# STANDARD OPERATING PROCEDURE

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| **SOP Number** | **3** | |
| **SOP Title** | Spheroid dissociation and cell counting method | |

| | NAME | TITLE | SIGNATURE | DATE |
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| **Reviewer** | | | | |
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## 1. PURPOSE

This protocol stems from the need to evaluate the cellularity of the 3D model developed as it must reproduce the cellularity of the corresponding tissue or tumour.

In addition, spheroids of different stage of growth could be examined with this method in order to correlate their size and dimension with the number of single cells after spheroid dissociation.

This method could be useful in order to obtain a good evaluation of the number of cells. Moreover, offers the advantage of taking the spheroid and dissociate it into single cells inside a capillary tube without any transfer, avoiding losing the cell content.

## 2. SCOPE

This protocol describes how to dissociate and count single cells from one spheroid harvested with a capillary tube.

## 3. DEFINITIONS

*Spheroid:* A 3D cell culture with an artificially created environment in which biological cells are permitted to grow or interact with their surroundings in all three dimensions.

## 4. MATERIALS

Capillary tube - *BRIS micro-haematocrit tubes* (1mm diameter)

Petri dishes 3.5 cm

Trypsin 1X

Specific medium for the 3D cell culture

Ultrasonic Bath – *VWR*

Parafilm

*Menzel-Gläser* - cover glass (24 x 40 mm)

Microscope slide

## 5. SPECIFIC PROCEDURE

1. Harvest spheroids from the culture (if cultured in a microstructured device, harvest spheroids by pipetting gently up and down in order to detach them from the surface without damaging)

2. Place spheroids in a 3.5 cm dish with 1.5 ml of fresh medium

3. Observe the spheroid culture under the microscope and with a p1000 micropipette (since is necessary to use a tip with a large diameter) collect one single spheroid from the culture, <u>making sure to collect the least amount of medium.</u>

4. Place the drop of medium containing the spheroid in an empty 3.5 cm dish as showed in the following image:
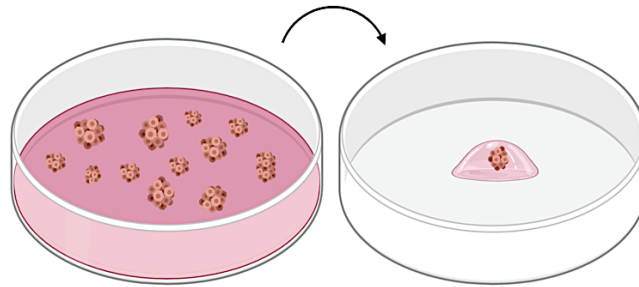
5. Add to the drop of medium 20µl of Trypsin 1X making sure that the spheroids is in contact with the solution

6. Connect the tip of a p200 micropipette to a small silicone tube and, on the other side of the tube, connect the capillary as showed in the following image:



silicon tube          capillary
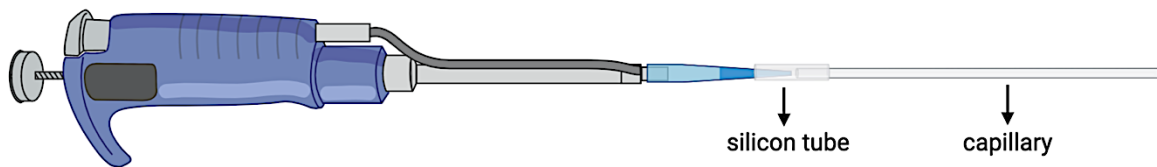
7. Harvest the spheroid under the microscope. In order to collect the least amount of medium, set the pipette at 20µl and then use the friction ring to slowly collect the spheroid

   *Note: do not use directly the pipette plunger since it will cause an excessive and less precise medium aspiration*

8. Once collected the spheroid, use the pipette friction ring to place the medium in the middle part of the capillary

9. Place a small cap made of parafilm to the capillary end

10. Slowly, remove the capillary from the silicon tube and cover the other end with parafilm cap

## 6. SPHEROID OBSERVATION TOOL

1. With instant glue paste two small plastic rectangles in the slide edges (the width of the plastic supports must allow the insertion of the capillary)

