



Best Experimental Practices for Live Cell Imaging with the 3D Cell Explorer

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Application Note by Nanolive SA

Abstract

Nanolive's 3D Cell Explorer allows for the creation of very powerful 3D images and 4D time-lapses of living cells with very high spatio-temporal resolution (x,y:180nm; z:400nm; t:1.7sec). However, to take full advantage of the microscope's live imaging capabilities, a proper setup of Nanolive's top stage incubator is necessary. The goal is to guarantee maximal experimental stability and to avoid any stress to the cells while they are in the top stage incubator.

In this application note we will describe how to properly set up Nanolive's top stage incubator such that mammalian cells can be imaged over a long period of time (up to weeks). To achieve this, we will guide you through the key steps for optimal humidity, CO₂ and temperature control as well as correct imaging regime. You should then be able to take advantage of the most impressive and unmatched 3D Cell Explorer capability: long-term, high-frequency live imaging.

1. Introduction

Long-term imaging of fine cellular dynamics is today's biggest challenge in cell biology (Frechin et al., 2015; Kruse & Jülicher, 2005; Kueh, Champhekhar, Nutt, Elowitz, & Rothenberg, 2013; Skylaki, Hilsenbeck, & Schroeder, 2016). The goal is not only to acquire snapshots of dynamic biological systems, but to actually see such active processes (Muzzey, Gómez-Uribe, Mettetal, & van Oudenaarden, 2009). However, fluorescence microscopy, which is the current method of choice in high-content live imaging approaches, induces phototoxicity when the sample is stimulated at various wavelengths. This stress induces cellular damages via radical-induced cellular structure alteration, which limits live imaging possibilities. Therefore, with the current live cell imaging strategies a tradeoff must be found between short live cell imaging with high-frequency acquisition or long-term live cell imaging with low-frequency acquisition.

On the one hand, high-frequency acquisition induces high phototoxic stress; if lucky, a researcher might be able to observe fine dynamics but cannot be sure that they are not perturbed by the imaging process. On the other hand, low-frequency acquisition might be more sustainable, however, fine dynamics would be lacking while the observed phenomenon, to a lesser extent, would likewise be perturbed by the imaging process.

The solution is in fact to perform long-term imaging at high frequency of acquisition! Because the 3D Cell Explorer's laser injects 100 times less energy into the sample than the gentlest of the current fluorescent imaging approaches, given that proper data management and environment control are set up correctly, it is technically possible to perform endless live imaging at the maximal acquisition speed (1 image per 1.7 seconds). This certainly exceeds any live cell imaging capabilities of any other microscope.

