Live Cell Imaging Tips

Imaging live cells is a complex task even for experienced microscopists. However, many research questions can only be addresed in living cells. For example studies of cellular dynamics and cell physiology require live observation over time. Additionally, microscopy of GFP-labeled proteins in living cells is both simpler and less prone to artifacts than microscopy of fixed cells. This guide is intended to help even novice users to be successful with live cell imaging and generate quality data at the ICBM.

What are the major issues in live cell imaging?

Perhaps the single most important issue in live cell imaging is growing the cells in a way that is optimal for live-cell microscopy. Although, choosing the correct microscope for your experiments, maintaining physiological conditions of the cells while they are on the microscope stage, and for studies involving repeated imaging over time, stability of the preparation are crucial as well.

How should I grow my cells for live cell microscopy?

For successful live cell imaging, you must plate your cells in dishes with coverslip bottoms. We most frequently use 35 mm No. 1.5 coverslip bottom dishes from MatTek. They come uncoated or coated with poly-D-lysine or collagen. Consult the literature in your field to see which coating is best for your cells. Descriptions and pricing can be found at: MatTek: http://www.glassbottomdishes.com/gbcustomerpriceweb.pdf. All of the microscopes at the ICBM have stage inserts available for 35 mm coverslip bottom dishes. Our stage heaters (covered in the next section) also are all sized for 35 mm coverslip bottom dishes, making this the best choice overall for live cell imaging at the ICBM.

We recommend use of a No. 1.5 coverslip because most microscope objectives are designed to be used with a cover glass that has a standard thickness of 0.17 millimeters, the average thickness of No. 1.5 coverslips. Some objectives have correction collars to compensate for coverslip thickness variations and most users find that using No. 1.5 coverslips and setting the correction collar to 0.17 is adequate. For maximum image quality, the thickness of each coverslip can be measured with a micrometer and the correction collar can be adjusted to this thickness. Plastic is not transparent enough for fluorescence and is frequently itself autofluorescent. Additionally, plastics polarize light and are not suitable for DIC imaging.

Both plastic and glass slides are too thick for use with high numerical aperture objectives. The numerical aperture of a microscope objective is a measure of its ability to gather light and resolve fine specimen detail. Higher numerical aperture objectives gather more light and resolve finer detail. These are desirable traits in a microscope objective, but the tradeoff is that the objective working distance decreases as magnification and numerical aperture increase. This means that the best objectives, high magnification, high numerical aperture objectives, have a short working distance. Most of the objectives at the ICBM are high magnification high numerical aperture objectives. If you use a dish or plate or slide that is too thick you will not be able to focus on your sample because your sample will be beyond the working distance of the objective. Some ask why they can focus on their cells just fine in a thick plastic dish on a lab scope but can't do it here? That is because the lab scope probably has lower magnification or lower numerical aperture objectives.

Things that will not work for live cell imaging the ICBM because they are too thick:

Plastic tissue culture dishes.
Plastic 6-well, 12-well, 24-well, 48-well, 96-well plates.
Tissue culture flasks.
Well-slides.

Although many well-slides claim to be good for imaging, most are still much too thick for use with high magnification high numerical aperture objectives. They must have coverslip thickness bottoms (0.17 to 0.18 mm).

The pH indicator phenol red can interfere with collection and interpretation of weak fluorescent signals. For best results, grow your cells in phenol red free medium.

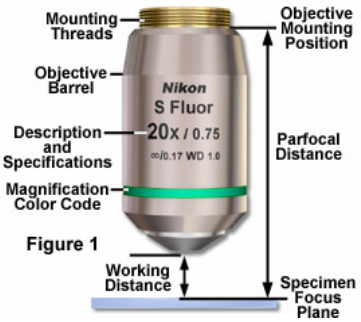


Figure 1

http://www.microscopyu.com/

Which microscope do I need to use for live cell microscopy?

Live cell imaging can be done on a number of different systems within the ICBM. Live cell imaging does require using an inverted microscope. But, there is no single "best" system for live cell imaging. Choice of microscope system depends on what you need to do. Detailed descriptions of the systems available at the ICBM can be found here: http://www.nephrology.iupui.edu/imaging/facilities.htm.

