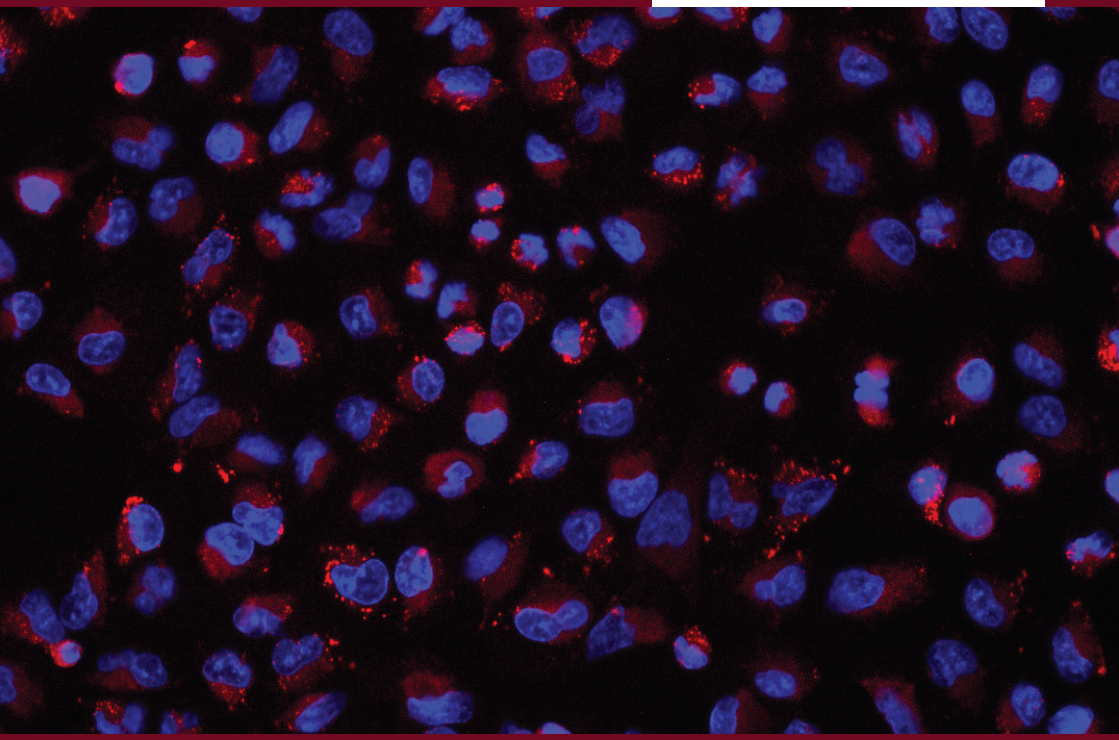
Current Protocols
The Fine Art of Experimentation



Live-Cell Imaging

Essential Knowledge Briefings

ThermoFisher SCIENTIFIC

COVER-IMAGE CAPTION: Hypoxia was studied in A549 cells labeled with Image-iT™ Hypoxia Reagent. Cells were exposed to 1% O_2 levels for a period of 15 minutes prior to imaging. Under normoxic conditions (20%), there was no signal from the Image-iT Hypoxia Reagent, but in response to the decrease in oxygen levels, the signal from the Image-iT Hypoxia Reagent increased, as shown by red, punctate staining in nearly all of the cells in this image. Nuclei (blue) were visualized by using NucBlue™ Live ReadyProbes™ Reagent. Cells were imaged on the EVOS™ FL Auto Imaging System with onstage incubator using a 20x objective.

Front cover image courtesy of Trillium Blackmer and Mike O'Grady (Molecular Probes, Thermo Fisher Scientific, Eugene, OR, USA).

Prepared for Wiley and Thermo Fisher Scientific, Inc. by EKB Editors, Mike May, Ph.D. and Gary Heebner, Ph.D.

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INTRODUCTION

Although prepared slides reveal many details about biological specimens, they always leave at least one question dangling: How does this look when it's alive? A biologist wonders how the organism or molecular component behaves in a dynamic, natural context, and how it interacts with its environment, making up the very foundation of live-cell imaging. In its most basic form, live-cell imaging allows biologists to examine and analyze living samples. This capability completely changes what can be learned about life, and how that information can be used—from basic research through biotechnology and medicine.

