Image live cells through time and space

Optimizing conditions for time-lapse fluorescence microscopy.

Fluorescence imaging of live cells is a very powerful approach to the study of dynamic cellular processes and events, yielding high spatial and temporal resolution. The availability of more stable and brighter fluorophores—organic dyes, fluorescent proteins, and nanocrystals—over the past several years has greatly expanded the toolbox for researchers exploring the frontiers of cell biology. Beyond fluorescent reagents, advances in optics, sensor technology, computing power, and refined software tools have been integrated into imaging systems that are both more powerful and straightforward to use than just a few years ago. Moreover, these imaging systems—such as the EVOS® FL Auto Imaging System—are available today for use by researchers who are not imaging specialists.

The result of these innovations has been widespread adoption of fluorescence imaging in cell-based research, where scientists can apply these new tools to areas from developmental and stem cell biology to medical research, and from drug discovery to environmental studies. It is no longer necessary to be an imaging expert to acquire high-quality images, and these imaging systems are becoming mainstays in laboratories that explore cellular processes and phenomena through an array of techniques and tools. Translational research is being enabled by a "democratization" of these sophisticated life science tools.

Live-cell imaging: Caveats and rewards

Fluorescence imaging of fixed cells is relatively straightforward (compared with live-cell imaging) and is used extensively, as it generally affords images of higher quality than those derived from live cells. Cell structure and morphology information gleaned from these fixed-cell studies can also be correlated to biological states and processes, such as cell health status or progression through developmental or disease-related pathways. Fluorescence imaging of live cells, however, is the method of choice for studying dynamic processes, and time-lapse imaging of live cells can provide unique insights into events that may unfold over hours or days (Figure 1).

Despite advances in both reagents and instrumentation, live-cell imaging remains challenging for two fundamental reasons: mammalian cells are highly sensitive to light, and they require stable environmental conditions. Utmost care must be taken to protect cells from excessive light, and to maintain stable conditions of temperature, levels of atmospheric gases (O_2 and CO_2), humidity, pH, and osmotic concentration (osmolarity) to ensure that the cells being observed remain healthy. Time-lapse imaging compounds these challenges because of the multiple light exposures and extended time out of the incubator. →

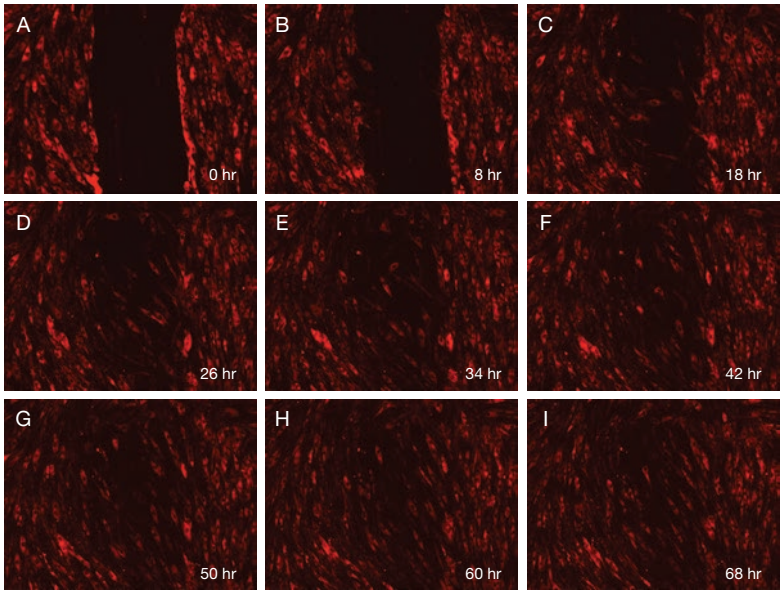


Figure 1. Time-lapse imaging of wound healing. Human dermal fibroblast cells were grown to confluence and labeled with 1 µM CellTracker™ Red CMTPX Dye (Cat. No. C34552). A scratch wound was made in the cells with a 20 µL pipette tip **(A)**. These representative frames from time-lapse imaging (sampled every 2 hours over a period of 70 hours) show the wound healing **(B–H)** until the wound is completely healed **(I)**. Wound healing was imaged over time on the EVOS® FL Auto Imaging System equipped with the EVOS® Onstage Incubator and Texas Red® optical filters.

Table 1. Factors to consider when fluorescently labeling cellular targets.