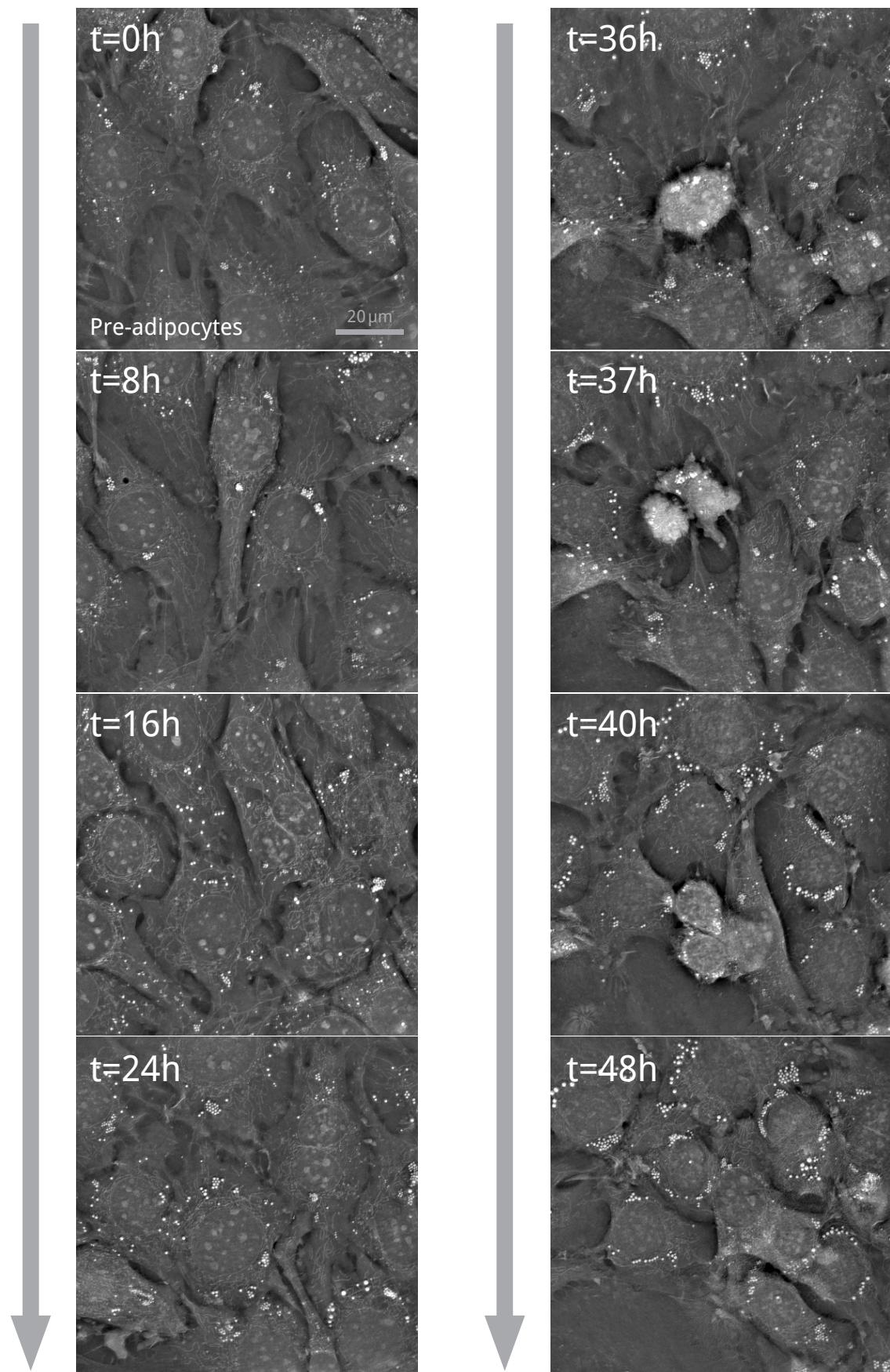


Figure 1: Live imaging of mouse pre-adipocytes for 48 hours. One image per minute. The 3D Cell Explorer generates no phototoxicity.

2. Prerequisites

First, you will need live mammalian cells, growing in a glass bottom dish compatible with the 3D Cell Explorer (<http://nanolive.ch/supporting-material/#labware>) inside a typical cell culture incubator. All animal cells grown in our recommended dishes, preferentially in monolayers, can be observed live with the 3D Cell Explorer. For simplicity, we will focus on the parameters that fit for mammalian cell culture. You can easily adapt this application note to any other cell culture (e.g. bacteria, yeast).

Secondly, you will need the Nanolive's top stage incubator equipment. This includes the top stage incubation chamber, a controller pad, and a humidity system. We recommend using our CO₂ mixer and air pump that will ensure a proper control of CO₂ proportions and will help you save some money on compressed air. Learn how to set up your Nanolive Top Stage Incubator here: <http://nanolive.ch/supporting-material/#setup-incubator>.



Figure 2: 3D Cell Explorer set up with its top stage incubator

Important note: the use of phenol-red free medium is preferred for best live cell imaging performances. Your favorite supplier certainly makes buffers optimized for live cell imaging.

3. Preparation of the environment control

a. Temperature

Letting the system equilibrate before use will ensure that the parameters will not drift out of your acceptable ranges. Our strongest recommendation is to first see how your top stage incubator reacts with your 3D Cell Explorer and with your imaging room, considering that each room has its own temperature and humidity. **We recommend limiting entry and exit from the room while the live cell imaging experiment is ongoing, and to be aware of automatic air conditioning programs in your room.**

Start the microscope and the computer, place the chamber on the microscope stage (if you just got it, remove the blue stickers within the removable part of the chamber that might impact the flatness of your field of view), connect it properly (see manual and set up video: <http://nanolive.ch/supporting-material/#setup-incubator>) and place the lid. Switch the controller on, and set the temperature to 38 degrees C°.



Figure 3: Top Stage Incubator Temperature Controller

Next, fill the humidity bottle with distilled water and start the injection of humid air at 1 liter per minute, which is when the small metal ball reaches the top of the mixer column. Leave the CO₂ off, it is not required yet. You can now wait ~2h so that the system equilibrates in temperature, particularly the microscope's stage.

