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Sample Preparation Manual for the 3D Cell Explorer

Contents

1. Important facts for the sample preparation

A. Cells need to be seeded on a glass surface as thin as a coverslip – we suggest either a glass bottom culture dish or on coverslip later mounted on a glass slide.

B. The instrument is suitable for imaging any kind of eukaryotic or prokaryotic cells. Thin tissue slices can be imaged as well.

C. There are no limitations in the cell confluency. Cells grown in monolayer or multilayers can be observed unless it exceeds the maximum thickness of sample structure (see specification table 1 and 2).

D. Optically transparent mounting media with a refractive index close to those shown in living material (RI~1.35) (i.e. live cell imaging solutions, PBS) are preferred. Slightly scattering medium (like culture medium with red phenol) can be used as well. Please be aware of the maximum medium volume (see specifications table 2).

E. The optical surfaces should be as clean as possible and cell holders should be carefully cleaned as to avoid that any kind of debris or remains are floating in the mounting medium.

2. Specifications overview

Specification table 1

Microscopy Slide		
Conditions	**Specification for 3D Cell Explorer**	**Comments**
Coverslip thickness	170 +/- 20 µm	www.hirschmannlab.com
Max. thickness of sample structure	30 µm	Suggested thickness: 5-10 µm Without paraffin Water based mounting medium (1.33 – 1.44 RI)

Specification table 2

Culture dish		
Conditions	**Specification for 3D Cell Explorer**	**Comments**
Glass bottom culture dishes diameter	35 mm or larger	http://nanolive.ch/wp-content/uploads/nanolive-ibidi-labware.pdf
Max. thickness of sample structure	30 µm	See suggested mounting media in section 3.3
Max. volume using optically clear medium	1,5 ml (in 35mm dish)	Depending on optical properties
Max. volume using scattering medium	1 ml (in 35mm dish)	List of tested media: Agar or agarose hydrogel

3. Sample preparation

The 3D Cell Explorer allows to measure the internal components of living cells offering the researcher the possibility to acquire high resolution 3D images within seconds. The instrument is suitable for any kind of eukaryotic or prokaryotic cells. Thin slices of tissues can be observed, too. Moreover, thanks to the extremely low power of the laser source and the compatibility with cell culture accessories (see section 5), the 3D Cell Explorer is suitable for long-term live cell imaging. This manual has the aim to guide researchers step by step through the sample preparation methods.

3.1 Compatible disposables

Imaging with the 3D Cell Explorer requires clean optical conditions throughout the whole optical system, which comprises the microscope and the sample. This means that the disposables' quality is crucial for optimal imaging results.

3.1.1 Microscopy slides and coverslips

The 3D Cell Explorer is compatible with samples mounted on optically transparent glass slides and coverslips. These glass disposables should have a thick bottom between 130-170 µm.

Recommended slides: Slide SuperFrost (e.g. BioSystems)
Recommended coverslips: Hirschmann (http://www.hirschmannlab.com/)

3.1.2 ibidi labware

The system is compatible with the following ibidi labware: dishes, multi-well chambers and channel slides.

Multi-well chambers:

- µ-Slide 2 Well
- µ-Slide 18 Well – Flat

Channel Slides

- µ-Slide I Luer
- µ-Slide y-shaped
- µ-Slide III, 3in1
- µ-Slide III 3D Perfusion
- µ-Slide VI – Flat
- µ-Slide VI 0.5 Glass Bottom

Microscopy dishes

- µ-Dish 35 mm, low
- µ-Dish 35 mm, high
- µ-Dish 35 mm, high Glass Bottom
- µ-Dish 35 mm, low Grid-500
- µ-Dish 35 mm, high Grid-500
- µ-Dish 35 mm, high Grid-500 Glass Bottom

Warning:

- During the 3D image acquisition process, use ibidi labware without lid.
- Please read our "ibidi labware compatibility report" (http://nanolive.ch/supporting-material/) before proceeding with your sample preparation.

Please note:

- Please visit "http://ibidi.com/nanolive-3d-cell-explorer/149-nanolive-3d-cell-explorer.html" to get more information about their compatible labware and possible applications.
- Thanks to the ibidi Free Sample Program you will have the possibility to choose up to 3 ibidi products as a free sample for testing.
- Only the above-mentioned disposables are guaranteed. If you want to use any other kind of disposables, please contact Nanolive's customer service (support@nanolive.ch) for more information.