One of the key requirements of this field is having live and healthy specimens, which can be imaged statically, like taking a snapshot of a living biological sample. Live-cell imaging can also involve time-lapse methods, which provide a biologist with dynamic information by periodically taking images over time, showing, for example, how a protein moves in a membrane over time.

In fact, cellular dynamics provides a keen area of interest. Applying live-cell imaging on a cellular level exposes biomolecular processes in action. That information—such as observing one protein interact with another—reveals functional information. To get the right information, though, the specimen must remain alive and as unperturbed as possible. The sample's environment must encourage the most natural—and unaltered—conditions that

can be maintained. Some advanced tools, including incubators, help biologists preserve a sample's natural conditions with the simultaneous ability to image ongoing processes. In addition, a biologist requires tools that label and help to track specific elements, like proteins, in a sample. The development of a broad range of fluorescent proteins completely transformed the ability to label very specific structures and track their location. Moreover, existing options makes these tools easy to use, even when tracking multiple targets.

To get started in live-cell imaging, a biologist uses several tools: reagents to keep the cells alive, dyes or other markers to label specific molecules or structures, an incubator to provide the right environment, plus a microscope and a digital imaging system. Many cells and tissue never experience light, so the tools used for live-cell imaging must be as gentle as possible—reducing phototoxicity to the sample. That means using a microscope that makes the best of the available light, and a detection system that creates images from the least light possible. The advances in basic digital cameras push the possibilities far beyond what was conceivable even a few years ago.

This Essential Knowledge Briefing introduces readers to the general field of live-cell imaging. It explains the fundamental challenges and solutions, as well as describing some of the applications of live-cell imaging through case studies, and it forecasts the key advances ahead.

HISTORY AND BACKGROUND

Live-cell imaging started long ago. On the simplest level it requires very little in terms of equipment. Using a magnifying glass to look at pond water, for example, is live-cell imaging. Things got much more interesting in the late 17th century when Dutch scientist Anton van Leeuwenhoek made a microscope that could magnify images by about 270 times. In fact, he used this microscope to reveal many of the microscopic creatures living in a drop of water.

To create a real image, though, scientists needed more than their own eyes. In 1839, English photography pioneer William Henry Fox Talbot made the first photomicrograph. That opened the door to making static images of living cells.

More than half a century passed before someone made the first dynamic, or time-lapse, images of live cells. In 1907, Swiss biologist Julius Ries filmed the fertilization and following development of a sea urchin egg. In fact, Ries made this film for teaching medical students that cells come from cells and that cells alone make up an organism.

In 1914, scientists could buy a microcinematographic device to more easily make movies of living organisms. In the early 1930s, American scientist Warren H. Lewis filmed the process of pinocytosis.

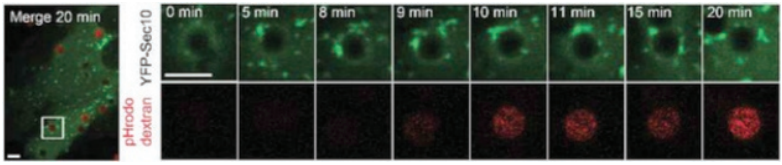
Instead of just watching living cells from the outside, phase-contrast microscopy let scientists see inside, observing the organelles. Kurt Michel of the Carl Zeiss microscope company brought this technique to time-lapse imaging in the early 1940s.

Not every scientist, however, appreciated live-cell imaging at that time. In the 1940s, for example, Nobelist Peter Medawar wrote

in his memoir that biologists making films of living cells have been "delighted, distracted, and beguiled by the sheer beauty" of the cells, but he did not think that such approaches would "solve biological problems."

Medawar's opinion aside, other scientists moved ahead. In the 1950s and 1960s, for instance, British cell biologist Michael Abercrombie used 16-millmeter films to record cell movement. This migration takes place in healthy ways, such as development, and during disease, such as metastasis of cancer.

