf tube etc

Response: Table is now included for material and reagents and all company names trademarks signs are removed and generic terms are used now.

6. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

Response: We have tried to address the short coming to provide detail protocol and this will also be seen in our video. However, we not using any special equipment apart from flow cytometer which in our case is Cytek Aurora (different from widely used flow cytometers such as BD flow cytometers). Therefore, we are happy to cover button clicks on flow cytometer in our video section.

7. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step.

Response: We have simplified the protocol now and each step have only 2-3 actions.

8. Step 1: please provide some guidelines for what volume is to be used for different tissue mass/volume/type.

Response: We have added a "Note" after step 1.2.2 to address this point.

9. Step 2: Please explain (notes are also permitted) the criteria for optimizing the amounts of Liberase/collagenase and DNase I.

The amount on enzyme can differ for type of tissue and we have mentioned this in a "Note" after step 1.2.3, and included a reference (Sadtler et al) that has described criteria in much more detail.

10. Step 2: please use mL and not ml.

Response: Addressed as suggested.

11. Step 3: please change 70 µM cell strainers to 70 µm cell strainers.

Response: Addressed as suggested

12. Please rewrite step 5; do you mean "…to use, and resuspend enzyme"?

Response: This point is corrected now.

13. Step 6: are the implants/tissue the samples? If so, please clarify; do you mean “place the implants/tissue in the plates, remove the plates from ice, and dice …”?

Response: Addressed now.

14. Step 6a, how do you evaluate viability and yield of cells? Please move information in step 17 to this point and cite references for using the hemocytometer and flow cytometry to count cells. How would these data help to optimize the dissociation technique for each tissue/material?

Response: This step is removed now and viability will only be determined by flow cytometry using Live/Blue dead reagent. But the total number of cells will be determined by using Invitrogen countless as mentioned in equipment table or by conventional way via haemocytometer. The total number of cells will help in determining the cell suspension needed for staining.

15. Step 13: Please add a space between 1x and PBS. Please define all abbreviations at first use. Please be consistent in using only small x for this purpose (refer to step 18a in line 180).

Response: Addressed now

16. Please remove the embedded figures and tables from the manuscript.
i) All figures should be uploaded separately to your Editorial Manager account. Each figure must be accompanied by a title and a description after the Representative Results of the manuscript text.

Response: Addressed as suggested

ii) All tables should be uploaded separately to your Editorial Manager account in the form of an .xls or .xlsx file. Each table must be accompanied by a title and a description after the Representative Results of the manuscript text.

Response: Addressed as suggested

17. Please use µL and not uL throughout the manuscript.

Response: This point is addressed now.

18. Please delete step 19 a, and combine that information with that in step 22.

Response: Step 19 is a separate step now (Step 4.7).

19. In Step 22, you say "…cocktail is shown below (Table 1)…" but the table below step 22 is labelled as Figure 2 and contains material that should be moved to the Table of Materials.

Response: Each antibody is now moved to Table of Material now and Figure 2 is changed as Table 2 and modified now.

20. Steps 35 & 40: Please specify how much of the intracellular antibody solution is to be used (dilution of the antibody, total volume of the solution), what dye is being used (how much and why), what is the volume of "one full drop"/"one drop", and please provide a specific example for filming of the protocol.

The intracellular staining protocol is listed for reference and the antibody type and dye will depend upon the end user's application. We chose here to describe a general protocol for intracellular staining that is important to put in context of the entire protocol, even if not utilized in the example dataset. We have added in text to include an example intraceullar marker and dilution.

21. Step 42: How much of the sample do you take for flow cytometry? If you plan to film this (especially because flow cytometry is the main focus of this protocol), please provide sufficient details to help filming. If you don't plan to film this, you can provide some details and also cite appropriate references.

Response: We planning to take 200 µL of sample as we have stated in step 4.16 and step 4.22.

22. Briefly explain the significance of looking for CD11, CD206, CD86, and F4/F80 in the representative results.

We have expanded our description of these markers in the representative results.

23. Lines 242-244: Please replace "was" with "what" in "Acknowledgement that was is considered a macrophage…".

Response: Replaced

24. Please do not abbreviate journal names in the reference list.
Response: Addressed as suggested

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

This article provides a detailed and accurate method for the flow cytometric analysis of the immune function of biological scaffold materials, which has good practicability and applicability. However there are still some problems needed to be solved.

