Protocol for obtaining, identifying, and quantifying lymphoid populations in gingival tissue by flow cytometry

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Abstract

Techniques for disaggregating gingival tissue to obtain cells in suspension involve mechanical and/or enzymatic processing. These techniques can affect cell biology, causing changes in cellular phenotype and viability or decreasing the number of cells obtained, thus affecting the characterization, identification, and quantification of lymphoid populations obtained from the gingival tissue of healthy individuals or those with periodontitis. These pitfalls should be completely avoided given the size of the tissue obtained. A protocol for processing gingival tissue from healthy subjects with gingivitis or with periodontitis and obtaining and characterizing the lymphoid populations by flow cytometry is described herein.

Keywords: Gingival tissue, disaggregation, cytometry, periodontitis, lymphocyte

Basic protocol: Preparation of the protocol and the acquisition method for identifying and quantifying lymphoid populations from the disaggregated gingival tissue of healthy individuals and individuals with periodontitis.

Support protocol 1: Preparation of gingival tissue biopsies from healthy subjects and subjects with periodontitis to obtain cells in suspension.

Support Protocol 2: Extraction of biopsies of gingival tissue from healthy subjects and subjects with periodontitis.

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INTRODUCTION

Three types of mucous membranes have been described in the oral cavity: masticatory, lining, and specialized. The functions of the masticatory mucosa, of which the gingiva and the hard palate are part, are to cover and protect the teeth and underlying tissues and thus to act as a seal to prevent microorganism penetration as well as a barrier against mechanical and/or chemical aggressions (Dale, 2002; Pöllänen, Salonen & Uitto, 2003; Schroeder & Listgarten, 1997). These functions are carried out by chemokines and cytokines, among other proteins, that can alert and attract other cell populations that mediate the defence of the host against dysbiosis of the dental biofilm, one of the causal agents of periodontal disease (Hajishengallis, 2015; Dutzan, Konkel, Greenwell-Wild & Moutsopoulos, 2016; Murakami, Mealey, Mariotti & Chapple, 2018).

Periodontal disease is a multifactorial inflammatory pathology resulting from the activation of the immune system against a dysbiotic microflora (Ford, Gamonal & Seymour, 2010; Hajishengallis, 2015; Murakami, Mealey, Mariotti & Chapple, 2018). In response to this activation, the epithelium plays an active role in the response to infection and the signalling that induces additional host responses, in which epithelial cells, cytokines, Langerhans cells, and dendritic cells integrate innate and adaptive immunity (Dale, 2002). This is characterized by the expression of T and B cells that play a critical role in the immunopathogenesis of periodontitis, since the activation of the different T and B subpopulations, as well as the cytokines that they express, can be crucial in the definition, stability, resolution, and progression of a lesion from gingivitis to periodontitis (Figueredo, Lira-Junior & Love, 2019; Ford, Gamonal & Seymour, 2010; Gonzales, 2015).

These cell populations have been identified with the help of techniques such as immunohistochemistry and (direct) immunofluorescence, allowing the use of up to two markers, and confocal microscopy, which allows for a greater number of markers and thus identifies possible lymphoid subpopulations (Gemmell, Yamazaki & Seymour, 2002; Yamazaki, Nakajima, Aoyagi & Hara, 1993). The advantage of these techniques is that they can determine the locations of cells within the different epithelia of the gingival tissue, but the quantification and identification of more than four populations in the same sample is difficult.

Flow cytometry (Afar, Engel & Clark, 1992; Vos, Simurdak, Davis, Myers & Brissette, 2003) is one of the most sensitive, specific, and versatile techniques but has the limitation that cells must be in suspension. For this reason, it is essential to disaggregate the tissue to obtain numerous free, viable cells without damaging their cellular integrity. Various methods have been described for obtaining and processing gingival tissue specimens and for obtaining cells in suspension for subsequent characterization and quantification by flow cytometry, but different studies have had

contrasting findings, and the proportions and viability of the various cell types have not been elucidated (Aramaki, Nagasawa, Ishikawa, Koseki & Ishikawa, 1998; Cardoso, Garlet, Moreira, Júnior, Rossi & Silva, 2008; López Barrera, Quijano Gómez, Suárez Londoño & Roa Molina, 2007; Mahanonda et al., 2016; Mizraji, Segev, Wilensky & Hovav, 2013; Oliver-Bell et al., 2015; Sugawara, Yamashita, Yoshie & Hara, 1992).

