

Automated Live Cell Imaging Systems Reveal Dynamic Cell Behavior

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*Automated time-lapsed microscopy provides unique research opportunities to visualize cells and subcellular components in experiments with time-dependent parameters. As accessibility to these systems is increasing, we review here their use in cell science with a focus on stem cell research. Although the use of time-lapsed imaging to answer biological questions dates back nearly 150 years, only recently have the use of an environmentally controlled chamber and robotic stage controllers allowed for high-throughput continuous imaging over long periods at the cell and subcellular levels. Numerous automated imaging systems are now available from both companies that specialize in live cell imaging and from major microscope manufacturers. We discuss the key components of robots used for time-lapsed live microscopic imaging, and the unique data that can be obtained from image analysis. We show how automated features enhance experimentation by providing examples of uniquely quantified proliferation and migration live cell imaging data. In addition to providing an efficient system that drastically reduces man-hours and consumes fewer laboratory resources, this technology greatly enhances cell science by providing a unique dataset of temporal changes in cell activity. © 2011 American Institute of Chemical Engineers *Biotechnol. Prog.*, 27: 913–924, 2011*

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Introduction

An early use of time-lapsed imaging to answer biological questions was performed by Eadweard J. Muybridge's creation of time-lapsed photographs in 1877 to answer the question of whether a galloping horse's hooves all left the ground at the same time. Subsequently, in the 1880–90s, Étienne-Jules Marey developed a camera to capture 12 frames per second, and later his high-quality, high-speed (60 images per second) technology was used for slow-motion cinematography of animal movements. Within 30 years of these inventions, time-lapsed imaging was applied at the microscopic level.^{1–5} To examine living cells and tissue, Sandison first developed a transparent chamber to microscopically examine live tissue in rabbit ears.⁶ At the same time, cell migration and cell division of blood cells observed with cinematography revealed a level of cell activity not previously described.^{7–9} And in 1929, Heinz Rosenberger designed and patented a microcinematographic apparatus for researchers to use as an automatic recording instrument. Although Rosenberger's system remarkably included most of the features in today's instruments, it is not clear if this design penetrated the market, as it appears he intended.¹⁰ By the 1950s, a variety of time-lapse approaches or filming were being used to study live cell behavior, such as UV time-lapsed images to follow mitosis in newt cells and film microscopy to study cell death in the presence of irradiation.^{1,4} However, it was not until later that the use of an environmentally controlled chamber and robotic stage controllers were introduced.

The use of a robotic microscope stage and environmental sensors and controllers now permits cells to be enclosed in an incubator with continuous imaging over longer periods of cell culture. The microscope stage automation enables researchers to examine numerous locations within one experimental plate or flask. For example, in stem cell studies, using one multiwell plate, different stem cell types can be compared or numerous small molecule drugs can be screened for their ability to induce a differentiated state. Control of the environment temperature and CO₂ allowed cells to be maintained in a physiologic atmosphere and examined over both short and long durations.

With automated control, high-throughput time-lapsed imaging has become an ideal platform for cell biology experiments with multiple variables and measurements of time-dependent parameters to track thousands of cells in a given experiment. Such measurements include the spatiotemporal quantification of cell mitosis,^{11,12} migration,^{13,14} cell death, and apoptosis.¹⁵ Cell lineage histories^{16,17} may also be constructed to identify events such as symmetric or asymmetric divisions and complex relationships among subpopulations. For example, stem cell migration and proliferation can be measured in studies of how growth factors may chemoattract cells or stimulate proliferation or migration of cells.¹⁸ Sequential high-resolution images collected at intervals on the order of minutes allows for analysis of cell velocity and cell division over extended periods of time.

Further hardware and software advances in more recent years include digital images with improved resolution, expanded regions of analysis, and sophisticated image analysis routines.^{16,19–24} Digital imaging and computer processing have greatly facilitated the task of analyzing cell activity. Digital images (jpeg, tif, and eps) are readily accessed, and several programs are available for automated and semiautomated image processing.

Here, we discuss the key components of automation for time-lapsed live microscopic imaging, and the unique data that can be obtained from image analysis. Many investigators have developed custom time-lapsed imaging systems^{14,25–30} yet, the details of the instrumentation to date has been limited.^{31,32} Numerous automated imaging systems are now available from both companies that specialize in live cell imaging and from major microscope manufacturers (Table 1). Here, we describe the key components of live cell imaging systems, using our own custom-built system as an example. Next, we describe how automated features enhance cell science, with a focus on stem cell studies, by providing examples from using both human and mouse stem cells.

Automated Instrumentation

Key components for high-throughput time-lapsed microscopy

Automated microscope-based imaging systems facilitate the observation of biological events under standard *in vitro* conditions. They can be used to analyze events that are very fast or very slow to be observed by the human eye. Fast events may be captured using a large number of frames (slow-motion imaging), and slow events are captured using a few number of frames (time-lapsed imaging). To date, live cell imaging systems are predominantly used for time-lapsed imaging, although slow-motion imaging has several applications in cell biology. Many cellular events such as cell division or migration occur on the order of magnitude of minutes, whereas finer time resolution into the seconds scale is necessary for molecular processes such as mitochondrial changes or protein translocation.

To observe events over extended periods of time greater than a few hours, a controlled environment is necessary to maintain conditions conducive for cell survival—temperature (37°C), CO₂ (5% greater than ambient atmosphere content), and humidity(100%). Further, the automation of image location recall and image acquisition allows for a large number of regions to be examined simultaneously.

The key hardware components for practical live automated cell imaging include: (1) a robotic microscope stage, (2) an incubator and environmental control system, and (3) camera and shutter control for image acquisition. Software for image analysis additionally plays a key role in data generation. A schematic of the components of a typical imaging system is shown in Figure 1A. The custom-built time-lapsed system of our lab, called live-automated cell imager (LACI), is shown in Figure 1B. Examples of commercially available systems and suppliers are listed in Table 1; however, details regarding each of these systems are beyond the scope of this review. Here, we will use the LACI system to illustrate the unique features of live cell imaging systems.