Major Concerns:
1. It is very important to have strict and consistent standards of obtaining the tissue and biomaterial samples, which is a prerequisite for accurate and reliable results of flow cytometry analysis. Is the standard consistent for different implant sites or different scaffold materials? This article does not describe in detail these parts.

The reviewer raises an important topic. Consistent standards, as with other techniques, are reliant upon the vigor imposed by the laboratory conducting the study. Here, we provide a general protocol to utilize for FACS prep that we have utilized in intraperitoneal implantation, volumetric muscle loss, and subcutaneous implantation. We have added an additional note after step 3.1 regarding material variations and considerations for dissection.

2. Is it appropriate to dissect the bio-scaffold material directly? Does the presence of the bio-material affect the preparation and staining of the single cell suspension? It is not discussed in the article.

We have used 70 µm cell strainer to filter cells out from biomaterial and as the biomaterial are generally large enough so its unlikely that they will pass through the strainer into the cell suspension. But if further purity is required then steps like density gradient separation can be performed. We have added this point as "Note" after step 3.9.

3. Splenic macrophages are different from the macrophages around the implant, and the macrophages around the implant play a vital role. Is the spleen as a control recommended in the article suitable?

We thank the reviewer for raising the important issue and indeed the macrophages around implants can be different from spleen but the purpose of using spleen is to obtain high number of immune cells to optimize flow cytometry setting such as forward scatter and side scatter and also to understand the autofluorescence of tissue resident immune cells such as macrophages which will feature more or less similar auto-florescence if isolated from any other tissue. Therefore, since our purpose is not measuring functional characteristics of cells, spleen provide sufficient cells for optimization and autofluorescence comparison. During development of panels, we note that is important to test and optimize on each tissue or implant type.

Minor Concerns:

1. Fig3 lacks ④.

Response: Corrected now.

Reviewer #2:
This manuscript describes the steps to isolate cells from dissected implant tissues and their subsequent analysis by flow cytometry. The manuscript is well written, with concise techniques reported

straightforwardly. These techniques are of the growing interest to members of the Immunology and flow cytometry community.

1. The title is very attractive but might mislead the readers on what was actually performed. The manuscript describes the cell isolation from dissected implant tissues and their subsequent analysis by flow cytometry. No scaffold (e.g., ECM) or in situ analyses were performed. Please change the title to represent the manuscript's data more accurately. Suggestion, "High-Dimensionality Flow Cytometry for Immune Function Analysis of dissected implant tissues."

Response: Title changed as suggested.

2. Please include a section for reagent preparation, including buffers for enzymatic digestion, staining, Viability eFluor780, and RPMI with 10 mM HEPES.

Response: Reagent preparation section added as section 1

3. Line 82. Please add an example of how to prepare the "enzyme solution volume." For 2g of spleen, one would prepare the "x" amount of …

Response: Added at Step 1.2.2.

4. It is a good practice to have Fc blocking prior to immunostaining. Why did the authors choose to have the Fc blocking concomitantly with the immunostaining step? Please clarify your choice in the discussion.

Fc block out-competes for non-specific binding of other antibodies providing the Fc block is added at sufficient concentration. There is no difference between adding Fc concomitantly and pre-incubation and decreases handling steps and processing time which is important for sample integrity.

Reviewer #3:
Manuscript Summary:
The manuscript provides a very straightforward methodology for cell isolation, processing and staining for immunophenotyping of myeloid and lymphoid origin present in biomaterials or scaffolds after implantation. Flow cytometry is widely used and a powerful tool to identify cell populations. Unfortunately, most existing flow cytometry protocols are designed for blood or tumoral tissues which are quite different when compared to biomaterial and engineered scaffolds. Most of us that use flow cytometry as a way to identify cell phenotypes in response to biomaterials/scaffolds had to invest significant time and resources adapting existing protocols and matching antibodies. The authors put together a well designed protocol that can be readily applied in every single lab with access to a flow cytometer and have selected a set of matching antibodies that have been reported previously.

Major Concerns:
No major concerns. This was a R1 by then I think that most concerns were addressed.

Minor Concerns:
Perhaps my only concern is that while cytometer setting and compensation set up is part of the protocol, a little more explanation can be provided. Perhaps this is part of the video. Additionally, it would be ideal that the authors provide some additional insights in respect to how to build the antibody panels and the gating strategy.

Response: We have briefly covered gating strategy in our representative result section and with reiterate these points cover along with panel designing in our video. We have expanded a note on compensation within the protocol and added in a discussion on iterative panel design. We will also discuss this in the video segment.