3.2 Cell Confluency

In sample preparation, no limitations in the cell confluency are given. Generally, the best image quality results can be obtained by imaging single cells or low confluent cell culture.

Cells grown in monolayer or multilayers can be observed as well, unless the maximum thickness of sample structure is exceeded (see specification table 1). Beyond this thickness limitation, low quality signal alert or failure message could appear.

3.3 Imaging medium

As a general requirement, any type of sample should be embedded in liquid mounting medium (e.g. PBS, culture medium, mounting solution etc.) during the imaging acquisition.

Dry samples cannot be imaged.

In order to have a high-quality image the liquid mounting medium should be optically clear, (i.e. no optical scattering of the laser beam).
Best are transparent liquids like:

- Live cell imaging solution specifically developed for live cell imaging applications (i.e. FluoroBrite DMEM Media A1896701).
- Tris-based buffer
- PBS

However, solutions with red phenol showed to be suitable for the image acquisition when a minimum amount of liquid is used to fully cover the bottom of the dish (not recommended).

Warning:

- The amount of liquid inside the culture dish should be enough to fill the entire bottom surface.
- High level of liquid could affect the acquisition quality, the mounting medium level should not exceed half of the dish height.

3.3.1 Suggested mounting media for living cells

The Refractive Index (RI) value of mammalian cells is similar to water based solutions with a range between 1.33 – 1.44 RI. Mounting media with RI values in this range are suitable for obtaining good quality acquisitions.

A list of compatible mounting media is proposed directly on STEVE software before the 3D acquisition takes place, as shown in Figure 1.

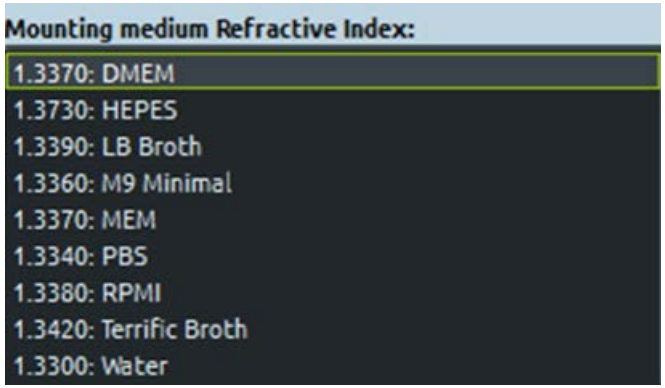


Figure 1. List of common mounting media RI values

Few other different biological samples (e.g. vegetal cells, fungi etc.) could present RI values out of this range. To obtain high quality images for these samples, the mounting medium RI value should be as close as possible to the RI components of the biological sample.

Example: By mixing different percentages of glycerol and PBS, it is possible to obtain a clear optical mounting medium with a specific RI.
The RI value graphic is reported on the Appendix chapter 6.

Please note that increasing glycerol concentration can be toxic for living cells.

3.3.2 Suggested mounting media for microscopy slides

- PBS (1.334 RI)
- Fluoromount-G Southern Biotech Assoc. (1.389 RI)
- ibidi Mounting Medium (1.42 – 1.44 RI)
- 75% glycerol/distilled water (1.44 RI)
- ProLong® Gold Antifade Mountant
  - Fresh (1.39 RI)
  - Cured for 1 day (1.40 RI)
  - Cured for 4 days (1.44 RI)

Please note:

- These solutions discussed in this manual serve as a starting point to establish and perfect your own preparation protocols.
- Not water-based mounting media are generally not compatible.
- OCT/paraffin are not fully transparent and they could interfere with the acquisitions, please remove the OCT or paraffin from the tissue sections before mounting the slide (please check section 3.5.3 for more details).

3.4 General rules for sample preparation and imaging

We are going to present in this section few key steps to prepare the three main sample categories:

- adherent live or fixed cells on ibidi dishes
- not adherent cells on ibidi dishes
- fixed cells on coverslips
- tissue slides

3.4.1 Adherent live or fixed cells on ibidi dishes

Please note: the following steps are optimized for ibidi dish 35mm_low borders (http://ibidi.com/dishes/14--dish-35-mm-high-ibitreat.html) please rescale the liquid quantities in case of different disposables.

for **fixed cells**:

1) Coat the culture dish with the minimum amount of coating material if needed (http://ibidi.com/img/cms/support/AN/AN08_Coating.pdf);
2) Seed cells according to ibidi procedure (http://ibidi.com/img/cms/products/labware/open_standard/D_801XX_Dish_35mmlow/IN_8013X_Dish35mm_low.pdf) in the appropriate culture medium for your cell line (see section 3.3.1);
3) Culture cells until they reach the desired confluency (<80% suggested);
4) Wash your cells with warm (37 °C) PBS and proceed with next fixation step in less than 30 seconds to limit the stress on the cells;
5) Fix cells with 1ml of 4%PFA and incubate for 15 minutes at room temperature; then wash 3 times with 1ml of PBS;
   Warning: Do not use methanol for fixation!
6) Cover the cells with the suggested amount of mounting medium solution (figure 1);
7) Proceed to image the sample or store it at 4°C (Ideally the acquisition is done just after).

for **living cells**:

Repeat the 1 - 3) steps described in the section 3.4.1

4) Exchange the culture medium if necessary with the suggested amount of pre-warmed living cell imaging medium.
5) Proceed to image the sample or store it in the incubator at 37°C.

Please note: The cells can be directly observed in a dish designed for observation after lid removal.

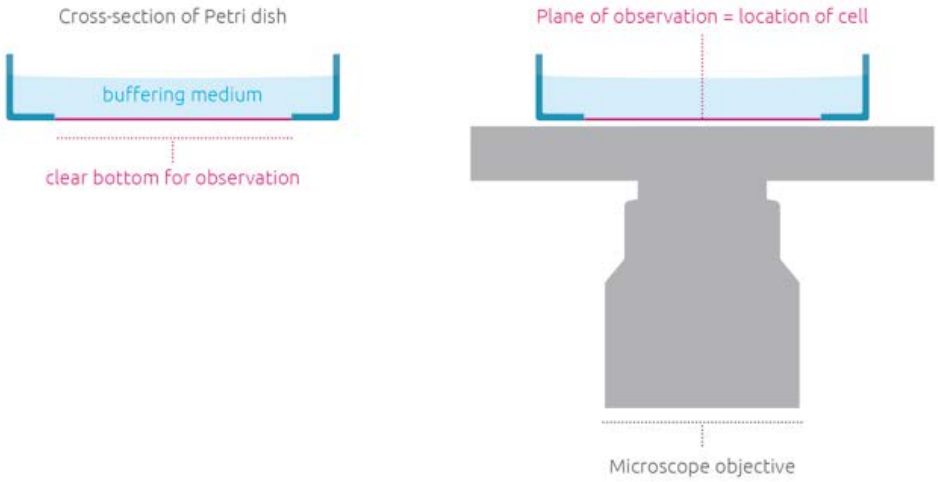


Figure 2. Schematic of observation of cells with the 3D Cell Explorer located in dish.

3.4.2 Not adherent cells on ibidi dishes

Please note: the following steps are optimized for ibidi dish 35mm_glass bottom (http://ibidi.com/labware/185-glass-bottom-dish-35-mm.html)

Not adherent cell culture (i.e. bacteria, yeast, red blood cells etc.) can be imaged in ibidi dishes as well.

1) Fill the dish with the recommended amount of mounting medium;
2) Add few drops of the suspension cells at the centre of the dish;
3) Wait 10min for the sedimentation process (acquisitions during this phase produces poor acquisition quality and high failing ratio);
4) Reduce the vibrations as much as possible, until the cells will be sitting stable in the bottom.
5) Proceed to image the sample

The following chemicals additives could help to fix the cells at the bottom without killing them:

- Protoslow, recommended for flagellates and ciliates (http://www.carolina.com/protist-viewing-supplies/protoslo-quieting-solution-laboratory-grade-15-ml/885141.pr):
- Cell-tak (https://catalog2.corning.com/LifeSciences/en-GB/Shopping/ProductDetails.aspx?productid=354240):
- Poly-d-lysin coating, recommended for floating blood cells.

3.4.3 Fixed cells on coverslips

General preparation procedure:

1) Place sterile coverslips at the bottom of a petri dish;
2) Coat the petri dish and the coverslips with appropriate coating material if needed;
3) Seed the desired number of cells using the cell culture medium of your choice;
4) Culture cells until they reach the desired confluency;
5) Wash your cells with warm (37 °C) PBS and proceed with next fixation step in less than 30 seconds to limit the stress on the cells;
6) Fix the cells with 2ml of 4%PFA and incubate for 15 minutes at room temperature;
7) Wash the cells 3 times with 2ml PBS;
8) Put the imaging spacer on the microscope slide and add a drop of suggested mounting medium (see section 3.3.2) on top to fill the imaging spacer;
9) Take the coverslip from PBS, position a tissue paper on the side and dry the coverslip by capillary force (**warning:** don't put tissue paper on top of it);
10) Position the coverslip on the spacer with the cells facing the microscope slide;
11) Proceed to image the sample or store it at 4°C.