Robotic microscope stage

Robotic motion control facilitates the full automation of cell biology experiments with multiple variables. Robotic motion allows the specimen plate to change positions relative to the location of the optics/objective. Or, in some systems, the optics/objectives move relative to the specimen plate. This is achieved through mechanized motion of either the stage or the objectives. To custom-build a time-lapsed imaging system, second-party manufacturers, such as Ludl

Table 1. Commercial Imaging Systems

Key Hardware Features of Time-lapsed Imaging Technologies			
Robotic stage movement		1–20	
Long-term incubation		1–15	
Adjustable magnification		1–3, 5–15	
Fluorescent capability		1–3, 5–15	
Commercial Suppliers of Equipment			
1. Delta Vision Core Applied Precision, Inc., Issaquah, Washington		16. Ludl Electronic Products, Hawthorne, NY	
2. BD Pathway BD Biosciences, Rockville, MD		17. Mad City Labs, Madison, WI	
3. Zeiss MicroImaging, Inc., Thornwood, NY		18. Prior Scientific, Rockland, MA	
4. IncuCyte FLR Essen Instruments Inc., Ann Arbor, Michigan		19. Applied Scientific Instrumentation, Eugene OR	
5. IN Cell Analyzer GE – Amersham		20. Biopetechs, Bioscience Tools Butler, PA	
6. CytoWorks Kairos Instruments, Inc., Harmar, PA		11. Opera LX PerkinElmer Life And Analytical Sciences, Inc., Waltham, MA	
7. Leica Microsystems, Inc. Bannockburn, IL		12. Pathology Devices, Inc., Westminster, MD	
8. ImageXpressMICRO MDS Analytical Technologies, Toronto, Canada		13. ArrayScan Thermo Scientific Cellomics, Pittsburgh, PA	
9. Biostation CT/IM Nikon Instruments, Melville, NY		14. Tokai Hit, Inc Shizuoka-ken, 418-0074 Japan	
10. Olympus America Inc., Center Valley, PA		15. WaferGen Biosystems, Inc., Fremont, CA	

Key features for time-lapsed microscopic imaging technology include robotic stage movement, long-term incubation control, and camera/imaging capabilities with both magnification adjustment and fluorescent capability. Many research labs utilize custom-built systems. More recently, several commercial suppliers have developed systems with a wide range of capabilities.

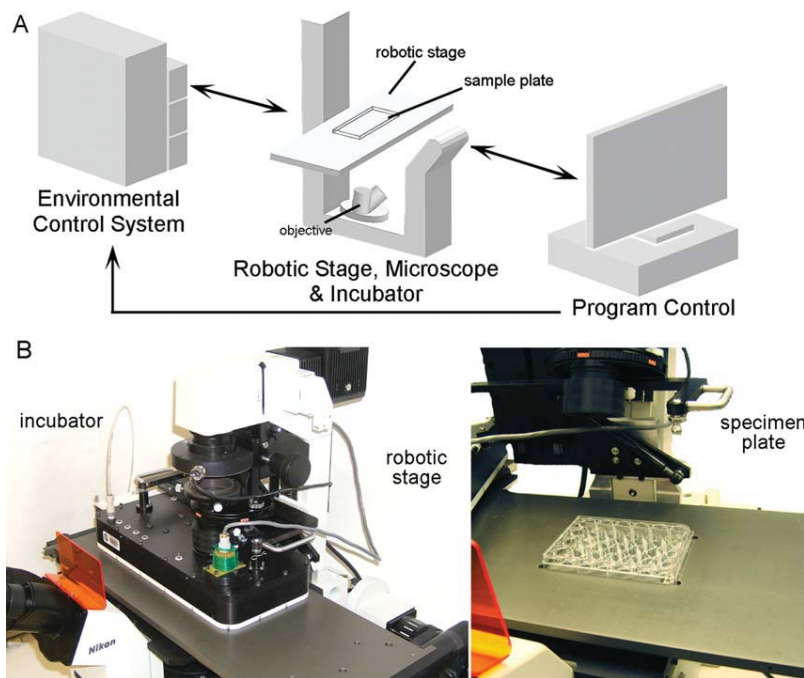


Figure 1. Time-lapsed imaging systems

(A) Schematic of key components of microscopic time-lapsed imaging systems. A robotic microscope stage and environmental control system for long-term incubation are central to time-lapsed imaging. Other key features include adjustable magnification, fluorescent capability and computerized program control. The sample or specimen plate is placed in the stage plate opening. In most systems, as shown here, the stage moves relative to the microscope optics or objectives. In other systems, the stage may be stationary, and the movement of the optics is automated. A control program allows the user to select numerous ROIs within the sample plate. An image is captured at each ROI for each scan interval. The environmental control system regulates temperature and levels of CO₂ or other gases in the incubator. The incubator, not pictured here, may surround the entire microscope system or it may be a smaller unit that surrounds only the sample plate. (B) Custom-built time-lapsed imaging system. The imaging system used in our laboratory includes a stage-top incubator (Kairos Instruments, Harmar PA) built around a Nikon TE2000 microscope.

Electronic Products, Mad City Labs, Prior Scientific, and Applied Scientific Instrumentation, make robotic stages adaptable to different microscope systems. Computer control of both stage position and camera image capture in the presence of environmental control allows investigators to screen a large number of conditions in a single experiment under biologically relevant conditions and time periods. In automated systems, user-defined conditions for each region of in-

terest (ROI) are created and stored by an acquisition program. At defined intervals, the system automatically returns to these locations and captures images per the designated processing type (transmitted light, fluorescent, exposure time controls, etc.). Some systems, such as Molecular Devices’s ImageXpress, also have features to automate the changing of objectives such that ROIs can be imaged at different magnifications.

Industry-leading laser autofocus

The industry-leading laser autofocus provides the fastest focusing available to compensate for focus drift produced by temperature fluctuations, gravity, viscosity changes in immersion media, vibration, plate irregularities, and biological variability.

The process control pathway of most commercially available systems is similar. Figure 2 illustrates the process control pathway of our LACI system.

Environmental control system

Many early studies of live cell activity occurred at room temperature and ambient atmospheric conditions. Yet, most cell biology questions require study over extended periods of time. Therefore, it is necessary to maintain cells in an environment controlled for temperature (to provide physiologic conditions), CO₂ content (to regulate pH levels), and humidity (to prevent evaporation).

Several systems use large enclosures that surround the entire microscope to control the environment. Other systems

use a smaller environmental chamber that is placed on top of the microscope stage, and the chamber encloses the cell specimen plate. In the smallest systems, disposable culture flasks with environmental control elements are used. Still other nonconventional microscope-like systems are compact enough to be inserted into an existing standard cell culture incubator.

Temperature control is achieved through a variety of designs, all require a system of control loops and detectors to monitor and adjust the temperature appropriately. For the custom-built LACI system in our lab,³³ control of temperature is performed through proportional-integrative derivative (PID) control loops that heat metal components of an environmental chamber, called the biobox, that rests on the microscope stage. The temperature of the LACI environment is maintained by controlling the temperature of the individual components of the chamber. Alternatively, other systems such as disposable culture flasks could use heating elements attached to similar PID control loops to maintain temperature and eliminate the need of add-on hardware.