You might think it is wrong to set the temperature to 38 degrees because mammalian cells grow at 37 degrees. The reason is that there is a difference between the temperature in the chamber, and the effective temperature in the well. We observed that in our conditions: a 38 degrees setting is suitable to obtain 37 degrees in the well. You can check this for your local conditions: after the two hour wait and without changing any of the parameters (gas, temperature), prepare a test well with culture medium in it and place it in the chamber with the green probe in the liquid, then close the chamber. You can immobilize the probe with tape if you cannot manage to keep it in. After 30 minutes, observe the temperature of the growth medium, it should be close to 37 degrees. If not, play with the temperature of the chamber and let it equilibrate. Be aware that the temperature you will find to generate a temperature of 37 degrees in the well is in function of many parameters such as the temperature and humidity of the room (be especially careful with air conditioning cycles), but also the flux of gas sent into the chamber. Therefore, we recommend to always leave the flux at 1 liter per minute.

Finally, do not get too preoccupied, few tenths of degrees away from the gold 37 degrees standard won't hurt (Watanabe & Okada, 1967).

b. Humidity

Humidity control is very important for long term live cell imaging. If you want to do an experiment for less than two days, you can directly proceed to the next section (c). For an experiment lasting more than two days, follow the steps in section a: The air humidity saturation is almost 100% when arriving in the chamber, however, almost is not enough. After two days, we observe a tiny loss of medium in the well because of evaporation and that can be extremely perturbing for sensitive cells. To counterbalance this loss, the incubator manufacturer, Okolab, recommends placing a 1 cm thick stack of Whatman paper all around the inner profiles of the chamber and soak it with water.

To make such a stack simply cut enough Whatman paper sheets according to figure 4. Because the absorbed water increases the thickness of the sheets, you will only need 2 to 6 sheets depending on your paper thickness.

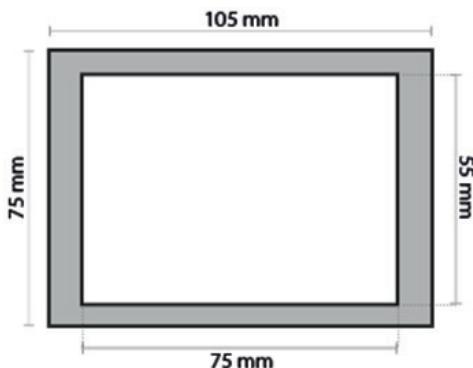


Figure 4: Dimensions for cutting a Whatman paper sponge

It is important to keep an eye on the amount of water contained within the paper. If necessary, you can open one access on the side of the chamber (remove a tiny screw) to easily re-humidify the Whatman paper without opening the chamber. We tested this solution and it ensures no medium loss for a week at least.

c. pH control

We recommend setting the CO₂ to 8-10% if you are using DMEM or other medium with carbonate buffers.



Figure 5: Manual Air / CO₂ Mixer

This percentage is higher than the usual 5% CO₂ in a standard incubator. The reason being that gas exchange is less efficient in the chamber of a top stage incubator since the chamber is not as hermetic as a standard 37 degrees incubator. Rest assured you will not asphyxiate your cells that are, in any culture conditions, facing at least an O₂ partial pressure ten times higher than in a normal tissue.

An additional very useful setup recommendation is to use the previously mentioned media optimized for live cell imaging. Those rely on HEPES buffer that do not depend on CO₂ content to maintain the pH at 7.4. We also strongly recommend using this buffer if you use premixed 5% CO₂/air.

Be aware it is likely that a long-term imaging experiment will make your cell population over confluent and that cells will increasingly enter apoptosis which will acidify your medium regardless of the strategies you use for controlling pH.

4. Starting the live imaging experiment

In summary, your temperature and humidity are under control, as well as your CO₂ mix. The chamber is ready for your living cells. Proceed with your experiment as quickly as possible to reduce the time cells are out of controlled environment as well as the time the chamber lid is open. Once the cells are in the chamber, we recommend waiting for at least 30 minutes to let them adapt but also to verify that everything is in order.

You are now ready to start a time-lapse.

5. During acquisition

If you followed the procedure described here, you should observe very little drift in the Z position of your sample. This drift is usually due to incorrect equilibration of the system's temperature. It can also be due to sudden changes in room temperature. Keep people out of the room and ask for stable air conditioning during the experiment.

If you do more than two days of acquisition, you will have to refill the water bottle. Do not touch anything else, just open the bottle and fill it while gas is on. This is a fast procedure and will have no impact on your experiment. However, if the bottle is on the same table as the microscope, you must avoid bumping into it or even leaning on the table. Any movement or vibration can alter the positioning of your sample.

6. General Hardware & Software Requirements

3D Cell Explorer models:

3D Cell Explorer

3D Cell Explorer-fluo

Incubation system:

Nanolive Top Stage Incubator

Microscope stage:

Normal 3D Cell Explorer stage

High grade 3D Cell Explorer stage

Software:

STEVE – all versions

7. References

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