Cell disaggregation techniques involve mechanical or enzymatic processing, separately and/or in combination. Enzymatic techniques generally use collagenase, hyaluronidase, dispase, trypsin, and DNase. Mechanical techniques are based on aspiration, vortexing, scraping, or tissue cutting (Cardoso, Garlet, Moreira, Júnior, Rossi & Silva, 2008; Cornacchiari et al., 1995). In the late 1990s, a cell disaggregation apparatus, the BD Medimachine™, was introduced for the isolation of epithelial specimens (Novelli et al., 1996). However, these methods and mechanisms can affect the cellular biology, generating changes or alterations in the cellular phenotype or causing a low recovery of viable cells (Flynn, Carton, Byrne, Kelehan, O'herlihy & O'farrelly, 1999; Novelli, Savoia, Cambieri, Ponti, Comessatti, Lisa & Bernengo, G., 2000; Van Damme et al., 2000).

Given the widespread use of tissue disaggregation protocols to obtain cells in suspension—protocols that yield inconsistent and nonreproducible results—it is suggested that the disaggregation protocols should be tailored the type of tissue and to the purpose of the sampling. For this reason, a protocol is presented herein for obtaining, identifying, and quantifying lymphoid populations in gingival tissue of healthy subjects and subjects with periodontitis that can capture the largest number of cells and preserve cell viability and integrity.

PREPARATION OF THE PROTOCOL AND THE ACQUISITION METHOD FOR IDENTIFYING AND QUANTIFYING LYMPHOID POPULATIONS FROM THE DISAGGREGATED GINGIVAL TISSUE OF HEALTHY INDIVIDUALS AND INDIVIDUALS WITH PERIODONTITIS

Given the special and scarce nature of the type of sample, there is a need to develop a standard disaggregation protocol that allows the recovery of as many cells as possible without affecting their immunophenotypic characteristics, with adequate cell viability, in a minimum amount of tissue, to guarantee a cell suspension of optimal quality to be input into the flow cytometer.

MATERIALS

Suspended gingival tissue cells (Support protocol 1)
Flow cytometer equipped with three lasers that allow excitation and detection of cells (BD FACSCanto II™).

- Blue: solid-state, air-cooled laser (488 nm and 20 mW of power)
- Red: HeNe laser (633 nm and 17 mW)
- Violet: solid-state diode laser (405 nm and 30 mW)

This is a 10-parameter cytometer, with forward scatter (FSC), side scatter (SSC), and eight fluorescences that allows simultaneous acquisition and analysis, with acquisition rates of up to 10,000 events/sec. The software for the acquisition of samples is BD FACSDiva™.

Preparation of the flow cytometer:

1. Equipment is turned on and the lasers are allowed to temper.
2. The state of the fluids and waste tank are checked.
3. Quality control is based on the clinical recommendations of the European Clinical EuroFlow Consortium.
   a. Daily reading of pearls (BD FACSDiva CS&T Research Beads™).
   b. Weekly pearl reading (BD OneFlow™ SetUp Beads).
   c. Monthly compensation (BD FC Beads 8-Colour Kit for BD OneFlow™ Assays) and running of applications by the acquisition software FACSCanto (BD FACS™ 7-Colour Setup Beads).

Preparation of the protocol:

4. Create the experiment in FACSDiva.
5. Each experiment has its instrument settings, which can be modified in the Inspector window (or imported if they have been saved from a previous experiment). Create as many specimens as desired. The specimen, designated by a syringe, encompasses a series of samples with similar characteristics and will contain as many tubes as there are samples.

With the Instrument and Inspector windows open, we have access to the voltages and parameters of the experiment (or of a particular tube, if we apply a specific voltage).

Acquisition of samples:

6. A gate or region is created on the selected lymphoid population, which allows the enrichment of only the population with FSC (size) and SSC (granularity) characteristics, according to the lymphoid population to be studied, which are small cells with low granularity, assuming no interference from other cell populations or debris.
7. The acquisition parameters were first calculated and later corroborated, according to previous experience with cell populations found in peripheral blood and according to possible positivity for the CD45 marker.
8. The selection threshold is greater than 100 on the forward (x) axis to discard the largest number of dead cells.
9. The parameters for each photodetector are determined by the negativity of the same unstained cells (negative control).