As the majority of mammalian cell culture systems use bicarbonate-based buffering systems, the technology to control carbon dioxide levels is relatively standard and approaches to maintain CO₂ levels on existing automated imaging systems have mainly incorporated this into the system design. In this case, CO₂ should be maintained at a level 5% above ambient atmospheric content. Alternatively, for cell growth in CO₂-independent systems, specialized flasks or media may be used, or continuous media perfusion may be used; although this may affect imaging by requiring movement of flasks or plates to purge the systems. For our LACI system, CO₂ levels are controlled in the biobox by another PID control loop, with CO₂ content detected by a nondispersive infrared sensor.

Finally, a humidified environment of >95% humidity is necessary for eukaryotic cell growth. Most incubators, including the LACI biobox, achieve this by heating a water bath in open air contact with the culture. Some systems such as the Wafergen system use fluid flow to maintain fluid levels. Other similar systems are available from Biopetechs and Bioscience Tools. Time-lapsed imaging systems that are equipped with an incubator to simulate physiological conditions allow for experiments whose duration is theoretically limited only by space and nutrients. Imaging systems without an incubation system are limited to cell activity occurring at ambient atmospheric conditions, which would lead to premature cell death and decrease the length of the study.

Camera and image acquisition

As in early years of time-lapsed microscopic imaging, a stand-alone microscope and camera may be used to collect images. Even today, many investigators will manually acquire time-lapsed images using a modern digital CCD camera by simply removing the flask from the culture incubator at set intervals and repositioning under the microscope to a defined location. Although this may be effective in answering many biological questions, automated control of image capture provides a robust round-the-clock dataset and expands the number of biological studies that can be conducted.

Within the specimen flask, ROIs are selected and the computer-controlled camera automatically captures images at the defined locations and time intervals. Autofocus features are

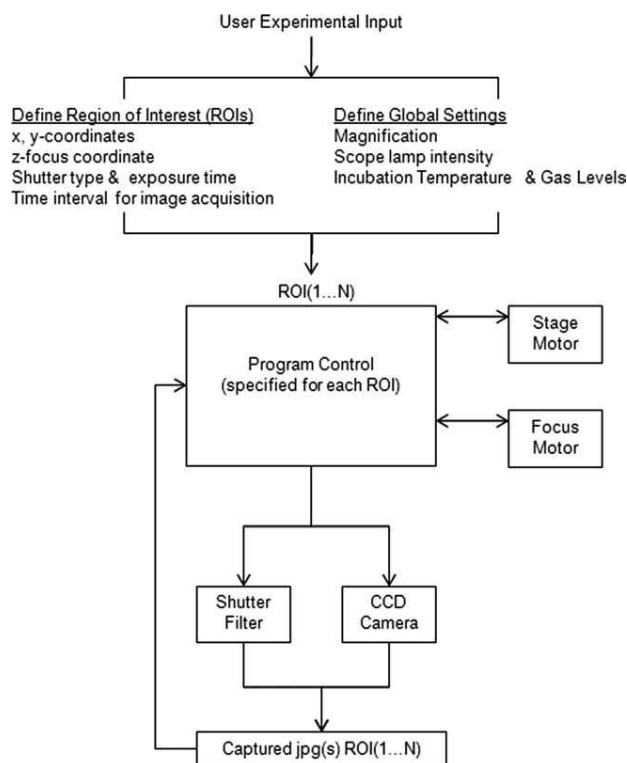


Figure 2. Control pathway.

The control pathway for our live automated cell imager (LACI) system is shown here. As in most systems, our system starts with user-defined input for each ROI—the *x-y* coordinates, the *z*-focus coordinate, the shutter type (visible and/or epifluorescence) and exposure times, and the time interval between image acquisitions. This data make up a single ROI. Global settings for all ROI include incubation conditions and magnification settings. To scan for *N* number of ROIs, the control Program examines the configuration of the ROIs on the specimen plate, and computes a path for stage movement. Stage motors supply positional data to compute the path to the next *x-y* coordinate and to set the appropriate focal plane. For each ROI, the shutter mode is specified and the CCD camera then captures a single image that is stored in an output file. The process is repeated *N* times until all the ROIs have been imaged for the scan interval. Data output consists of serial jpeg images of each ROI at all time intervals specified by the user.

Table 2. Biological Measurements of Time-Lapse Image Analysis

Parameters	Biological Relevance
<i>Cell Proliferation</i>	
Colony count, object count	To determine the growth rates of the cell population as a whole or for individual cells, e.g., in response to treatment with small molecules or drugs that may stimulate or inhibit cell growth.
Cell division time, mitotic fraction	
Proliferation count	
Population doubling time	
<i>Cell Morphology</i>	
Area, Circular radius,	To determine the health or differentiation status of the cells in response to small molecules, drugs or other variable. Cell shape and changes in cell shape may indicate viability or functional cell type.
Elongation, Perimeter,	
Max diameter, Min diameter,	
Roundness, Convex Perimeter,	
Compactness, Roughness	
<i>Cell Motility</i>	
Velocity, Linearity, Straightness,	To determine the motility of cells, e.g., as it may relate to cell chemoattraction to a specific agent or metastatic potential.
Percent motile,	
Unique Track Index,	
Track Boundary, Track Size	
Persistence	
<i>Cell Fluorescence</i>	
Fluorescent marker channel	To determine the temporal and spatial expression of signals by individual cells. Fluorescent tools are used to identify which cells show expression, e.g., of specific proteins or transcription factors.
Intensity	
<i>Cellular Ethology</i>	
Cell-Cell Interactions-frequency,	To determine how specific cells within the population interact with each other. This is the study of cell behaviors to understand the population as a whole.
proximity, subpopulation / subtype interactions,	
Duration of Proximity	

Numerous measurements are obtained from time-lapse images. Parameters generally fall into five categories—cell proliferation, cell morphology, cell motility, cell fluorescence, and cellular ethology. The biological questions that can be addressed are numerous. Here, we list examples of the biological relevance of each category.

available in most systems. Microscope magnification is varied according to the biological process of interest. For example, many cellular processes such as the migration of cells, the extension of cell pseudopodia, and cell division to two daughter cells, occurs on the scale of 10 μm to 1 mm, and 4 $\times$, 10 $\times$, and 20 $\times$ objectives are adequate. Imaging of bimolecular processes, such as translocation of transcription factors or changes in mitochondria membrane potential occurs on a scale of 10² nanometers and requires higher power 40 $\times$, 60 $\times$, and higher objectives. Fluorescent probes specific for these processes are powerful tools for tracking of such events.