Target and probe considerations
Relative target abundance
Specificity of probe for cellular target
Target distribution (dispersed or concentrated)
Fluorescent label considerations *
Fluorophore photostability
Fluorescence output per dye
Spectral separation of dyes

* See a discussion of fluorescent organelle probes in the "Journal Club" article on page 35.

Controlling light factors

Live cells are highly sensitive to light, which can cause a range of deleterious effects [1]. Of these, phototoxicity from UV light and free radicals can be a significant problem when performing fluorescence microscopy, especially time-lapse fluorescence imaging. Phototoxicity can result in cell damage and artifacts, and can complicate interpretation of results (see the "Tips for minimizing phototoxicity in time-lapse fluorescence imaging" box on page 25). Excess light can also destroy the fluorophores (i.e., photobleaching) being used to report on the functions or structures under study.

A number of probe- and illumination-related factors should be considered when designing time-lapse fluorescence imaging experiments. First, care should be taken to optimize labeling of cellular targets (a nucleus is far easier to label than is a low-abundance protein), and no target should be labeled excessively (Table 1). The absolute signal is less relevant than the signal-to-noise ratio (S/N). Uniform labeling is particularly challenging when mixing fluorophore categories (e.g., organic dyes, fluorescent proteins, and nanocrystals), though one useful approach is to match more brightly fluorescent probes with less abundant or more disperse targets. Excessive fluorescent labeling of cellular targets can lead to:

- Nonspecific staining with increased background signals
- Physiological artifacts and structural perturbations
- Cytotoxicity

And second, image sampling should be kept to a minimum to reduce phototoxicity and photobleaching. Cell damage must be avoided and cells maintained in a healthy state to obtain data that faithfully reflect the process under study. One advantage of LED-based illumination, such as in the EVOS® Imaging Systems, is the negligible UV light emission and near-elimination of cellular UV exposure.

Controlling environmental factors

Cell biologists are adept at culturing cells that are maintained in a cell culture incubator. However, maintaining the same conditions on a microscope stage during time-lapse imaging is far more challenging. Although mammalian cells vary widely in their tolerance of nonphysiological conditions, transformed cells lines are generally more tolerant of environmental fluctuations than are primary cells (e.g., oocytes and stem cells). Maintaining environmental conditions that are close to physiological on the microscope stage is therefore of utmost importance in time-lapse imaging experiments; in the absence of environmental control, conditions will slip out of the physiological range within a few minutes. How well the environment is controlled on the microscope stage is one of the most critical factors in successful time-lapse imaging and may, in fact, decide the success or failure of an experiment.

For instance, many cell culture media are carbonate buffered and, once they are removed from the incubator, the relatively lower CO_2 concentration of air will cause an increase in the medium pH within an hour. For cell imaging on microscope stages lacking an environmental incubator, we have developed an optically clear, physiological solution (Live Cell Imaging Solution, or LCIS) that will maintain physiological pH and osmolarity for up to 4 hours at ambient atmosphere and temperature. For longer-term imaging, particularly in a microscope-stage incubator, we offer a clear cell culture medium—Gibco® FluoroBrite™ DMEM—that is based on DMEM to help preserve cell health while also providing low background fluorescence (comparable to that of phosphate-buffered saline and 90% lower than standard phenol red–free DMEM).

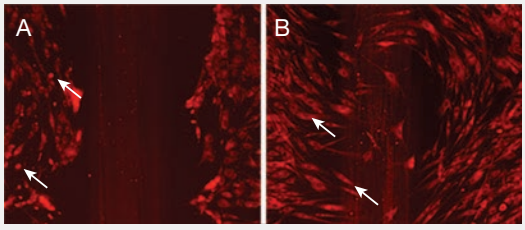
A stage-top live-cell chamber: The EVOS® Onstage Incubator

For imaging over extended periods, the temperature and atmospheric conditions (gases and humidity) must be tightly maintained. Among available options, a stage-top live-cell chamber that allows control of temperature and atmospheric conditions is often the best choice. We recently introduced the EVOS® Onstage Incubator, an environmental chamber that was specifically developed as an accessory to the EVOS® FL Auto Imaging System and fits on that system's automated X-Y stage. A small, separate control unit supplies power, gas (air, CO_2, and N_2 for hypoxia experiments), and control of humidity and temperature. Several vessel holders are available to accommodate a range of culture vessels and permit easy and convenient time-lapse imaging of cells right from the incubator.