10. All the cells of the tube are acquired to determine the final number obtained by the disaggregation protocol.
11. The data analysis is performed in FlowJo 8.7 (BD)

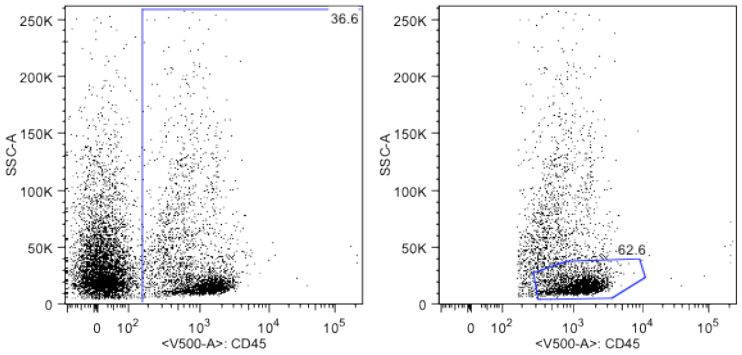

Figure 1. Lymphocyte acquisition region. Dotted image representative of the selected region at the time of cell acquisition, corresponding to the lymphocyte region by size and granularity and by the positivity of the CD45+ marker.

Gate strategies:

12. The enrichment gate is used as a strategy to include only the population of interest and exclude possible interfering cell populations and debris. Depending on the cellular recovery obtained after disaggregation, more than three million events can be acquired in each gate. In addition to the gate, it is advisable to have CD45 labelling in this case because the population of interest is leukocytes, specifically the lymphoid population.

NOTE: Cells larger than lymphocytes are not taken into account, and populations such as fibroblasts that could be evidenced in the suspension to be acquired are discarded. These are identified by their positivity for CD14.