Most systems listed in Table 1 can accommodate both transmitted light and epifluorescent imaging. Fluorescent probes are numerous, e.g., the green fluorescent protein (GFP, from the jellyfish *Aequorea victoria*) may be linked to most proteins of interest, or the molecule propidium iodide that intercalates with DNA and fluoresces red may be used to illuminate cell nuclei. These probes can reveal which cells are positive for the specific expression and can be used to localize the position of the positive signal (i.e., nuclear, cytoplasmic, or colocalized with additional markers). Pixel intensity differences in digital images also may be measured. The use of fluorescent molecular probes greatly expands cell biology studies to include mechanistic investigations of specific factors. Most commercial systems include automated filter and shutters, which are computer controlled by a custom program, to permit visible and epifluorescent images to be acquired in multiple wavelengths using specific filters.

The custom LACI system used in our lab consists of an inverted Nikon Eclipse TE 2000 U microscope and Photometric ES Cool Snap CCD camera, which captures both transmitted and fluorescent light. Depending on the experimental design, we use templates of preprogrammed locations and autofocus, or we manually select ROIs and focus (*x*, *y*, and *z* control) with a joystick control. A monitor displays a

live view of the optical field. Stacks of jpeg images are then acquired at user-defined intervals for up to 384 different locations. Some commercially available systems can be adapted to an existing microscope in a laboratory, whereas others are an integrated microscope system.

Image analysis

Several image analysis programs can provide cell object recognition and objective cell measurements from a sequence of images such as jpeg or tiff files. Measurements of cell activities are obtained by a number of commercially available packages (ImageProPlus, MetaMorph, Northern Eclipse, etc.), freely available open-source packages (ImageJ, CellProfiler), or user developed custom programs. At this time, fluorescent image processing is much more feasible than visible image processing, although several user-interactive programs provide robust imaging data. Analysis of the time-lapsed images provide data of (1) proliferation, e.g., live cell number, total cell number, apoptotic cell number;^{11,15} (2) migration, e.g., individual cell short-term and long-term motility: curvilinear, straight-line, angular, time-smoothed, and instantaneous velocities as well as persistence and speed;^{33–37} (3) individual cell morphology: area/size, length, breadth, best-fit ellipse, number of pseudopodia;^{33,38,39} (4) individual cell fluorescent molecular expression assigned to various probes, and (5) cellular ethology, e.g., dynamic cell–cell interactions (Table 2). Some approaches to the measurement of cell proliferation, morphology, and migration are shown in images of Figures 3–5. In the next section, we provide examples of the use of live cell imaging in stem cell biology experiments.

Visualization of Unique Cell Activity

Automated time-lapsed microscopy systems can reveal unique data on dynamic cell behavior. A number of studies

can be performed using these systems including: (1) cell growth kinetic assays, (2) migration and velocity assays, (3) cell differentiation assays, and (4) cell signaling assays—all of which may be performed using a high-throughput approach, and may measure multiple parameters simultaneously. Here, we describe assays that benefit from time-lapsed imaging data. We use several examples from stem cell biology experiments to illustrate these assays. In addition, the examples presented here, time-lapsed imaging has been a powerful tool to examine stem cell lineage histories/cell fate,^{15,17,40–43} population heterogeneity,^{15,39} stem cell asymmetry,^{15,17,41} and coculture effects.^{41,43,44}

Cell growth kinetic assays

Most notably, one can observe the increase in cell numbers in time-lapsed analysis of healthy growing populations. Although changes in total cell numbers over time are straightforward to collect,^{11,45,46} more subtle characteristics of cell proliferation kinetics may be recorded with time-lapsed imaging data.^{15,17,28,47} Investigators may directly measure the cell division time (CDT) for individual cells. This is the time-lapsed between cytokinetic events and generally takes 12–24 h depending on cell type. The actual event of cell splitting, cytokinesis, is visible over a 10- to 30-min interval (Figure 4A). No other biological assay provides as accurate a measure of cell division as direct observation of time-lapsed images. Other methods such as labeling with bromodeoxyuridine or tritiated thymidine pro-

vide only indirect measures, as does CDT estimation based on the exponential growth equation [the CDT is often indirectly estimated, and sometimes confused, as the population doubling time (PDT)]. The PDT is readily calculated from time-lapsed data and the exponential equation

$$N_i = N_0 * e^{k*t_i} \quad (1)$$

where $k = \ln 2 / \text{PDT}$, N_i is the number of cells at time t_i , N_0 is the initial number of cells and, therefore, $\text{CDT} \leq \text{PDT}$.

In addition, a variation of this Eq. 1 may be used to obtain information about subsets of nondividing cells that may exist among cell populations; and their percent distribution may be measured. For example, quiescent nondividing cells are critical cells in a stem cell population and may be directly identified based on the lack of cell division of the cells, or their percentage may be calculated from the Sherley equation

$$N_i = N_0 \left(0.5 + \frac{1 - (2\alpha)^{(t/\text{CDT})+1}}{2 - 4\alpha} \right) \quad (2)$$

where α is the mitotic fraction and $1 - \alpha$ is the nondividing fraction.^{11,48} To date, there are no protein or molecular markers, which directly identify quiescent cells. Therefore, the ability to identify this subpopulation through its nondividing behavior, as revealed through time-lapsed imaging, presents a unique and valuable approach to study these cells.

Time-lapsed imaging systems allow researchers to scale down conventional cell biology assays (10^0 – 10^3 per cm^2),

