10x Genomics®

Single Cell Protocols
Cell Preparation Guide
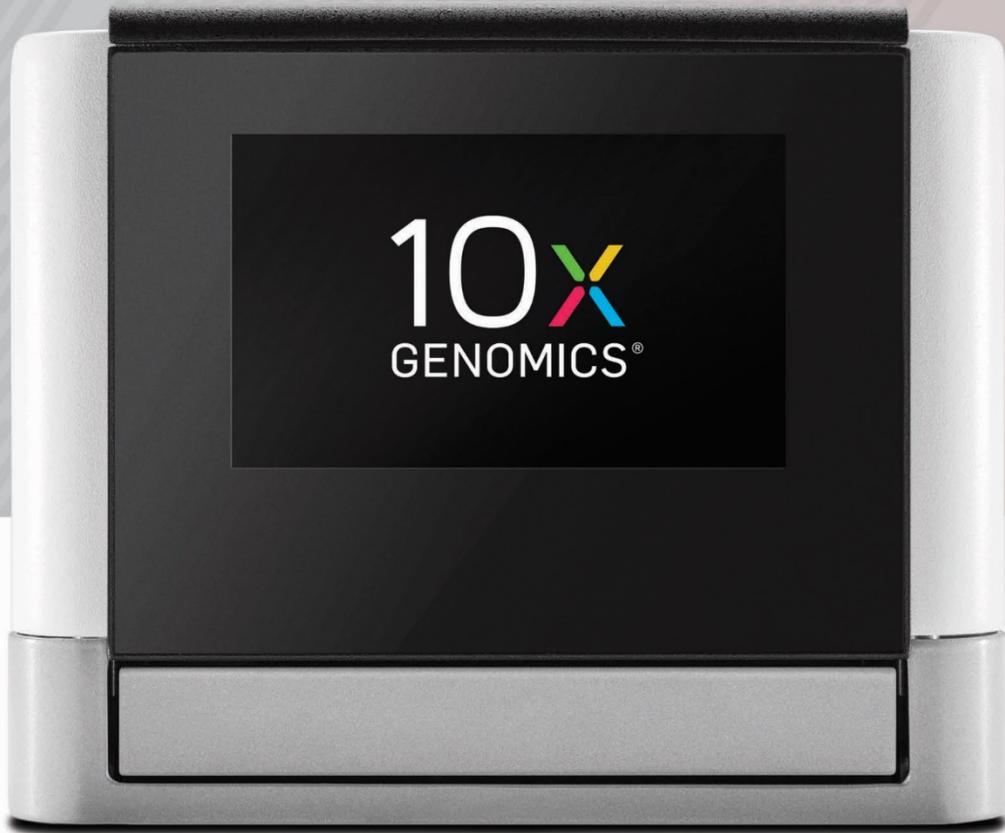
Notices

Manual Part Number

CG00053   Rev C

Legal Notices

Customer Information and Feedback

For technical information or advice, please contact our Customer Technical Support Division online at any time.

Email: support@10xgenomics.com

10x Genomics

7068 Koll Center Parkway

Suite 401

Pleasanton, CA 94566 USA

_____

Table of Contents

Introduction

Introduction

Recommended Reagents & Equipment

Introduction

Single Cell Suspension for Optimal Performance

10x Genomics® Single Cell Protocols require a suspension of viable single cells as input. Minimizing the presence of cellular aggregates, dead cells, non-cellular nucleic acids and potential inhibitors of reverse transcription is critical to obtaining high quality data.

This Cell Preparation Guide describes best practices and general protocols for washing, counting and concentrating cells from both abundant and limited cell suspensions (greater than or less than 100000 total cells, respectively) in preparation for use in 10x Genomics Single Cell Protocols.

The general protocols described here are expected to be compatible with many, but not all, cell and sample types. Additional optimization may be required for the preparation of cell types that are particularly sensitive to suspension composition or handling technique. Preparation of single cells from solid tissues or cryopreserved samples may also require additional dissociation and handling strategies not covered here. For additional information on preparation of specific sample types, consult 10x Genomics Demonstrated Protocols available on the 10x Support site: support.10xgenomics.com.