In parallel with improvements in imaging technology, crucial changes in labeling started in the late 1800s, when scientists started to develop synthetic fluorescent dyes. By the 1920s, biologists started using these with fluorescence microscopy to view bacteria and other samples. The development of cell-permeable acetomethoxy forms of dyes allowed scientists to image processes such as migration, mitochondrial dynamics and calcium or pH changes. Attaching dyes to ligands—for example, transferrin or epidermal growth factor (EGF)—facilitated their internalization/uptake to be studied.



Knockdown of exocyst and Rab11 impairs the acidification of endothelial cell phagosomes. HUVEC expressing YFP-Sec10 were preloaded with pHrodo™ dextran for 15 minutes, reacted with invasin-coated beads and imaged by spinning disc live-cell microscopy. Enlarged still frames from a video (not shown) depict a single phagosome the position of which is indicated in the Merge 20 min picture. Scale bars, 5 µm. (From: Rauch L, Hennings K, Aepfelbacher M. 2014. A role for exocyst in maturation and bactericidal function of *Staphylococci*-containing endothelial cell phagosomes. *Traffic* 15:1083–1098. doi: 10.1111/tra.12189.)

Perhaps the most transformative step in live-cell imaging came from a jellyfish, *Aequorea victoria*, which gave biologists green fluorescent protein (GFP). The path to this fluorescent marker started in the 1960s and culminated in American biologist Martin Chalfie's 1994 article in *Science* that reported the expression of wild-type GFP in *E. coli* and *C. elegans*. These findings suggested that this marker could provide *in vivo* fluorescence in a wide variety of cells and organisms. For example, scientists could label specific proteins with GFP in living cells. Consequently, GFP allows the analysis of protein-protein interactions in live cells.

The work on GFP earned the 2008 Nobel Prize in Chemistry for Chalfie, Roger Tsien of the University of California at San Diego and Osamu Shimomura of Boston University.

One fluorescent marker's huge impact set off the search for others. For one thing, mutations in GFP change its spectral properties—both the wavelength that excites the marker and the wavelength that it produces. As a result, scientists developed *Aequorea*-based markers in blue, cyan, green and yellow. Despite vast genetic-engineering efforts, scientists could not make a useful red marker from *Aequorea*.

Other organisms, though, use proteins similar to GFP. For example, Anthozoa corals come in many colors, and scientists isolated proteins that cover the entire visible light spectrum from them. In particular, this organism produces a red marker, DsRed, which provides many useful qualities, including photostability and its red-shifted emission of fluorescence. Nonetheless, the wild-type version of this marker tends to form tetramers that can change the behavior of the attached organism. Through 33

amino-acid substitutions, Tsien and his colleagues created mRFP1, which is a monomeric red-FP.

Further modifications of the amino acids of mRFP1 created markers that emit a range of fluorescent colors, and these labels are now known as mFruit: mHoneydew, mBanana, mOrange, mTangerine, mStrawberry and mCherry—named for fruits with colors that resemble the emitted wavelength.

The complete range of existing fluorescent proteins (FPs) is a long list with equally broad physical and biological characteristics. Moreover, new markers and modifications of existing FPs keep emerging.

In addition, today's FPs offer a growing number of features for tracking dynamics in live cells, especially in super-resolution microscopy, which exceeds the diffraction limit that traditionally restricted the resolution of light microscopy. These phototransformable FPs come primarily in three categories: photoactivation, photoconversion and photoswitching. With a photoactivatable FP, violet or ultraviolet light more or less turns it on—increasing its very low emission to a high level. In photoconversion, light can be used to switch the bandwidth of an FP's fluorescent emission. Last, in an FP that can be photoswitched, specific wavelengths of light turn it on or off, reversibly, and these can be used in live-cell super-resolution imaging, for protein tracking and even to control a protein's activity with optical signals.

In the early 2000s, live-cell imaging became more mainstream, especially as FPs became widely available. A search of "fluorescent protein" on PubMed.gov for 2000–2014, for example, returned nearly 34,000 articles.