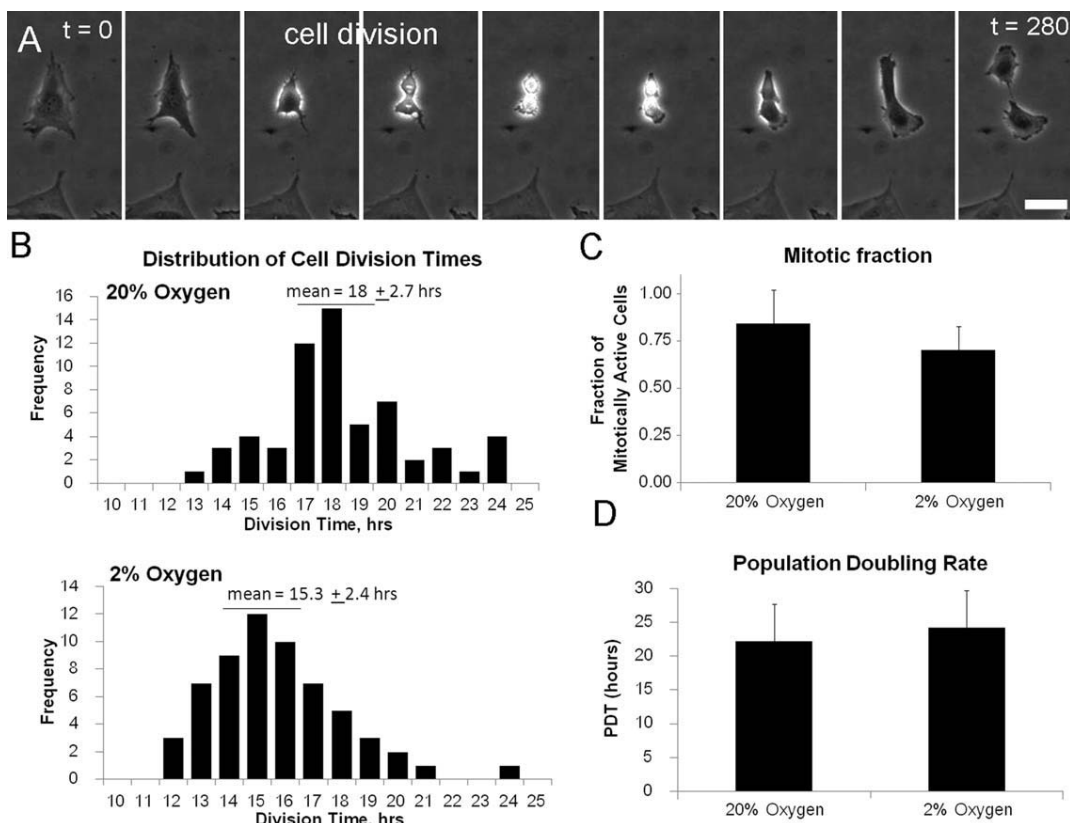


Figure 3. Time-lapse data of cell proliferation behavior.

(A) Cell division occurring in a human mesenchymal stem cell population. Total time lapsed is 280 minutes, Scale bar represents $50 \mu\text{m}$. (B) The distribution of CDTs, or cell cycle times, in human mesenchymal stem cells grown in 20% and 2% oxygen environments. Direct cell cycle measurement is uniquely possible with time-lapse images. (C) The mitotic fraction or fraction of dividing cells in the population is also uniquely estimated here and is based on the growth data and CDT. (D) Cell population doubling time (PDT) is readily calculated from the growth curve data.

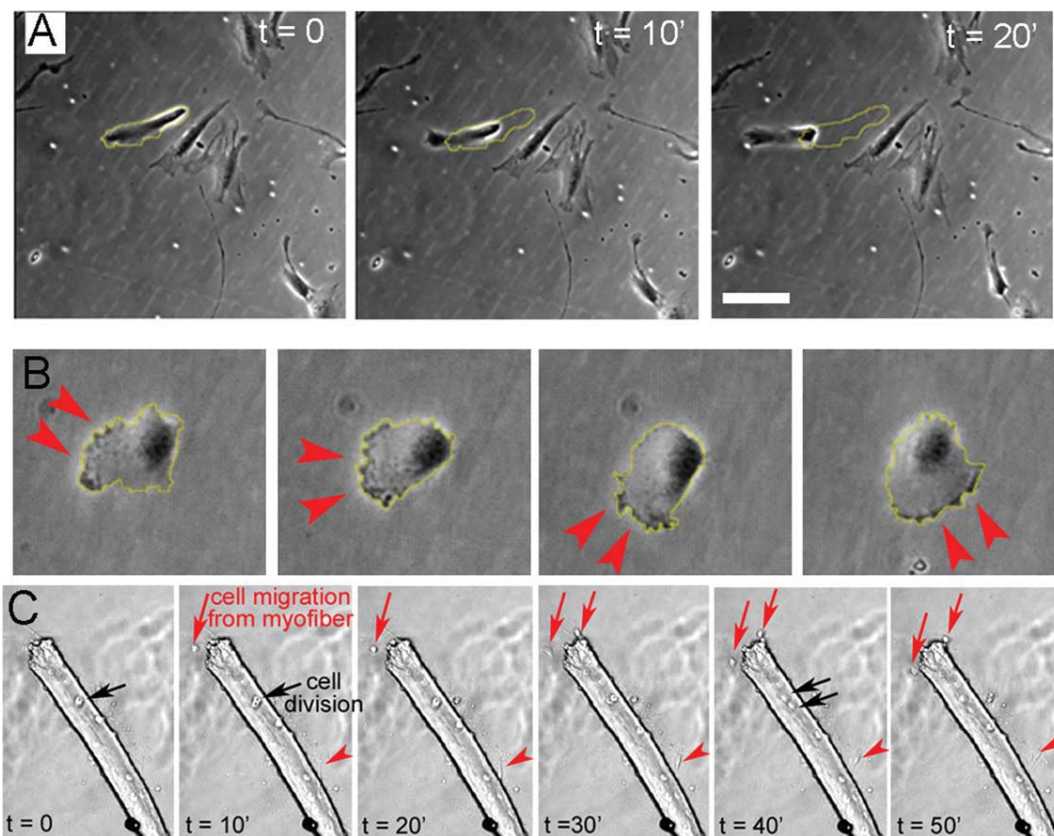


Figure 4. Time-lapse images of cell migration.

(A) At $t = 0$, a yellow line trace shows the cells original morphology and location. At $t = 10$ minutes and $t = 20$ minutes, the cell migration can be observed relative to the original position (yellow line trace). Scale bar represents $100 \mu\text{m}$. (B) Higher magnification shows pseudopodia (red arrowheads). Movement of pseudopodia may be quantified from morphological changes that are quantified from yellow trace data. Open access programs like ImageJ (National Institutes of Health) can be used to collect data regarding the cells morphology and migration. (C) Time-lapse activity may be observed with explants tissue. A skeletal muscle fiber was dissected from muscle tissue and adhered to the flask using extracellular Matrigel. Cells on the fiber (black arrow) are classically called muscle satellite cells and can be observed to migrate and divide on the membrane of the explanted myofiber. Other cells migrate out from the muscle fiber (red arrows).

which typically operate with larger numbers of cells ($>10^3$ – 10^5 per cm^2). The conventional approach to study growth kinetics is to use multiple flasks, each flask being used for a different timepoint. Cells are counted in a hemocytometer or flow cytometer, both of which require high cell numbers on a scale of 10^4 – 10^5 cells. Conversely, the multichamber plates used in most time-lapsed imaging systems are of small surface area and allows for the use of small quantities of cells, 1–1,000 cells. For example, in a 24-well plate, 2,000 stem cells per well is often sufficient.¹¹ In fact, many key assays require examining single cells that may give rise to a more numerous colony. With the correct optics, these systems can monitor contact inhibition and densities, which range from a single cell to 100% confluency.