Recommended Reagents & Equipment

Supplier	Description	Part Number (US)
–	Refrigerated Benchtop Centrifuge for 15 ml and 50 ml tubes	–
	Microcentrifuge for 2 ml LoBind tubes	–
	Inverted tissue culture microscope with 10X/20X magnification and fluorescence imaging capability	–
Rainin	Tips LTS 1ML Filter RT-L1000FLR	17007954
	Tips LTS W-O 1ML Fltr RT-L1000WFLR	17014297
	Pipet-Lite LTS Pipette L-1000XLS+	17014382
VWR	Sterile Polypropylene Centrifuge Tubes with Flat Caps, 50 ml	82018-050
	Sterile Polypropylene Centrifuge Tubes with Flat Caps, 15 ml	21008-103
	10 ml Serological Pipette	89130-898
Integra	PIPETBOY acu 2	155018
Eppendorf	DNA LoBind Tubes, 2.0 ml*	022431048
Corning Cellgro	Phosphate-Buffered Saline (PBS) 1X without calcium & magnesium	21-040-CV
Sigma-Aldrich	Phosphate-Buffered Saline (PBS) with 10% Bovine Albumin (alternative to Thermo Fisher product)	SRE0036
Thermo Fisher Scientific	UltraPure™ Bovine Serum Albumin (BSA, 50 mg/ml) (alternative to Sigma-Aldrich product)	AM2616
	Trypan Blue Stain (0.4%) for use with the Countess® Automated Cell Counter	T10282
	Countess® II Automated Cell Counter†	AMQAX1000
	Countess® II Automated Cell Counting Chamber Slides†	C10228
	Live/DEAD Viability/Cytotoxicity Kit for mammalian cells	L3224
EMD Millipore	Scepter™ 2.0 Handheld Automated Cell Counter†	PHCC20060
	Scepter™ Cell Counter Sensors, 60 µm†	PHCC60050
	Scepter™ Cell Counter Sensors, 40 µm†	PHCC40050
iNCYTO	Hemocytometer C chip†	DHC-N01
Miltenyi Biotec	MACS SmartStrainers, 30 µm	130-098-458
Bel-Art	Flowmi™ Cell Strainer, 40 µm	H13680-0040
ibidi	µ-Slide VI - Flat	80621

*No substitutions are allowed. Items have been validated by 10x Genomics® and are required for Single Cell workflow, training and system operations. †10x Genomics recommends the Countess II Cell Counter for most counting applications, but users should determine the best system for specific sample types.

Tips

1
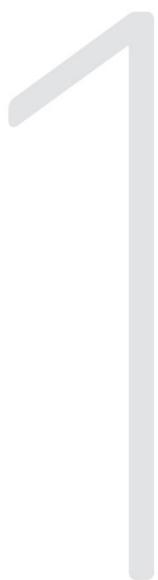
1.   Tips

1.1.   Best Practices for Handling Biological Samples

Best practices for handling any sample type include following all applicable biohazard safety regulations, using sterile technique, nuclease-free reagents and consumables, minimizing pipetting steps, and using wide-bore pipette tips where possible to minimize cell damage.

1.2.   Concentration for Optimal Performance

The total number of cells required in the suspension used as input to 10x Genomics® Single Cell Solutions is determined by the cell recovery target defined by the user. Refer to the applicable 10x Genomics Single Cell Protocol to determine this relationship.

If possible, it is recommended to bring the input cell suspension to a concentration that allows pipetting between 2.5 and 15 µl into each reaction. Using suspension volumes less than 2.5 µl increases variance due to pipetting inaccuracy, while using volumes larger than 15 µl increases the risk of introducing unwanted debris or inhibitors into the reactions. For example, if the total number of input cells required is 1700, then the optimal cell concentration is in the range of ~200 to 700 cells/µl.

The cell concentration should always be determined based on the total number of cells, not just viable cells. Moreover, some loss is inevitable from every washing and resuspension step due to cells adhering to tube walls and pipette tips. It is therefore critical to estimate the number of input cells from the final single cell suspension.

1.3.   Factors Influencing Cell Recovery

To recover the expected number of cells, it is critical to maximize viability, minimize the cell preparation time, accurately measure the input cell concentration and pipette the correct volume into each reaction.

Ideally, input cell suspensions should contain more than 90% viable cells. Non-viable and dying cells generally contain less and more fragmented RNA that may not be efficiently captured by 10x Genomics Single Cell Solutions. The presence of a high fraction of non-viable cells in the input suspension may therefore decrease the apparent efficiency of cell partitioning and recovery.