The Spinning Disk system is the best system to use for time-lapse fluorescence. It is equipped with an EMCCD camera, which is capable of detecting very low levels of light. It is the fastest system with the best signal to noise ratio, the least phototoxicity and the least photobleaching. Under optimal conditions images can be acquired with exposure times as short as 30 ms per plane,

which makes it possible to image the entire volume of a cell with z-steps in the submicron range in as little as 1-2 seconds per channel. This level of temporal and spatial resolution allows you to follow high speed subcellular events in 3D over time.

When very high speed is not required both the Zeiss LSM 510 UV and the BioRad systems are excellent for confocal imaging of living cells. With the Zeiss system it is also possible to concurrently collect both fluorescent and DIC images.

Live cell imaging can also be done using one of our widefield epifluorescent systems. These systems are at their best when used to image very thin single cell preparations. The Nikon Eclipse TE200 Inverted Microscope equipped with the Orca-ER Cooled CCD camera is capable of very low light detection with minimal phototoxicity and photobleaching. High power DIC images can be acquired along with fluorescence. Top acquisition speeds for this system are in the 2-8 frame per second range, if the sample has adequate signal. The Nikon Diaphot 200 Inverted Microsope with the SPOT color camera has limited fluorescence sensitivity, but is optimal for collection of DIC and phase contrast images. The low power DIC objectives on the SPOT system are ideal for following movement of cells such as in chemotaxis experiments, or even to development of whole organisms such as zebrafish embryos.

Doing the study: How do I keep my cells happy on the microscope stage?

When you perform live cell imaging experiments your goal should be to maintain the cells in a healthy state with normal function while they are on the microscope stage. Control of the cells' environment is a critical component in the success or failure of your live cell experiments. Cells that appear even slightly unhealthy should not be used for imaging and data collection.

• *Maintain pH*

Cells are typically grown and maintained in a cell incubator at 37°C with 5% CO_2. The pH value of $NaHCO_3$ buffered media depends on the CO_2 content of the incubator atmosphere. When the CO_2 supply to an incubator fails, media becomes alkaline and cells are adversely affected and may die. You can image cells in their normal growth medium if you supply 5% CO_2 to the dish on the microscope stage. However, the easiest solution is to use HEPES buffered medium.

HEPES buffer does not require a controlled atmosphere. Buffer strength for cell culture applications is usually in the range of 10 to 25 mM. HEPES can be added to a phenol red free version of your normal media. While most cell types do well in HEPES buffered medium, it should still be evaluated by the researcher prior to use for live cell imaging. Do not image live cells in PBS. For further information, consult the literature in your field. If extended time lapse (many hours to days) is necessary you may need to use CO_2 and your usual media on the stage. This is possible but currently requires a more elaborate setup. We are working towards simplifying the use of warmed humidified CO_2.

A recipe for a HEPES buffered imaging medium is included below. (Brown et al. 2000 Traffic 1:124-140)

Imaging Medium Stock (5x) pH 7.4

750 mM NaCl
100 mM HEPES
5 mM $CaCl_2$
25 mM KCl
5 mM $MgCl_2$

Use at 1x. On day of use add 95 mg glucose and 95 mg albumin to 50 ml of medium. Warm to 37°C. Keep cells in incubator in their usual medium until immediately before imaging. When ready to image, remove their usual medium and replace it with imaging medium. Cells can normally be kept on the stage in this medium for 30-60 min. A cell culture incubator is available in the ICBM in room E243D for use during imaging. Please remove all of your cells from the incubator at the end of each day's experiment.

• *Maintain Temperature*

Cellular function is exquisitely sensitive to temperature. Objective warmers and stage warmers for 35 mm dishes are available for all of the microscopes in the ICBM. When you sign up for microscope time on the calendar, indicate that you will need a stage warmer and an objective warmer for your live cell imaging. Set both to the same temperature. Allow as long as 30 min for the temperature to come to equilibrium. Heating of both the dish and the objective prevents temperature gradients across the dish. Ask for help with setup the first time that you use the dish and objective warmers.

For maximum temperature stability fill your 35mm coverslip bottom dish at least halfway with medium. The medium serves as thermal mass and damps temperature fluctuations. In live cell imaging experiments special care is required to prevent spills of medium from the cell culture dish onto the objective or the body of the scope. If you do have a spill, please inform facility personnel immediately. This can help prevent damage to the microscopes.