2. Stick on the plastic supports a cover glass (24 x 40 mm)

3. Once glue has dried, insert the capillary in the small compartment between the slide and the cover glass

4. Add a small amount of water in the compartment in order to immerge the capillary middle part containing the medium as illustrated in the following image:

plastic support

cover glass

parafilm cap
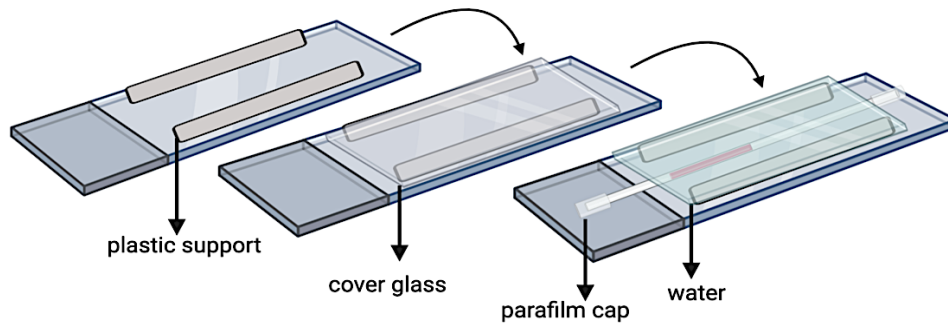
water

## 7. INTACT SPHEROID MAGE ACQUISITION

1. Position the mentioned above slide support under the microscope.

2. The capillary outer part must be immersed in water, specifically in the area where the spheroid has been collected

3. Check the entire length of the capillary to be sure to have collected one single spheroid

4. Acquire different images of the spheroid, gently rotating the capillary without moving the support. This will allow, then, to estimate the volume of the intact spheroid
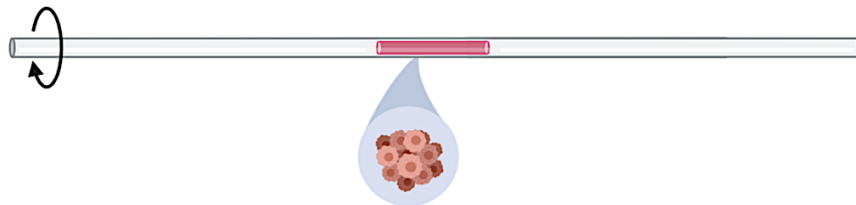


5. Dry the water contained in the slide support with absorbent paper and then remove the capillary taking care not to remove the parafilm caps

## 8. CAPILLARY SONICATION

1. Place the capillary in a ultra sonicator bath, it must be completely immersed in water, check that parafilm caps completely cover the capillary ends

2. Perform a **5 minutes** sonication

3. Check the spheroid dissociation under the microscope

4. Repeat the sonication if the spheroid has not been dissociated properly

## 5. DISSOCIATED CELLS MAGE ACQUISITION

1. Choose one side of the capillary tube to start with, generally 20X objective allows obtaining the least image number, being able to recognize and distinguish the capillary contained elements

2. Observe the capillary length and start acquiring the first image once observed dissociated cells as showed in the following image:
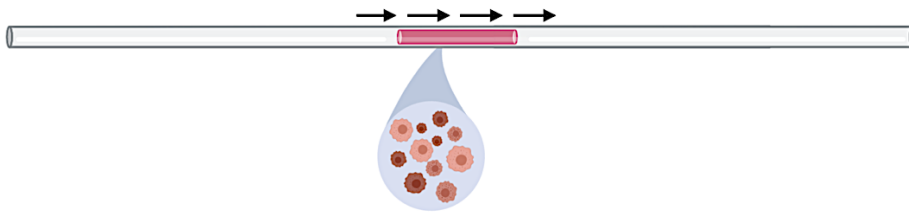