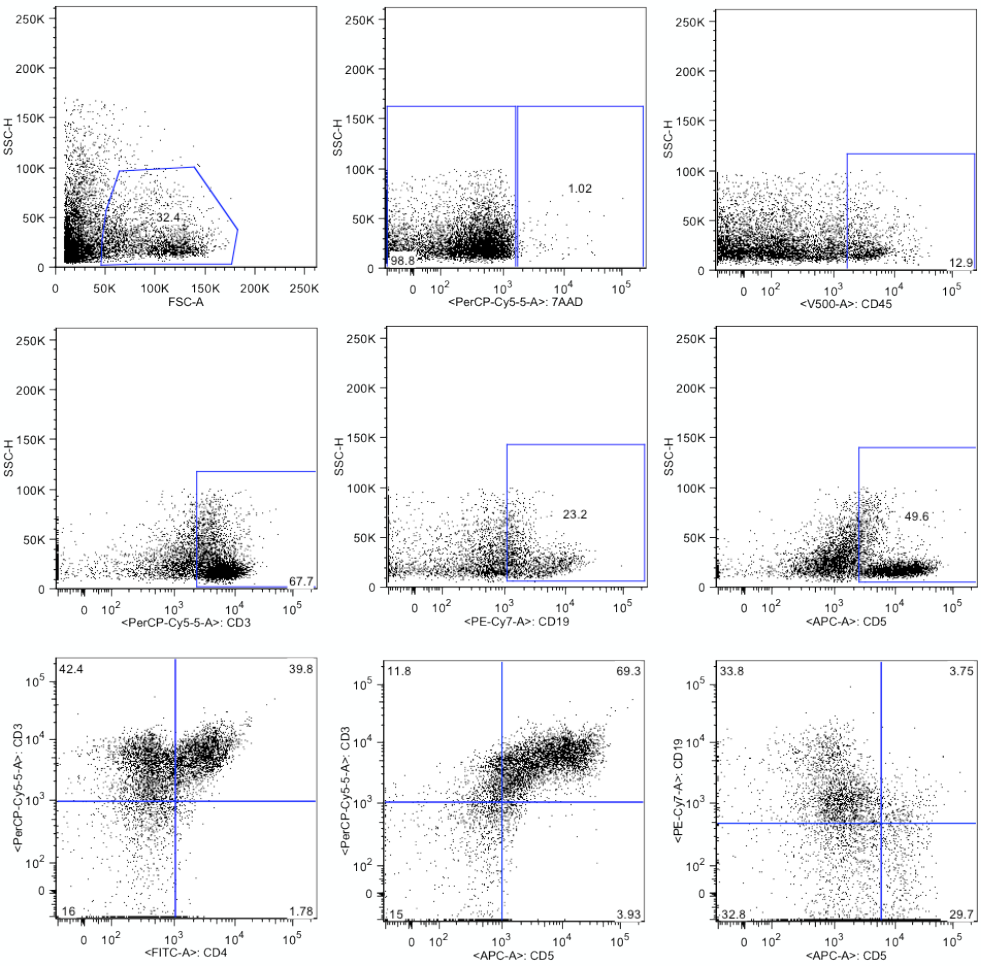

Figure 2. Lymphoid cells obtained following an optimized gingival tissue disaggregation protocol. Dotted image is representative of lymphoid populations from the enriched lymphocyte region. Markers 7-AAD, CD45, CD3, CD4, CD19, and CD5 were used, and in each image, the number of cells that could be quantified and analysed is observed.

PREPARATION OF GINGIVAL TISSUE BIOPSIES FROM HEALTHY SUBJECTS AND SUBJECTS WITH PERIODONTITIS TO OBTAIN CELLS IN SUSPENSION

This protocol describes the preparation of biopsies of gingival tissue obtained from patients diagnosed as healthy or with gingivitis (H/G) and from patients with periodontitis (P) by mechanical disaggregation under magnification (MC-M1232, D. F. Vasconcellos) and the labelling of the tissue for flow cytometry.

MATERIALS

Biopsy of gingival tissue (Support protocol II)
Physiological saline solution
Falcon 15-mL centrifuge tube

Roswell Park Memorial Institute (RPMI) medium with 10% FBS (foetal bovine serum)
Scalpel handle and 15c blade
Tissue clamp
Microsurgery microscope (MC-M1232, D. F. Vasconcellos)
Sterile plastic Petri dish
Centrifuge
Filcon 50-μm filter (BD Biosciences)
Falcon™ polystyrene tubes for flow cytometry
Fluorescence-activated cell sorting (FACS) antibody buffer
IntraStain, Fixation + Perm kit (Buffer A - Buffer B) (DAKO - Agilent Technologies)
FACSFlow™ (BD)
Perm/Wash Buffer™ (BD)

Transport of the sample to obtain cells in suspension:

1. The biopsy specimen is washed with saline to remove excess blood and debris.
2. Transport of biopsy specimen at 4°C in a 15-mL Falcon tube containing 1 mL of RPMI medium with 10% FBS

Disaggregation of the sample to obtain cells in suspension for staining:

3. A microsurgery microscope (MC-M1232, D. F. Vasconcellos) is adjusted to 10× magnification.
4. In a Petri dish with a 15c scalpel blade and tweezers, the tissue is completely disaggregated.
5. The Petri dish is washed with RPMI with 10% FBS as well as the scalpel blade, anticipating that cells from this disaggregation can be lodged there.
6. The washing and disaggregation product is stored in a 15-mL Falcon centrifuge tube and centrifuged twice at 2000 rpm for 10 minutes at 4°C.
7. The entire centrifuged content is filtered through a 50-μm Filcon filter
8. Flow cytometry staining is performed.

Note: First, a Neubauer chamber is used to count the cells, and then the number of events is determined by flow cytometry.

Sample staining (surface markers):

11. Cells are dispensed into three cytometry tubes in equal amounts (labelled for reading of the Treg, Th17, and B lymphocyte phenotypes)
12. An additional tube of unstained cells is placed as a fluorescence-negative control.
13. The four tubes are centrifuged at 2000 rpm for 5 minutes at 4°C.
14. The supernatant is discarded.
15. The cells are resuspended in 50 μL of FACS buffer.

16. Specific antibodies are added for the different surface markers according to the amount previously titrated following the manufacturer's instructions. For each cellular subphenotype, the combinations of antibodies used are as follows:

Regulatory T cells (Treg): CD3 APC-H7 (3 µµL) (BD), CD4 FITC (10 µL) (BD), CD25 PE-Cy7 1 µL (BD), CD127 PE µL1 µL (BD), CD45 V500 (µL3 µL) (BD), 7AAD (µL1 µL) (BD) and intracellular staining antibodies: FoxP3 V450 (5 µµL) (BD), Helios-Alexa Fluor 647 (0.25 µµL) (BD).