Depending on cell type, viability may significantly decrease when cells are kept in suspension for a prolonged period of time before use. Some cells types, such as peripheral blood mononuclear cells (PBMCs), can form clumps when they are kept in PBS for an extended period of time. Such clumps are not easily dissociated into single cells by pipette mixing and can therefore decrease the effective concentration of single cells in the input suspension. Cell suspensions should therefore be loaded as soon as possible after preparation, ideally within 30 min.

Clumps, debris and fibers can also interfere with accurate cell counting, especially when using an automated cell counter. Thus, minimization of cell clumps, debris and fibers by filtering or other techniques is necessary for accurate cell counting and loading (see Section 1.7).

1.4.   Regular-Bore versus Wide-Bore Pipette Tips

To minimize physical damage to cells from shearing forces, it is critical to pipette gently and slowly during cell resuspensions. Using roughly treated cells as input will compromise system performance. It is particularly important to pipette cell suspensions gently when using a regular-bore pipette tip as its smaller diameter is more likely to shear and damage cells. The speed at which the cell suspension is passing through the opening of the pipette tip is also an important factor – the faster it passes through, the more damaging the process.

The use of wide-bore pipette tips is therefore recommended for most pipetting steps. However, generating single cell suspensions from pellets or clumps is best achieved using a regular-bore pipette tip. The protocols in this guide therefore call for regular-bore pipette tips when resuspending cells after the final wash step.

1.5.   Centrifugation Conditions

Cell number, concentration, size and density all influence pelleting efficiency at a given centrifugation speed, time, and temperature. Thus, the optimal centrifugation conditions may need to be optimized for specific sample types.

The ideal centrifugation conditions will generate a solid, but not too tightly packed, cell pellet with minimal cells remaining in the supernatant. Minimizing cells in the supernatant is especially important for maintaining an accurate representation of heterogeneous samples that contain cells of variable sizes. Excessive centrifugation may decrease viability. Moreover, a tightly packed cell pellet may require extra pipetting to resuspend the cells, which can also damage cells due to shearing effects.

Recommended initial centrifugation conditions are 150 rcf for 3 min at room temperature for larger cells such as most immortalized cell lines and 300 rcf for 5 min at room temperature for smaller cells such as PBMCs. When working with a new sample type, save and count cells in the supernatants to ensure that losses are low.

1.6.   Washing & Resuspension

The recommended cell washing and resuspension solution is 1X PBS (calcium and magnesium free) containing 0.04% weight/volume BSA (400 µg/ml). BSA is added to minimize cell losses and aggregation. Primary cells, stem cells, and other sensitive cell types may require washing and suspension in alternative buffers to maximize viability. If necessary, PBS can be replaced with most common cell culture buffers.

The following alternative buffers have been verified to not influence performance of 10x Genomics® Single Cell Protocols when profiling mixtures of human 293T/17 and mouse NIH/3T3 cell lines:

- Dulbecco's Phosphate-Buffered Saline (DPBS)
- Hank's Balanced Salt Solution (HBSS)

If cell viability cannot be maintained in one of these buffers, it is also possible to wash and resuspend in most common cell culture media, with or without serum. The following media have been verified to be compatible with 10x Genomics Single Cell Protocols with little or no loss of performance when profiling mixtures of human 293T/17 and mouse NIH/3T3 cell lines:

- Eagle's Minimum Essential Medium (EMEM) + 10% FBS
- Dulbecco's Modified Eagle Medium (DMEM) + 10% FBS

_____

TIPS

- Iscove's Modified Eagle Medium (IMEM) + 10% FBS
- Roswell Park Memorial Institute (RPMI) + 10% FBS
- Ham's F12 + 10% FBS
- 1:1 DMEM/F12 +10% FBS
- M199

When washing and resuspending cells, always use sufficient volumes to maintain concentrations at less than 5000 cells/μl (*e.g.* less than 5 million cells per tube when using 1 ml washing solution in 2 ml LoBind tubes). Maintaining cells at higher concentrations can cause aggregation and clumping that will interfere with generation of ideal single cell suspensions.

1.7.   Straining

GEM generation occurs in microfluidic channels that are narrower than the typical human hair (*i.e.* <100 μm). To ensure optimal system performance, care should be taken to avoid introduction of debris, fibers or clumped cells into these channels. Removing debris and clumps is also important for accurate cell counting with automated counters.

Filtering cell suspensions with an appropriate cell strainer is helpful for removing large clumps and debris. The strainer should have a pore size that is larger than the maximum cell diameter in the sample, but small enough to catch larger clumps.