Time-lapsed imaging also significantly reduces man-hours by automating location recall and image acquisition. The system captures and stores images without any human input after the initial selection of the ROIs. This enables the experiment to be completed over several days without the constant watch of the experimenter. For example, an experiment that examines cell proliferation among five different cell types/donors or treatments over a course of three time-points, would require ~ 10 h of cell culture work after setup. The same experiment would require ~ 3 h of image analysis work after setup (and the time-lapsed record would be available for any future analysis such as migration measurements).

Stem cell kinetics must be understood for ex vivo expansion and biomanufacturing of stem cell products and design *in vivo* delivery systems. To illustrate the use of time-lapsed imaging for measuring cell proliferation, human umbilical cord mesenchymal stem cells (UC-MSCs as previously characterized in detail⁴⁹) were examined in the LACI imaging system to compare growth in 2% vs. 20% oxygen conditions (controllable oxygen environment). As stem cells used therapeutically will be in a physiologic low-oxygen environment ($\sim 2\%$ in most cases), it is important to understand how cells proliferate under these conditions. Cell division distributions illustrate the range of CDTs was significantly different under these two oxygen environments, at 2% O_2 , the mean CDT was 15.3 ± 2.3 h and at 20% O_2 , the mean CDT was 18 ± 2.7 h (Figure 3B, $P < 0.05$). Next, the mitotic fraction, or fraction of cells within the population, which are actively dividing, can then be calculated by performing a nonlinear regression best fit of the experimental cell growth data (N vs. t) using Eq. 2 developed by Sherley et al. and the measured CDT.^{15,48} Here, we observed no significant difference but a trend toward more dividing cells in the UC-MSCs grown in 20% O_2 (Figure 3C). Together, these values may be used to calculate the PDT as previously described¹¹ or more simply PDT may be calculated from the growth data and the use of Eq. 1. The PDT depends on both the CDT and the mitotic fraction, and in this analysis, we observed no overall difference in PDT between cells grown in 20% vs. 2% for 48 h (Figure 3D).

Migration and velocity assays

Cell migration is examined in variety of experiments designed to test the role of specific small molecules or growth factors on cell motility, or the mechanics of cell movement on different substrata, or the metastatic potential of genetic changes within cells, to name a few.^{18,50–53}

From 2D images, the x - y position of cells can be assigned according to the 2D centroid of the cell, and this is obtained from object/cell traces (Figure 4A). Changes in x - y position between imaging intervals or sequential frames are used to calculate cell displacement, cell velocity, total distance traveled, and similar measures. A particularly interesting parameter of migration is persistence time, or the amount of time that a cell moves with directional persistence before it changes direction by more than 60°:

$$\langle d^2(t) \rangle = 2S^2[Pt - P^2(1 - e^{-t/P})] \quad (3)$$

where d^2 is the mean square displacement, S is the root mean square speed, and P is the persistence time.^{37,54}

Pseudopodia movement may also be examined via time-lapsed imaging (Figure 4B). The cell as a whole moves slower than its leading edge lamellipodia, and, therefore, scan intervals of less than 5 min may be used to study the mechanics of lamellipodia movement.

Other unique experimental questions regarding cell migration and activity may be answered using time-lapsed imaging, especially when direct observation of cell behavior provides the most definitive evidence. For example, time-lapsed imaging was used to examine the dynamics between muscle fibers (>100 μ m diameter) dissected from skeletal muscle tissue and the satellite stem cells (<10 μ m diameter) that reside on the fiber *in vivo*. Both migration and division could be studied (Figure 4C).

The “scratch wound” assay is an example of how time-lapsed analysis may be used to understand stem cell migration. In the “scratch wound” assay, a confluent layer of cells is wounded by manually scratching a region of the plate with a glass or plastic cell scraper to remove all cells, then, the movement of cells into the wound is studied (Figure 5A,B). Variables in these experiments may include different stem or progenitor cells different supplements to the growth media or alterations in the binding proteins on the cell surface. In this example, we examined endothelial progenitor cell migration into an $\sim$ 800- μ m-wide scratch wound over the course of 36 h. Time-lapsed image sets were quantitatively analyzed using custom-developed programs (Cytotracker, Kairos Instruments). At time = 0, we selected individual cells from two different regions to be tracked. Cells in Region 1 were at the edge of the scratch wound, whereas cells in Region 2 were $\sim$ 200 μ m away from the scratch edge (Figure 5C). We tracked the x - y position of the cell centroid for each 10-min interval frame over the course of 36 h (or 216 image frames per cell). The x - y data was used to determine total area traveled, mean cell velocity, and mean cell persistence for cells in each region. Cells at the free edge had a significantly larger mean cell velocity (0.34 ± 0.04 μ m/min) when compared with cells within the confluent Region 2 away from the edge (0.29 ± 0.03 μ m/min, $P = 0.046$, two-tailed t -test, Figure 5D). We also detected a significant decrease in cell velocity over time for cells in the confluent region (0.31 ± 0.03 μ m/min during the first 12 h when compared

with 0.25 ± 0.04 μ m/min during the final 12 h, $P = 0.036$, Figure 5E). However, there was no change in cell velocity for cells at the scratch wound edge during the course of the 36 h. Finally, although there were differences in cell velocity within the two regions, there were no differences in the persistence of cell migration (Figure 5F). The mean directional persistence time for cells at the scratch wound margin was 2.5 ± 0.8 min, when compared with a mean persistence time of 2.6 ± 0.9 min for cells within the confluent Region 2. This example illustrates the capability of time-lapsed imaging to obtain several measures of cell migration and in particular it detected differences in the migration characteristics depending on the location of the cell relative to the wound. Because the automated systems allow for examination of multiple wells within an experiment, a number of cell types can be examined in side-by-side analysis.

Understanding how stem cells migrate at the site of delivery after direct transplantation, or after transplantation via a scaffold or other carrier, is a major challenge in stem cell research. In some applications, cell dispersal and migration to the wound site is desirable. In other applications, the goal is to keep transplanted cells on the carrier material. The *in vitro* information related to migration characteristics can be incorporated into design variables for tissue-engineered constructs.