3. Acquire more images from different focal planes of the same point of the capillary tube (ex. starting with images of cells in focus positioned at the capillary edges up to those in the center)

4. Name images with sequential numbers so that they can be re-ordered

5. While acquiring sequential images of the capillary, remember that the first edge of the second image must show the last edge of the first one. This is important in order to have a good image processing with ImageJ software

## 6. CELL COUNTING METHOD RECAPITULATION
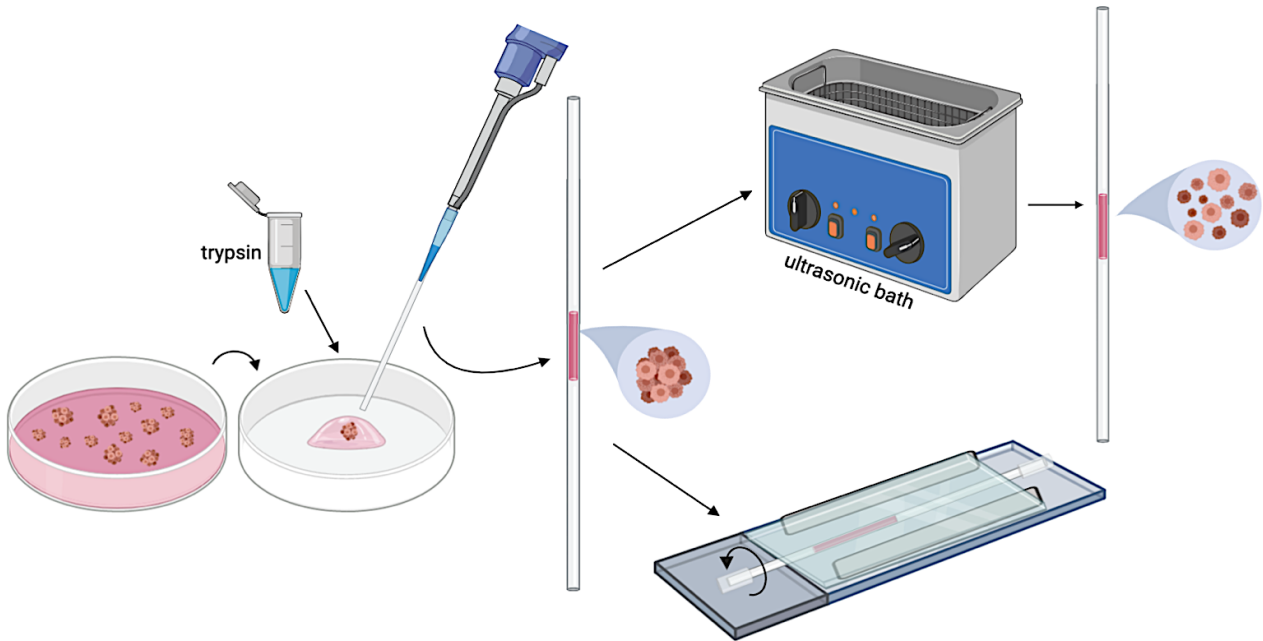
## 5. IMAGE PROCESSING (*FIJI IMAGEJ SOFTWARE*)

1. Import image files

2. Open first images obtained from the same point of the capillary tube

3. **Image > Stacks > Images to Stack** (multiple images will be converted in a single stack image).

4. **Plugins > Stack Focuser**

   *Options setting: enter the (n>2) for n x n kernel; enter 100 value*

   *Note: The plugin is a very simple algorithm of patching a "focused" image from a stack of images corresponding to different focal planes. Plugin provides optional generation of an 8-bit greyscale "height" map, i.e. image of heights at which focused elements are located. It is very important that images in the stack are of the same brightness; otherwise pasting together pieces of different images will create artificial edges*

5. Name the obtained image and save it as **.tiff** file

6. Once obtained two images of two sequential capillary segments, **crop** the scale bar, if present

7. **Plugin > Stitching** (if < or = 2 Pairwise Stitching)