Th17: CD3 APC-H7 (µL3 µL) (BD), CD4 FITC (µL10 µL) (BD), CD25 PE-Cy7 (µL1 µL) (BD), CD161 APC (µL10 µL) (BD), RIL-23 PE (µL10 µL) (R&D System), CD45 V500 (µL3 µL) (BD), 7AAD (µL1 µL) (BD).

BL: CD19 PE-Cy7 (µL3 µL) (BD), CD5 APC (µL3 µL) (BD), CD1d PE (20 µµL) (BD), CD3 APC-H7 (µL3 µL) (BD), CD45 V500 (µL3 µL) (BD), 7AAD (µL1 µL) (BD).

17. Each sample is incubated for 15 minutes at room temperature in the dark.
18.1 500 µL of FACS buffer is added.
19.1 The tube is centrifuged at 2000 rpm for 5 minutes at 4°C
20.1 The pellet is resuspended in 200 µL of 4% paraformaldehyde/PBS.

Sample staining (intracellular markers):

18.2 100 µµL of lysis buffer A to lyse the red blood cells and incubate for 15 minutes at room temperature in the dark.
18.2.1 Cells are washed with 2 mL of FACS buffer and centrifuged at 2000 rpm for 10 minutes at 4°C.
18.2.2 The supernatant is discarded and resuspended in 100 µL of lysis buffer B, surface antibody binding buffer, and membrane permeabilizer.
18.2.3 Antibodies for intracellular labelling (FoxP3 V450, Helios-Alexa Fluor) are added and incubated for 20 minutes at room temperature in the dark.
19.2 Two washes are performed each with 2 mL of FACS buffer and centrifuged at 2000 rpm for 5 minutes at 4°C.
20.2 The supernatant is discarded, and the cells are resuspended in 200 µL of buffer per wash.
22. Cells are acquired and read with the BD FACSCanto II™ and FACSDiva acquisition software.

EXTRACTION OF BIOPSIES OF GINGIVAL TISSUE FROM HEALTHY SUBJECTS AND SUBJECTS WITH PERIODONTITIS

This section describes the protocol for obtaining biopsies of gingival tissue from patients diagnosed as healthy or with gingivitis and subjects with periodontitis following the new classification of periodontal disease (Lang & Bartold, 2018; Papapanou et al., 2018).

MATERIALS

Scalpel handle
Bard-Parker® no. 15 scalpel blade
Hu-Friedy® periodontal probe (15 UNC COLOR-CODED)

Sampling

1. Selection of patients who meet the inclusion criteria
2. Probing with the Hu-Friedy® periodontal probe is corroborated after the subject signs informed consent for tissue donation.
3. The sample is collected using the following procedures:
    1. Internal bevel incision to with a no. 15 Bard-Parker® scalpel blade, technique modified from the original by Matsuki et a. (1993), which consists of an internal bevel incision of 2 mm, as deep as possible to obtain junctional or pocket epithelium, without relaxants.

NOTE: In patients diagnosed with periodontitis, the biopsy is obtained from teeth that were to be subjected to open-field root scraping and planing or tooth extraction as indicated by their poor prognosis given their periodontal conditions. The samples of H/G tissue were taken from tooth biopsies, which for aesthetic or functional reasons required an increase in the size of the clinical crown or had an extraction indicated by orthodontic treatment.

REAGENTS AND SOLUTIONS

RPMI with 10% FBS

RPMI (1640 Gibco® Life Technologies) complemented with 10% FBS (Gibco®), with HEPES (Gibco®), 1% L-glutamine (Gibco®), and 1% penicillin-streptomycin (Gibco®).

FACS buffer

PBS with 2% FBS and 0.02% sodium azide

COMMENTS

Context

With the protocols presented in this document, we seek to demonstrate that manual mechanical disaggregation under magnification is the most efficient, reproducible, and viable method for obtaining lymphoid cells in suspension of gingival tissue from healthy patients or with periodontitis for reading by flow cytometry. Therefore, the protocol can be used later in various studies to characterize the lymphoid populations present in scarce tissues, such as gingival and

even peri-implant tissues. It can also be used in other types of tissue that may be affected by the use of enzymes or mechanical means, such as BD Medimachine™, in their processing.