Sealing method:

The medium chamber can be sealed to avoid liquid drying or leakage. There are two ways of possible sealing:

- Recommended: tape imaging spacer (SS1X9-SecureSeal Imaging Spacer, Grace Biolabs, Product #654002)
- Nail polish (as second choice since it frequently overflows on the coverslip).

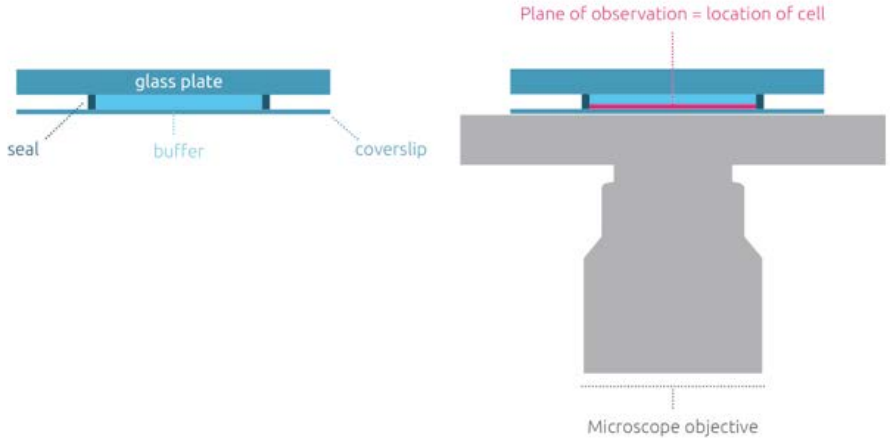


Figure 3. Schematic of observation of cells with the 3D Cell Explorer located between glass plate and coverslips

3.4.4 Tissue slides

The 3D Cell Explorer is also suitable for imaging slices of tissues by respecting the following conditions:

- Remove the OCT or paraffin from the tissue sections before mounting the slide (OCT/paraffin are not fully transparent and they interfere with the acquisitions);
- The best results can be obtained with very thin tissue sections (5-10 microns). The maximal tissue thickness should be less than the maximal thickness of sample structure (see Specification table 1);
- The mounting medium should be an “aqueous mounting medium” with RI value in the range of 1.33 - 1.44. You can find the RI value of mounting medium in the product data sheet;
- Observe the slide upside down (i.e. with the coverslip slide facing the objective) (Figure 3).

4. Cleanness of sample

The cleanness of the sample is crucial to insure good quality of the image.
Due to the rotating illumination system, debris (either floating or out-of-focus) can be hit by the beam. Therefore, optical surfaces must be as clean as possible and cell holders should be carefully cleaned so that dead cells or any kind of remains are not floating in the mounting medium.

Depending on the support used for the experiment, there are two procedures to follow before starting the acquisition with the 3D Cell Explorer:

- Coverslip: lens tissues (like Thorlabs™, #MC-50E) are recommended to clean the surface of the coverslip in contact with the objective.
  Lens tissues are ideal for removing all dust or fingerprints from the coverslip without leaving any trace of lint or fibers. To clean the coverslip, wet the tissue with a few drops of ethanol and gently rub the surface.
- Glass bottom culture dish: to remove any dead cells or cell debris wash the cells 3 times with PBS and then add the appropriate mounting medium; using the same procedure described above for the coverslip, clean the external surface of the glass bottom dish which should be in contact with the objective.

Due to the field of view of the microscope and the depth of field (for exact specifications, please check the technical specification sheet in the user's manual or on our web site http://nanolive.ch/hardware/) dimensions of the cell should be inferior to these values to be fully reconstructed.

5. Accessories

The 3D Cell Explorer, in combination with a top stage incubator, allows for non-invasive 4D long-term live cell imaging for several days in physiological environmental conditions.