Depending on the degree of clumping and the strainer type, the number of cells and the amount of wash solution retained in the strainer can vary. For general use, the MACS SmartFilter is recommended as it generally causes minimal changes to the cell concentration. However, a volume loss of 100 μl or more can occur. For low cell suspension volumes, the Flowmi™ Tip Strainer is recommended to minimize volume losses. However, the cell concentration can decrease by 30% or more. It is therefore important to measure the cell concentration after straining for an accurate cell count.

General Sample Preparation

2

2. General Sample Preparation

2.1. Overview

This Protocol is intended as general and initial guidance on straining, washing and concentrating input cell suspensions for 10x Genomics® Single Cell Protocols when working with unfamiliar or uncharacterized cells. Solid tissues and other large cell aggregates must be dissociated using mechanical or enzymatic dissociation, cell sorting, or other cell isolation techniques before proceeding with this Protocol. Further optimization – such as adjusting the number of wash steps, the composition of the wash solution, centrifugation conditions, and type of strainer – may be required to remove ambient RNA and contaminants while maximizing the purity and recovery for a specific cell type (refer to Section 4 for advice on how to assess the concentration and quality of a cell suspension).

The Protocol is best suited for abundant samples for which avoiding cell losses during washing and straining is not critical. If the initial cell suspension is estimated to contain less than 100000 cells in total, refer to Section 3 for recommendations on the preparation of limited samples.

2.2. General Cell Preparation Protocol

NOTE

This Protocol assumes that cells are already in a largely single cell suspension from cell culture, dissociated tissues, cell sorting, or other cell isolation methods.

a) Obtain a vial containing cells of interest in suspension.

b) Using a **wide-bore** pipette tip, gently and thoroughly mix the cells. Determine the cell concentration using a Countess® II Automated Cell Counter or other counting device (see Section 4 for more information on cell counting).

NOTE

Quantification at this point enables an approximation of the appropriate volume for the subsequent resuspension in order to obtain target concentrations. When determining the volume for resuspension, assume ~25% of cells will be lost during wash steps.

c) If significant amounts of cell clumps or debris are observed, gently mix cells by pipetting up and down 10 – 15 times and filter cells using a cell strainer with an appropriate pore size.

d) Centrifuge cells at **300 rcf** for **5 min**.

In general, recommended initial centrifugation conditions are 300 rcf for 5 min at room temperature for smaller cells such as many primary cells, and 150 rcf for 3 min at room temperature for larger cells such as many immortalized cell lines.

NOTE

Larger cells need to be centrifuged at lower speed or for shorter time to preserve the cell integrity. Some very small cells may need to be centrifuged at even higher speeds (e.g. up to 400 rcf) or for longer time (e.g. up to 10 or 15 min) to minimize cell loss due to inefficient pelleting. However, excessive centrifugation speed can compromise the cell integrity and viability.

CRITICAL!

Depending on the rotor type, the cell pellet forms on the side or on the bottom of the conical vial. Know the expected position of the pellet, especially when working with small or limited cells, as the pellet can be difficult to see.

_____

e) Remove supernatant without disrupting the cell pellet. Save the removed supernatant in another tube until the Protocol is complete.

f) Using a **wide-bore** pipette tip, add **1 ml** 1X PBS with 0.04% BSA to the tube. Gently pipette mix 5 times and invert tubes to resuspend the cell pellet.

g) Centrifuge cells at **300 rcf** for **5 min**.

h) Remove supernatant without disrupting the cell pellet. Save the removed supernatant in another tube until the Protocol is complete.

i) Add the appropriate volume of 1X PBS with 0.04% BSA to achieve the target cell concentration. Gently pipette mix 10 – 15 times or until the cells are completely suspended.

NOTE

Do not invert the tube in this step as cells can stick to the sides of the tube, thereby changing the cell concentration.

j) Use a cell strainer to remove any remaining cell debris and large clumps. For low cell suspension volumes, a Flowmi™ Tip Strainer is recommended for minimal loss of additional sample volume.

k) Determine the cell concentration using a Countess® II Automated Cell Counter or other cell counting device.

NOTE

If cell concentration is much lower than expected, check the concentration of saved supernatants and re-pellet the supernatant at a higher centrifugation speed. It is possible that cells were not efficiently pelleted at the initial speed or were lost to the cell strainer.

l) If necessary, adjust the volume to obtain the target cell concentration.

m) Once the target cell concentration is obtained, place the cells on ice.

n) Proceed with the 10x Genomics® Single Cell Protocol.