Critical parameters

Before starting the implementation of this protocol, it should be considered that one of its major limitations is the size of the tissue and the previous standardization of the sample collection method, with which all the epithelia available in the gingival tissue were obtained, comprising tissue from the junctional epithelium or pocket epithelium to the oral epithelium. Calibration must be performed for the use of microsurgery. Additionally, a beginning adjustment to the settings of the flow cytometer is recommended.

Solving problems

PROBLEMS WITH CYTOMETRY READING

The standardization of this protocol led us to focus much more on the pre-analytical part of the sample, since with satisfactory disaggregation, quality results that benefit research and the patient can be obtained.

Quantifying the cell suspension is important to determine the state of the tissue after disaggregation.

Adjusting the cytometer settings such as FSC and SSC and including negative and positive controls in each fluorescence reading was important because each cell population can have its own baseline level of autofluorescence. Knowing this allowed us to adjust the settings to a particular population and type of sample, which made the configuration customizable.

Understanding the results

The proposal of this protocol is motivated by the possible continuous interaction of the different enzymes and mechanical means (Medimachine) described in the literature to address the processing of not only gingival tissues but also most other tissues that persistently report a low number of cells in flow cytometry counts and low cell viability. By eliminating these mechanisms (enzymatic and mechanical disaggregation by Medimachine), the above problems were completely eliminated, as 450,000 cells were recovered by flow cytometry counting, of which 97% were viable. From this group of viable cells the populations could be characterized as follows: 4.03% were $CD45^+$, and 60% were alive. The lymphocyte region was 48.4%, and the markers were CD3 68.1%, CD19 60.3%, CD5 9.78%, and CD4 8.27%. A total of 3% of the cells were 7-AAD^+.

Given the results obtained in this initial test, which met expectations, a test was performed with two tissues: one with a diagnosis of periodontitis and two tissues with a diagnosis of H/G under

the same conditions. The results shown below are taken from 7-AAD⁻ cells, that is, viable cells, which made up between 80 and 95%. The numbers are given as a percentage after analysis in FlowJo 8.7. Figure 3 shows the strategy for analysing each cellular subphenotype.

For the regulatory T phenotype:

Patient	Lymphocyte region	CD45+	CD3	CD4	CD4 $CD127^{low}$	CD4 $CD25^{high}$	CD4 FoxP3
P	35,9%	52,8%	45,8%	50,1%	62,7%	12,8%	78,9%
S/G 1	73.7%	9.03%	52%	69.8%	40.8%	20.3%	69.2%
S/G 2	51.7%	29.1%	50.4%	65.9%	29.3%	13.2%	41.7%

For the Th17 phenotype:

Patient	Lymphocyte region	CD45+	CD3	CD4	CD4 CD161	CD4 CD161 rIL-23	CD4 CD25
P	46,1%	58,9%	39,2%	50,5%	39,6%	95,8%	15%
S/G 1	66,3%	14,5%	42,7%	67,2%	32,5%	100%	15,4%
S/G 2	78,2%	18%	46,7%	59,1%	36,4%	100%	22,8%

For B10 or B1a phenotype:

Patient	Lymphocyte region	CD45+	CD3	CD19	CD19 CD5+	CD19 CD5+ CD1d+	CD19 CD1d
P A	69,5%	50%	29,8%	14,9%	44,7%	83,6%	16,4%
S/G 1	79,2%	19%	39,8%	15,4%	28%	39,4%	52,8%
S/G 2	80,7%	19%	39,3%	13,07%	37,9%	37,3%	61,2%

Although it is recognized that it is not possible to make associations to previously published studies, due to the limitations of this study, which focussed on standardization to obtain reliable and reproducible protocols, it is important to emphasize the relevance of conducting a study that

allows to evaluate these cellular subtypes in both healthy periodontium (H/G) and in diseased periodontium (P) to elucidate the regulatory, effector or autoreactive role of the different cellular subtypes.

Time considerations

It is recommended to process the sample immediately after the biopsy; if not, it is possible to wait a maximum of 3 days, storing the biopsy in RPMI with 10% FBS at 4°C.

Labelling with the 7-AAD viability dye is recommended when working with samples obtained from disaggregation in order to determine the percentage of viable cells to be analysed.

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