IN PRACTICE

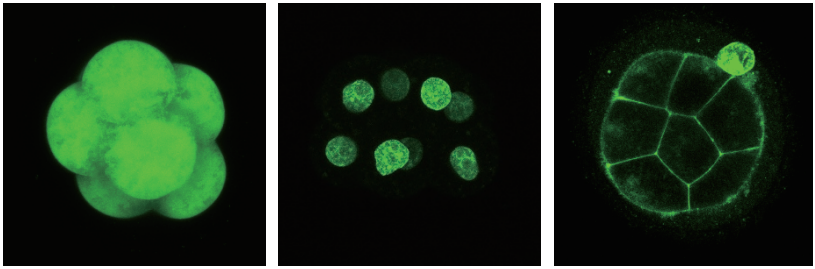
Live-cell imaging applies to a wide range of biological applications, including basic and medical research. A large amount of the research involves proteins. These molecules always move, and only live-cell techniques can track them dynamically—at least without making many assumptions. With molecular probes, specific proteins can be tracked in space and time in live cells. This kind of research reveals how proteins move and interact.

Tracking the movements of proteins helps biologists understand cell signaling; documenting proteins involved in a variety of reactions. In addition, the movement of proteins in membranes can be studied through live-cell imaging. For example, proteins and lipids diffuse in the cell membrane, and that movement depends on the structure of the membrane, including the cytoskeleton inside the cell, and the particular biomolecules being tracked. This movement in the membrane also plays a role in cell signaling. Using FPs, these protein movements can be tracked. In fact, multiple proteins can be simultaneously tracked in the membrane of live cells.

Beyond simply tracking proteins moving in a membrane, live-cell imaging can also be used to investigate membrane recycling, including endocytosis and exocytosis. For example, total internal reflection fluorescence (TIRF) microscopy can be combined with FPs to study plasma-membrane events. In addition, biologists often use fluorescent dyes for research on membrane turnover.

The movement of proteins outside of live cells also plays a fundamental role in development. For example, morphogenic gradients in embryos guide many growth processes, such as the

pathways followed by growing neurons. Tracking proteins in live cells can help biologists understand these processes.

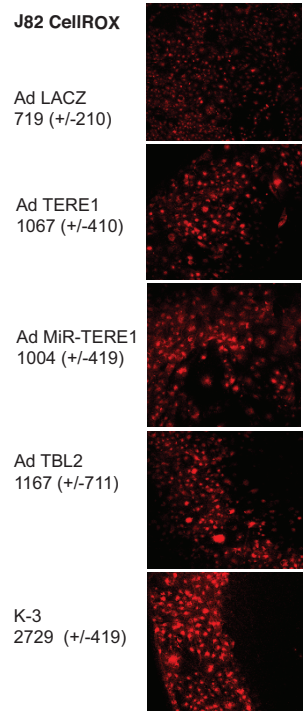


Characteristics of nucleus markers and membrane markers. Images of 8-cell-stage embryos. The left panel is a *CAG-Venus* mouse embryo expressing a native fluorescent protein Venus, the middle panel is *R26-H2B-EGFP* expressing a nucleus marker, and the right is *R26-Lyn-Venus* expressing a membrane marker. (From: Abe T, Fujimori T. 2013, Reporter mouse lines for fluorescence imaging. *Development, Growth & Differentiation* 55:390–405. doi: 10.1111/dgd.12062.)

Even the most fundamental process in development—mitosis—can be studied with live-cell imaging. For example, researchers could use an FP to label key structures in the process, such as the tubulin in the mitotic spindles. Then, with live-cell imaging the function of the labeled molecule can be studied under normal and experimentally altered conditions, such as using taxol to suppress the action of the microtubules.

Medical researchers also use live-cell imaging. In fact, live-cell imaging can be applied to a wide range of medical-research questions. In many cases, the boundary between basic and medical research gets blurry with live-cell imaging. For example, biologists can study exocytosis to see how it impacts a cell, and problems with exocytosis appear in a range of medical problems, from allergies and hormonal conditions to neurodegeneration.

With the right experimental setup, researchers can explore the potential medical consequences of a live cell's environment. Something as simple as controlling the oxygen levels of a live cell being imaged can reveal mechanisms of disease or possible treatments. For instance, so-called hypoxia inducible transcription factors play a role in a range of diseases, including cancer. By manipulating a live cell's oxygen environment, these factors can be studied. In addition, biopharmaceutical researchers explore these factors as potential therapeutic targets.