_____

Preparation of Limited Samples

3

3. Preparation of Limited Samples

3.1. Overview

This Protocol outlines how to process cells when cell number is <100000 in order to maximize the recovery yield while minimizing the cell damage. It is important to handle cells as little as possible and save all the supernatants until the Protocol is complete. Washing the cells once is recommended, but it may be possible to skip washing completely if the number of available cells is very small and there is little visible debris in the suspension. If cells are unstable in PBS, the buffer can be replaced with most common cell culture buffers and media with minimal reduction in performance (Refer to Section 1.6 for more information).

3.2. Limited Sample Preparation Protocol

NOTE

This Protocol assumes that cells are single cells suspended from tissue dissociation, cell isolation, or FACS-sorted into PBS, other buffers, or media.

a) Using a **wide-bore** pipette tip, gently and thoroughly mix the cells. Determine the cell concentration using a Countess® II Automated Cell Counter or other cell counting device (see Section 4 for more information on cell counting).

NOTE

Quantification at this point enables an approximation of the appropriate volume for the subsequent resuspension in order to obtain target concentrations. When determining the volume for resuspension, assume ~25% of cells will be lost during wash steps.

b) If cell clumps or cell debris are observed, gently mix cells by pipetting up and down 10 – 15 times and filter cells using a cell strainer with an appropriate pore size.

c) Centrifuge cells at **300 rcf** for **5 min**.

NOTE

Some cells need to be centrifuged at higher speed (e.g. up to 400 rcf) or for longer time (e.g. up to 10 or 15 min) to minimize cell loss due to inefficient pelleting. However, be careful not to over-centrifuge as increased centrifugation speed can compromise the cell integrity and viability.

CRITICAL!

Depending on the rotor type, the cell pellet forms on the side or on the bottom of the conical vial. Know the expected position of the pellet, especially when working with small or limited cells, as the pellet can be difficult to see.

d) Remove the supernatant without disrupting the cell pellet, leaving ~**50 µl** or an appropriate volume of supernatant to achieve the target cell concentration. Save the removed supernatant in another tube until the Protocol is complete.

e) Using a **regular-bore** pipette tip, resuspend the cell pellet in the leftover supernatant by gently pipetting up and down 10 – 15 times.

NOTE

If losing more cells can be tolerated, it is recommended to wash cells at least once before counting to remove any ambient RNA and contaminants. For washing, add 1 ml PBS with 0.04% BSA, mix gently, and repeat steps c – e.

f) Determine the cell concentration using a Countess® II Automated Cell Counter or other cell counting device.

_____

If cell concentration is much lower than expected, check the concentration of saved supernatants and re-pellet the supernatant at a higher centrifugation speed. It is possible that cells were not efficiently pelleted at the initial speed.

g)   If necessary, adjust the volume to obtain the target cell concentration.

h)   Once the target cell concentration is obtained, place the cells on ice.

i)   Proceed with the 10x Genomics® Single Cell Protocol.

_____

Cell Counting & Quality Control

4

4. Cell Counting & Quality Control

4.1. Overview

Visualization of cell suspensions is critical for accurate determination of cell concentration and viability, suspension quality and cell sizes prior to use in 10x Genomics® Single Cell Solutions.

To determine accurate cell counts, best practices include sampling the cell suspension at least twice and at least two counts on each sample (i.e. a minimum of four counts in total, based on two independent draws from the cell suspension).

The Countess® II Automated Cell Counter is recommended for determining cell concentrations for most applications. Sample types with very small cells, highly variable cell sizes or high levels of aggregation may require alternative counting methods.

When characterizing a sample type for the first time, it is recommended to perform two different counting and viability assays. The first method is to stain cells with trypan blue and count the cell concentration and viability using the Countess® II (Section 4.2). The second method is to stain cells with fluorescent dyes and measure cell viability using a tissue culture microscope and automated cell counting software (Section 4.4). The latter method is particularly useful for accurate characterization of very small and heterogeneous cell populations such as PBMCs. If a tissue culture microscope and imaging solution is not available, manual counting using a hemocytometer can also be a useful control.

Cell suspensions should also be checked visually for debris or cell aggregates as these can clog microfluidic channels. If observed, additional pipetting or filtering may be required to obtain optimal performance. Pipetting should be done gently and carefully to avoid lysing or otherwise damaging cells. In general, filtering or straining does not decrease cell viability.