Cell differentiation assays

A key characteristic of stem cells is their ability to differentiate to other cell types. Morphology may be used to identify cell types, e.g., endothelial cells, myotubes or neurons all have distinctive morphologies. Morphological changes may be measured in time-lapsed images to determine if stem cell differentiation has occurred; visual changes in the cells' morphology may be observed over time using freely available programs such as ImageJ.^{39,55} In cases where there is no clear morphological change, fluorescent signaling may be used to illuminate those cells that express a specific marker. These approaches allow for quantification of cell differentiation from time-lapsed imaging data. Further, the high-throughput capability of the automated systems greatly facilitates the screening of a large number of conditions that may induce differentiation states. As a number of factors still need to be explored in the area of induction of stem cell differentiation—including variations in biochemical signal concentrations and types of biochemical signals and variations in substrate concentration and substrate type—time-lapsed imaging technologies provide an ideal screening platform.⁵⁶

Automated time-lapsed systems also have greatly facilitated the understanding of stem cell heterogeneity.^{15,57–59} Stem cell populations may be composed of cells at different stages of differentiation, or entirely different cell types. Studies that aim to discover the exact cell that is responsible for a particular activity are critical and involve clonal experiments, which are initiated with a single cell. Heterogeneous stem cell populations may be physically separated into single cells using fluorescence-activated cell sorting or limiting dilutions in which 1 cell is delivered into the chamber well of a multiwell plate. Using conventional methods, each well (sometimes >300 wells) are manually examined and periodically re examined for 2–3 weeks for proliferation or differentiation. The clonal technique is streamlined and definitive evidence of clonality is provided by time-lapsed images.

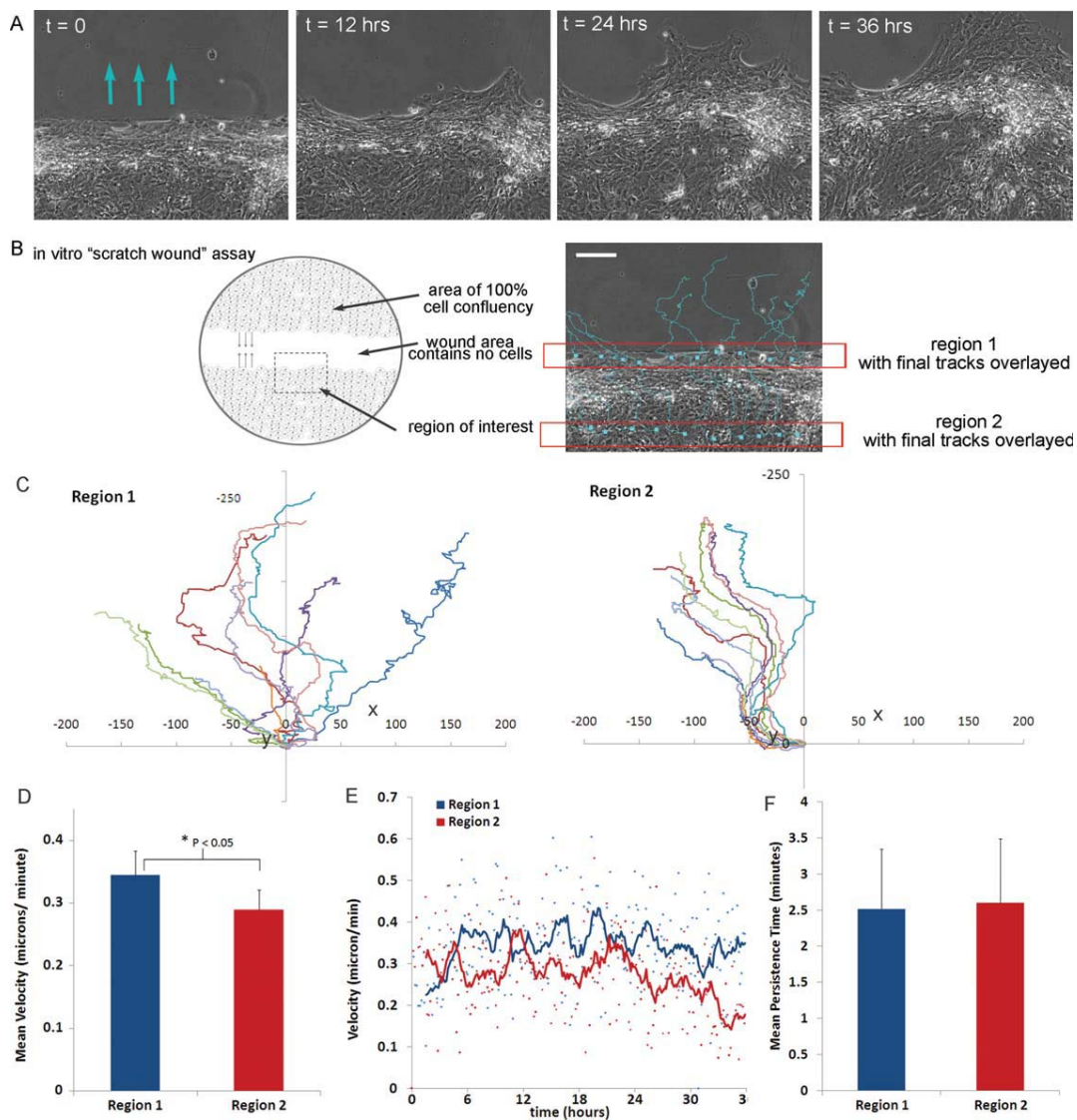


Figure 5. Time-lapse data of cell migration behavior.

(A) In the “scratch wound” assay, a confluent layer of cells is wounded by manually scratching a region of the plate to remove all cells. In this analysis, cell migration into the “wound” occurs over several days. In this example, the total wound area was $\sim 800\text{-}\mu\text{m}$ -wide (entire wound not shown) and we observed cells on the edge of the wound to infiltrate about one-third of the scratch wound area by 36 h. (B) Schematic of assay. We quantified the movement of individual cells in two different regions—cells in Region 1 were at the edge of the scratch wound, whereas cells in Region 2 were $\sim 200\text{ }\mu\text{m}$ away from the scratch edge. (C) Cell tracks were obtained from the x-y positions of each cell for each 10-min interval frame over the course of 36 h (216 image frames per cell). Cells in both regions moved in the direction towards the scratch wound, with no cells migrating inwards toward the confluent region. (D) The mean cell velocity for cells at the free edge had a significantly faster mean cell velocity ($0.34 \pm 0.04\text{ }\mu\text{m}/\text{min}$) as compared to cells within the confluent Region 2 away from the edge ($0.29 \pm 0.03\text{ }\mu\text{m}/\text{min}$, $p = 0.046$). (E) There was no change in cell velocity for cells at the scratch wound edge during the course of the 36 hours, however, there was a significant decrease in cell velocity over time for cells in the confluent Region 2 ($p = 0.036$). (F) There was no difference in the mean directional persistence of cell migration for the two regions (Region 1; 2.5 ± 0.8 minutes, and Region 2; 2.6 ± 0.9 minutes).