Tips for minimizing phototoxicity in time-lapse fluorescence imaging

Phototoxicity—an effect commonly associated with the photochemical destruction of a fluorescent dye—can ruin even the best-designed fluorescence experiments and is a critical factor in time-lapse microscopy. Here are a few simple tips to help reduce or prevent phototoxicity in your experiments:

- Minimize illumination exposure times and keep the light source power on the lowest possible setting that will afford satisfactory signal-to-noise ratios and generate accurate data [1]. Maximize the camera gain to reduce light intensity and exposure times.
- Don't over-sample. Image cells with a sampling rate relevant to the process or phenomenon under study.



Phototoxicity during imaging of wound healing. Human dermal fibroblasts were labeled with CellTracker™ Red CMTPX Dye (Cat. No. C34552) in a wound healing experiment where **(A)** extreme light exposure caused phototoxicity, resulting in a "balled-up" morphology (indicated by arrows) characteristic of unhealthy cells or **(B)** minimal light exposure resulted in a fibrous cell morphology (indicated by arrows) characteristic of healthy fibroblasts. Wound healing was imaged over time on the EVOS® FL Auto Imaging System equipped with the EVOS® Onstage Incubator and Texas Red® optical filters.

- For long-duration time-lapse imaging, use an onstage incubator when possible to keep temperature and CO_2 levels constant. Don't rely on buffer additives like HEPES to maintain pH for long periods. HEPES works well for short-term time-lapse imaging; however, when exposed to intense light over long durations, it can produce hydrogen peroxide, a cell toxin [2].
- Limit the number of channels that you use to only those absolutely necessary for the experiment. Each additional channel increases the sample's exposure to light.
- When possible, avoid using autofocus for every image taken during time-lapse imaging. Autofocus can increase the amount of light hitting your sample by as much as 10 times. With the EVOS® FL Auto Imaging System, it is straightforward to set focus "beacons" for the field or well section to be imaged.
- Minimize the time that the sample is being exposed to light while you are setting up the experiment. If you are imaging in multiple channels, your sample may be exposed to significant levels of light before the time-lapse image acquisition even begins.
- Choose the correct imaging system, including the objective lens and camera [3], for the experiment you are performing.

References

1. Frigault MM, Lacoste J, Swift JL, Brown CM (2009) Live-cell microscopy—tips and tools. *J Cell Science* 122:753–767.
2. Lepe-Zuniga JL, Zigler JS Jr, Gery I (1987) Toxicity of light-exposed Hepes media. *J Immunol Methods* 103:145.
3. Waters JC (2013) Live-cell fluorescence imaging. *Methods Cell Biol* 114:125–150.

The EVOS® Onstage Incubator controls temperature with a precision of ±0.1°C, which helps maintain cell health and reduces the risk of temperature-related biological artifacts. It also minimizes thermal contractions or expansions that may cause focus instability or drift, while the EVOS® FL Auto autofocus system helps ensure that focus is maintained during time-lapse studies when cell movement may shift the focus plane. Additional focusing options for time-lapse imaging include automatic fine and coarse focus, beacons with fixed focus points, or the use of autofocus only in the transmitted light channel to minimize light damage. The preferred option will depend on cell type, staining, duration, and other imaging parameters.

Together, the EVOS® FL Auto Imaging System and EVOS® Onstage Incubator enable precise control of temperature, humidity, and three gases for time-lapse imaging of live cells under both physiological and nonphysiological conditions. Environmental settings and image acquisition parameters are all seamlessly integrated into the EVOS® FL Auto interface, creating a high-performance inverted imaging system with unmatched flexibility, ease of use, and optics for demanding time-lapse fluorescence imaging experiments.

Time-lapse fluorescence imaging is within your reach

To find out more about the EVOS® FL Auto Imaging System and EVOS® Onstage Incubator, visit **lifetechnologies.com/evosflautobp70**. ■

Reference

1. Schneckenburger H, Weber P, Wagner M et al. (2012) *J Microsc* 245:311–318.

Product	Quantity	Cat. No.
EVOS® FL Auto Imaging System	1 each	AMAFD1000
EVOS® Onstage Incubator	1 each	AMC1000
Live Cell Imaging Solution (LCIS)	500 mL	A14291DJ
FluoroBrite™ DMEM	500 mL	A1896701