Visual estimation of cell size (diameter when in suspension) can also be useful for setting expectations about relative cDNA yields and transcript counts from different sample types. The number of distinct cDNA molecules captured from a cell is correlated to its RNA content. In general, the RNA content of a cell correlates with its size. Thus, larger cells are expected to yield more cDNA than smaller cells. For example, 1200 NIH/3T3 cells (average cell diameter = ~16 μm) contain significantly more total RNA (~16 pg/cell) than 1200 PBMCs (mean diameter = ~8 μm, ~1 pg/cell), which means that the former are expected to yield significantly more cDNA and higher complexity libraries.

_____

4.2.   Using a Countess® II Automated Cell Counter

NOTE   *Refer to the manufacturer's user guide for details and advice on the operation of the Countess® II Automated Cell Counter. The optimal range of cell concentration for this device is 100 to 4000 cells/µl.*

a)   Thoroughly vortex 0.4% trypan blue stain and centrifuge briefly.

b)   Using a **wide-bore** pipette tip, gently and thoroughly mix the cells.

NOTE   *It is critical that the cell suspension is homogeneous to minimize sampling error. Also ensure that cells are free of debris and fibers as these can interfere with cell counting.*

c)   Immediately, aliquot **10 µl** cells and mix with **10 µl** 0.4% trypan blue stain. Gently pipette mix.

d)   Transfer **10 µl** trypan blue stained cells to a chamber on Countess® II Cell Counting Slide.

e)   Insert the slide into the Countess® II Cell Counter, and determine the cell concentration and cell viability.

NOTE   *Optimization of focusing and light exposure are critical for accurate counting.*

4.3.   Using Other Automated & Manual Cell Counters

The following table summarizes the advantages and disadvantages of the Countess® II and two alternative methods: the EMD Millipore Scepter™ Handheld Automated Cell Counter and manual hemocytometers.

Instrument	Advantages	Disadvantages	Cost	Cell Size Range
Countess® II	Fast Automated	Cannot measure viability of very small cells	$$	7 – 60 µm
Scepter™	Fast Automated	Limited dynamic range Needs multiple sensors for different cell sizes Cannot visualize cells	$$	3 – 18 µm 8 – 25 µm
Hemocytometer	Flexible	Slow, manual	$	Any

4.4.   Using Fluorescent Viability Stains & Microscopy

The Thermo Fisher Scientific LIVE/DEAD® Viability/Cytotoxic kit for mammalian cells is recommended for measuring viability of mammalian cells. Refer to the manufacturer's user guide for detailed instructions and advice. Other kits or dyes may require fluoresence filters with alternative excitation and emission ranges.

Prepare fresh staining solution immdiately before use.

a)   Thaw Calcein AM (4 mM) and Ethidium homodimer-1 (EthD-1, 2 mM) tubes to room temperature.

b)   Vortex both tubes and briefly centrifuge.

c)   Add **2 μl** EthD-1 to **100 μl** 1X PBS (calcium, magnesium free) and vortex for **5 sec**. Centrifuge briefly.

d)   Add **1 μl** Calcein AM to the tube containing EthD-1 and vortex. Centrifuge briefly.

e)   Using a **wide-bore** pipette tip, gently and thoroughly mix cells.

f)   Immediately, aliquot **15 μl** cells and mix with **15 μl** fluorescent staining solution. Pipette gently to mix.

The volume can be adjusted down to 5 μl of cells and 5 μl of staining solution (1:1 ratio).

g)   Load **25 μl** (or less) of the stained cells to a slide (*e.g.* ibidi μ-Slide VI - Flat).

h)   Wait ~**5 – 10 min** for Calcein AM to be digested and cells to settle down in the slide.

i)   Image cells using a microscope with a 10X or 20X objective (See Section 4.5).

4.5.   Microscopy & Automated Image Analysis

This Protocol describes the use of a Nikon Ti-E (Eclipse) inverted microscope with specific objectives and fluorescence imaging capabilities. Any equivalent microscope solution can be used as an alternative.