Systems can be fully automated to position each well location for image acquisition. Most systems also provide auto-focusing for each location. In addition, the incubator allows for the plates to remain in the system for extended periods. Automated systems provide continual repositioning and monitoring of the plate, to set locations, focus the field of view and acquire images over defined time intervals. As only a low-percentage of single cells may form clones, it is necessary to maximize the number of initiating cells, and the investigator is spared the tedious task of relocating hundreds of locations. In addition, when the interval is set for frequent image acquisition, unique information regarding cell division, and cell migration can be obtained. The resulting cell colonies can then be followed in screening assays, which

examine cell activity (with minimal to no cell flask disturbance) after exposure to various compounds, using cell proliferation or cell differentiation assays.

Cell signaling assays

Fluorescent tools allow signaling networks within stem cells to be understood. Knowledge of the signaling pathways within stem cells can establish targets to allow for directing cell fate toward proliferation or differentiation after *in vivo* transplantation. Fluorescent probes may be used to visualize the presence of specific cell surface receptors or intracellular or nuclear factors during proliferation, differentiation, or migration activities. The genetic tools provide a fluorescent

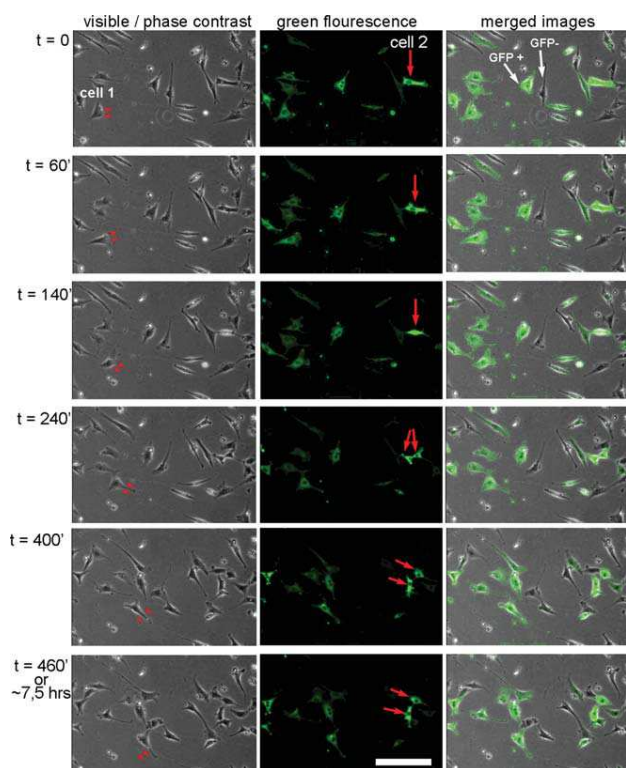


Figure 6. Transmitted light and green fluorescent time-lapsed imaging of cell growth.

Fluorescent images, captured within 0.25 s of the phase contrast image, show only cells which are expressing the GFP in a demonstration of how signaling pathways may be studied. Further measurements of cell morphology, migration and proliferation illustrate the complexity of the data. Phase contrast images in column 1 show heterogeneity in cell shape or morphology. Individual cells may be tracked and their migration paths are recorded to obtain several measures related to velocity and direction of movement. Red arrowheads indicate the direction of movement of Cell 1. Cell division is also visible in fluorescent (and phase contrast) images; red arrow shows Cell 2 before cell division, and upon cell division, daughter cells are shown by 2 red arrows. Visible and fluorescent images are merged to show clearly the heterogeneity of GFP expression. From images such as these, a number of parameters of cell activity can be measured, as listed in Table 2. Scale bar represents 100 μm .

signal that not only reveals which cells may be expressing the given factor, but the probes may also reveal the location of the signal, e.g., cytosolic, mitochondrial, or nuclear. GFP is commonly used to tag specific proteins. This may be in the form of specially designed vectors where GFP is under the control of a specific promoter of a protein of interest, or the GFP may be a fusion protein that is physically associated with the protein of interest. Also, dyes such as annexin-V or propidium iodide may be used to identify cell apoptosis or cell necrosis.

Fluorescent images may be captured within 0.25 sec of the phase contrast image, in the LACI system. Figure 6 illustrates the population heterogeneity that may occur in stem cell biology assays. Visible and fluorescent images are merged to show clearly the heterogeneity of GFP expression. Molecular and genetic tools can be designed such that cells temporal and spatial fluorescent signals indicate when a specific transcription factor is expressed or when it is phosphorylated or dephosphorylated for example. Additional colors may be acquired simultaneously, as aided by an automated filter wheel. Long-term phototoxicity may be controlled by

adjusting the fluorescence exposure time or the frequency of fluorescence capture or by the use of red or infrared light. This example illustrates a subset of cells, which express GFP and also shows the dynamics of morphology, migration and proliferation that may be measured simultaneously.

Future Directions for In Vitro Live Cell Imaging. Although the hardware components for this technology have greatly advanced its use in cell research, further developments in the areas of fluorescent biological tools and image analysis programs will add to the power of this approach. There are numerous fluorescent tags available to label dead cells, subcellular components such as mitochondria, or charged free radicals in the cytoplasm; however, much more effort is involved to label specific proteins or transcription factors. These specific labels often involve fusing a fluorescent protein to the protein of interest, and this introduces questions regarding how the biology, especially in a live cell imaging setting, is affected. Surprisingly, there has yet to be developed a nontoxic nuclear label comparable to DAPI, 7AAD or propidium iodide that can readily label the nucleus of live cells. On the upside of this, the imaging tools to automate the analysis of fluorescent images are well developed. Because of the clear contrast that is created in these images, there are numerous programs available for these time-lapsed images. Phase contrast and brightfield images, on the other hand, have less image contrast, are harder to segment, and subsequently, there are far fewer programs available for this approach. Recently, algorithms developed for other applications are being applied to phase contrast time-lapsed image analysis with promising success.^{17,19}

Although there is also great interest in tracking cells in a three-dimensional (3D) environment, programs to acquire such images are not readily available. Autofocus features are available on most commercial live cell imaging systems, yet reconstruction of cell movement in the 3D environment is not readily available. Certainly, the future of time-lapsed imaging in cell science will rely on improvements in phase contrast/brightfield imaging and 3D cell tracking.

Conclusions

Live-cell imaging provides a tool to capture unique data of cell behavior. The technological advancements in computer processing, digital imaging, automated controls, and data handling, create a robust tool for basic and applied biomedical research. Several commercial systems are available, and many existing laboratory microscopes are adaptable for live-cell imaging capabilities. We highlight, here, the common hardware components of the various systems and we describe the benefit of these features to cell biology experiments. Experimental questions related to cell proliferation, migration, cell-cell interactions, and cell signaling may be addressed using these systems. This technology greatly enhances cell science by providing a unique dataset to quantify temporal changes in cell populations. Practically, the technology also drastically reduces man-hours, consumes fewer laboratory resources, and provides standardization to assays for cell research.

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