   *Note: The Stitching Plugin is able to reconstruct big images/stacks from an arbitrary number of tiled input images/stacks. The Pairwise Stitching first queries for two input images that you intend to stitch. They can contain rectangular ROIs which limit the search to those areas, however, the full images will be stitched together. Once you selected the input images it will show the actual dialog for the Pairwise Stitching. The*

*dialog will depend on the dimensionality of the input images. Please note that RGB input images will be converted into 8-Bit composite images*

8.  In the Pairwise Stitching dialog select ***Linear Binding*** as fusion method. Do not change other default options in the dialog

## 6. AUTOMATIC CELL RECOGNITION AND COUNTING

1.  Start with a saved **Stack Focused Image** (*.tiff*) of the first part of the capillary segment

2.  **Image > Crop**  for removing all the image outlines

    *Note: in order to perform properly the automatic cell counting it will be necessary to crop all the image edges even if are present single cells which will be counted in a second moment manually to estimate the final cells number*

3.  **Image > Adjust > Brightness/Contrast** in order to increase contrast, distinguish single cells from the background and to make visualization of the image easier

4.  **Process > Filters > Mean** the pixel is replaced with the average of itself and its neighbours within the specified radius. Use the 2.0 radius

5.  **Image > Adjust > Auto Local Threshold**

    *Note: This plugin binarises 8-bit images using various local thresholding methods. 'Local' means that the threshold is computed for each pixel according to the image characteristings within a window of radius r (in pixel units) around it.*

6.  **Method** selects the algorithm to be applied

7.  Use **Bernsen** method as in the following dialog window

    *Note: The method uses a user-provided contrast threshold. If the local contrast (max-min) is above or equal to the contrast threshold, the threshold is set at the local midgrey value (the mean of the minimum and maximum grey values in the local window). If the local contrast is below the contrast threshold the neighbourhood is considered to consist only of one class and the pixel is set to object or background depending on the value of the midgrey.*
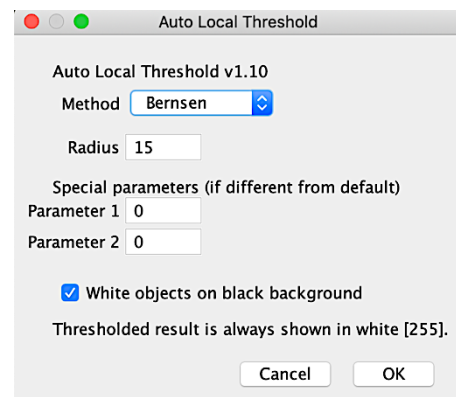
8. **Analyze > Analyze Particles** This will provide information about each particle in the image.

9. **Size:** choose the most suitable size range of the cells to be detected

10. **Circularity:** 0.4 - 1.00

    *Note: Set the minimum size and maximum pixel area size to exclude anything that is not an object of interest in the image. Roundness values between 0.0 and 1.0 can also be selected to help exclude unwanted objects. Select the Show: Outlines option to display an image of the detected objects.*
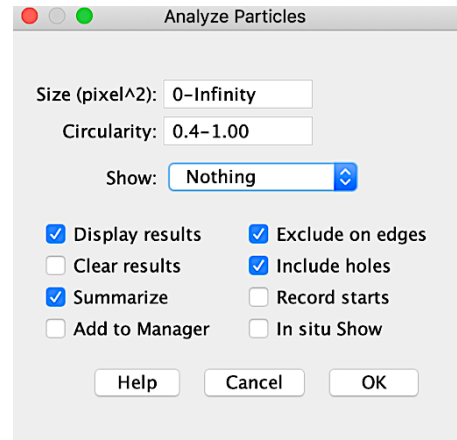
Once estimated the number of cells from the automatic count, use:

**Plugins › Analyze › Cell Counter › Cell Counter** to count cells present on the image edges that have not been counted with the mentioned before method

## 1. INTERNAL AND EXTERNAL REFERENCES

https://imagej.net

## 2. CHANGE HISTORY

| SOP no. | Effective Date | Significant Changes | Previous SOP no. |
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