Microscope Settings

- Nikon Ti-E (Eclipse) inverted microscope
- 10X or 20X magnification
  - The majority of a chamber can be imaged with ~5 images under 10X magnification (scrolling along the length of the chamber after each image)
  - About half of a chamber can be imaged with ~7 images under 20X magnification (scrolling along the length of the chamber after each image)
- Light source for fluorescence microscopy (mercury vapor lamp or xenon arc lamp)
- Filter cubes (excitation/emission ranges):
  - Nikon B-2E/C (λ 465-495 / λ 515-555), or equivalent, for live stain
 - Nikon G-2E/C (λ 528-553 / λ 590-650), or equivalent, for dead stain

Microscope Imaging

a) Place the ibidi® μ-Slide onto the microscope and focus under bright field illumination.

b) Switch to the Nikon B-2E/C (λ 465-495 / λ 515-555) filter cube, or equivalent, for the live stain and re-focus if necessary.

c) Save an image of the field of view.

d) Switch to the Nikon G-2E/C (λ 528-553 / λ 590-650), or equivalent, for the dead stain and save an image in the field of view.

e) Repeat steps b – d until the entire chamber has been imaged.

_____

Automated Counting of Live/Dead Cells Images Using Image-J or Fiji

This sequence of steps allows cells that have been stained with the Live and Dead stains (Section 4.4) to be counted automatically, using ImageJ (http://imagej.net) or Fiji (http://fiji.sc). Open each pair of images for a given field of view, one in the Live wavelength and the other in the Dead wavelength, and apply the following steps to both:

a) Select Image -> Type -> 8-bit

b) Select Image -> Adjust -> Threshold

c) Recommended threshold settings for Dead and Live channel images settings are shown below. Depending on the intensity of the fluorescence signal captured in the images, the lower threshold may need to be adjusted. Increase the threshold to avoid retaining background fluorescence (for high intensity) and decrease the threshold to avoid the loss of cells (for low intensity).

Dead   Live
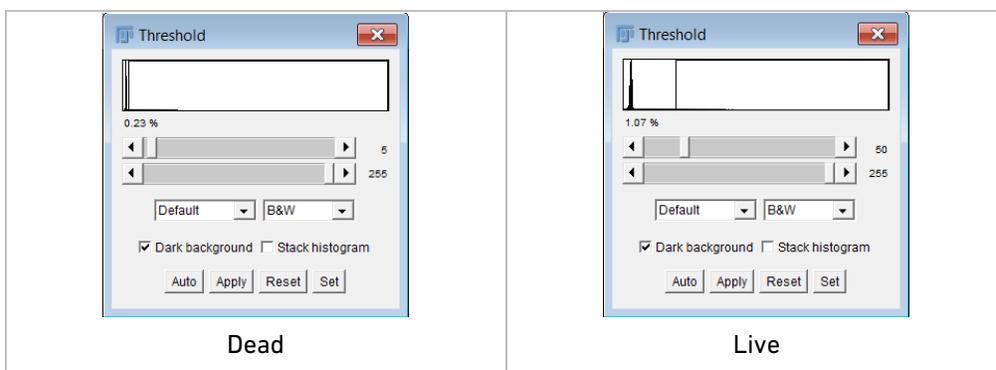
d) Select Process -> Binary -> Make Binary

e) Select Process -> Binary -> Open

f) Select Process -> Binary -> Fill Holes

g) Select Process -> Binary -> Watershed

h) Select Analyze -> Analyze Particles

i) Use the analyze particles settings shown below for the automated counting of cells. Record the number of cells counted in each image, found in the Results window.
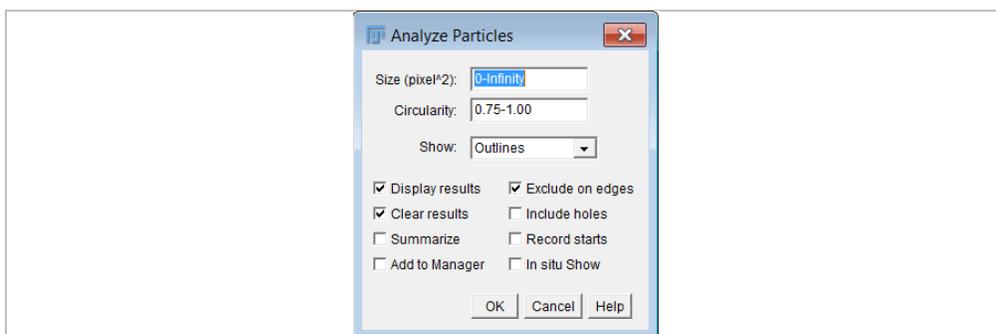
j) To determine cell viability %, divide number of live cells by the sum of live and dead cells and multiply by 100.

_____