Microscope users sometimes complain of instability of z-positioning over time when doing live cell imaging. This focus drift is nearly always due to thermal expansion that occurs due to a temperature gradient. When using high NA objectives, the dish is thermally coupled to the objective by the immersion media. This is why it is necessary to use both a stage warmer and an objective heater if z-stability is required for an experiment.

• *Minimize phototoxicity and photobleaching*

Photobleaching, also called fading, occurs when a fluorophore undergoes an irreversible covalent modification and loses its ability to fluoresce. Different fluorophores can undergo different numbers of exitation emission cycles before photobleaching. Phototoxicity largely results from the formation of oxygen radicals due to non-radiative energy transfer. These oxygen radicals can be toxic to the cells. To minimize both photoxicity and photobleaching, minimize the energy level of the exitation light and the duration of exitation. Use as little light as possible, especially if you are acquiring an extended time series. The spinning disc system is capable of detecting very low levels of light, which allows use of minimal exitation light resulting in the reduced phototoxicity and photobleaching.

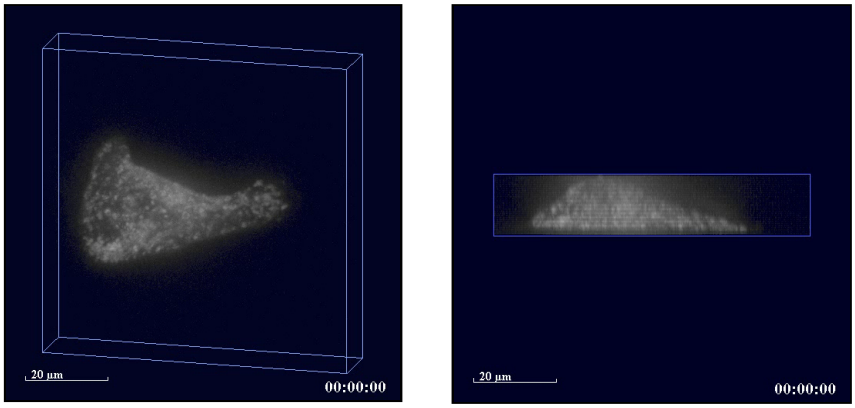
If you do all of this, here's the kind of results you can expect

Example : Spinning disc microscope, 5 hour time lapse

This example of time lapse live cell imaging was done using the spinning disk microscope system. Cells imaged had been transiently transfected with rab7-eGFP. Rab7 is present on endosomes that traffic rapidly about the cell. The rab7-eGFP signal is limited, so illumination was adjusted to give adequate signal with minimal exitation intensity and duration. Even slight disruption of cell temperature or pH would have slowed or stopped the rab7-labeled endosome movement, so the vigorous intracellular motion provided a convenient visual readout of the health and happiness of the cells over time. For this time lapse sequence, cells were maintained on the stage for a period of 5 hours by using a stage heater, an objective heater and warmed humidified CO_2.

For the first half of each movie below, 120 image stacks were collected at a rate of one image stack every 2 seconds. For the second half of each movie below, 120 additional image stacks were collected at a rate of one image stack every minute. Exposure time for each image plane was 31 ms. Acquisition speed was 16 frames per second. When imaging of this cell began, the dish had already been maintained on the microscope stage for three hours. Endosomes were visible zipping merrily around the cell. At the end of this 240 image stack sequence (6,960 image planes!!!) photobleaching was minimal, endosomes continued to zip about and the cell was crawling, indicating that the cell had remained happy on the stage for over 5 hours.

The preparation was remarkably stable both horizontally and vertically. Observation of these time lapse sequences from the x,z perspective shows that the cells remained stable in the z-plane throughout a 2 hour period of imaging. There was no focus drift. This study demonstrates that cell physiology and preparation stability can be maintained even under the most extreme conditions.



Images were acquired using the ICBM Spinning Disk Microscope equipped with a 100x Oil NA 1.4 objective. Each image is 512 x 512 pixels with 29 image planes per stack. Pixel dimensions in x,y = 0.15 micron and the z step = 0.5 micron. Exposure time was 31 ms per image plane.

Links:

http://www.microscopyu.com/articles/livecellimaging/index.html

References:

Goldman, R., and Spector, D. (2005) Live Cell Imaging: A Laboratory Manual. Cold Spring Harbor, NY: Cold Spring Harbor Press.