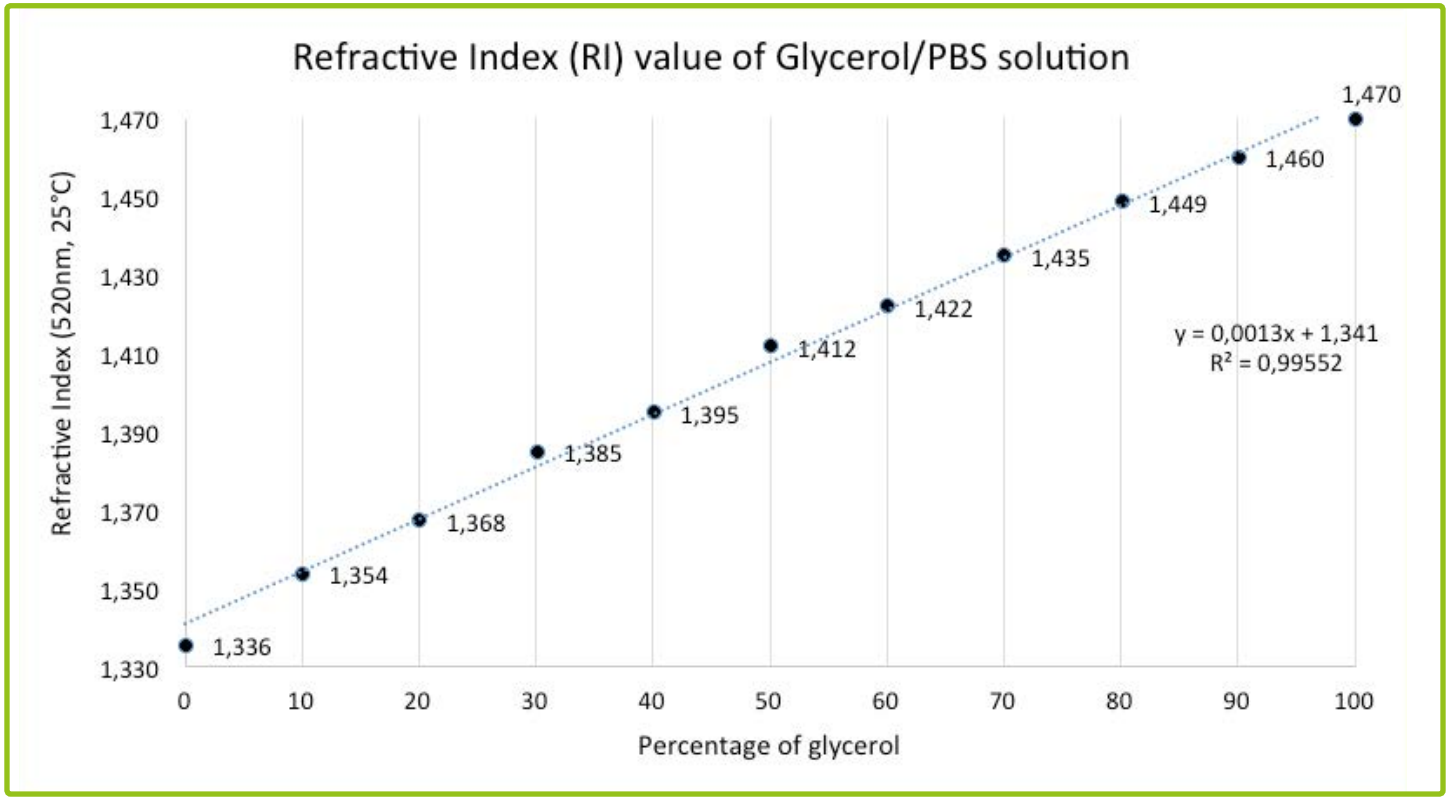
5.1 Nanolive-ibidi top stage incubator

The Nanolive-ibidi top-stage incubator brings all the required incubation controls directly on the stage of your 3D Cell Explorer: temperature, humidity and pH can be meticulously regulated during your time-lapse cell experiments to give you the most accurate living cell results.

The Nanolive-ibidi top-stage incubator is our recommended model. Please visit our website (http://nanolive.ch/accessories/) to get a quote and find the technical specifications.

If you want to learn more about ibidi and their product portfolio, please visit www.ibidi.com.

6. Appendix



Graph 1. Refractive Index (RI) value of Glycerol/PBS solution

Glycerol reference: G5516 – Sigma Aldrich
Instrument: AR4 Series Abbe Refractometer (KRUSS OPTRONIC)