Ectopic expression of TERE1, TBL2, or vitamins K-2 or K-3 elevate cellular ROS. J82 cells were treated for 60 h with Ad-LACZ, Ad-TERE1, or Ad-TBL2 or incubated for 1 h with vitamins K-2 or K-3 (30 µM). Confocal imaging was performed after loading cells with 5 µM of CellROX™ deep red or dihydrorhodamine 123 fluorogenic probes. Cellular fluorescence intensities were quantified after off-cell background subtraction. (From: Fredericks WJ, *et al.* 2013. The TERE1 protein interacts with mitochondrial TBL2: Regulation of trans-membrane potential, ROS/RNS and SXR target genes. *Journal of Cellular Biochemistry* 114:2170–2187. doi: 10.1002/jcb.24567.)

The following case studies portray several specific uses of live-cell imaging.

CASE STUDY ONE

Phagocytes provide part of the innate immune response by engulfing and internalizing foreign material—such as cells and microbes—through a process called phagocytosis. A protein called high mobility group box 1 (HMGB1) participates in many disease-related functions, including modulating inflammation and ingesting dead cells. Past research showed that extracellular HMGB1 inhibits phagocytosis, and Edward Abraham of the department of medicine from the University of Alabama at Birmingham in the United States and his colleagues wondered if this protein also had an intracellular impact.

To explore this concept, the scientists combined bone marrow–derived macrophages (BMDMs) with apoptotic cells, which were labeled directly through N-hydroxysuccinimide ester chemistry with a pH-sensitive dye. This detects the phagocytosis of the apoptotic cells by macrophages. In these experiments, the cells were fixed and processed for immunocytochemistry (ICC) using a green dye attached to a species-specific secondary antibody. In other experiments in this paper, two different proteins were detected, using ICC, with green and red dyes. In addition proteins were also detected using ICC (green dye) and red dye–conjugated phalloidin, which labels F-actin.

The results showed that HMGB1 started largely in the nucleus, and its levels increased in the cytoplasm after the BMDMs ingested the apoptotic cells. These studies also showed HMGB1 in the cell membrane, as this protein moved from the nucleus to the cytoplasm to the extracellular space.

After seeing that HMGB1 increased in the cytoplasm and extracellular space after phagocytosis, the scientists wondered if this protein impacted the process in both compartments. In following experiments that blocked the expression of HMGB1, phagocytosis increased. The researchers showed that even fibroblasts—which the scientists described as "not professional phagocytes"—participated in more phagocytic activity when HMGB1's expression was limited.

Abraham's team drilled down further by showing that reducing the levels of HMGB1 freed other phagocytic-related processes inside of cells, including the activity of a protein, Rac-1, that drives some of the changes in a cell's cytoskeleton during phagocytosis.

This work shows the value of using a live-cell reporter followed by ICC, which uses dyes.

[*Journal of Immunology*, 2011, **187**, 4686–4694 (DOI: 10.4049/jimmunol.1101500)]

CASE STUDY TWO

Around 1950, physicians started using positive-pressure mechanical ventilation, which reduced mortality for polio patients who needed assistance breathing, but wide use of this technology revealed the potential to cause lung damage. So-called ventilator-induced lung injury can be deadly in someone with adult respiratory disease syndrome. Bioengineer Susan Margulies at the University of Pennsylvania in the United States studies the fate of cells damaged through mechanical deformation, including ventilator-induced lung injury.

Using rat alveolar epithelial cells (RAECs), Margulies and her colleagues studied the ventilator-induced oxidative stress on the permeability of the epithelium in the lung's alveoli. It had already been shown that cyclical stretching of cells can produce reactive oxygen species (ROS). Nonetheless, such a relationship between stretching and ROS production had not been shown in RAECs, which maintain the proper permeability of the lung's epithelium.

Other work indicated that NF-kB (nuclear factor kappa-light-chain-enhancer of activated B cells) activation, which arises in response to a variety of cellular stresses, could be involved. Also, work by Margulies had shown that cyclic stretch causes the phosphorylation of ERK (extracellular signal–regulated kinase). Her team wanted to see if the NF-kB pathway and ERK played a role in changing the permeability of RAECs.

The researchers mechanically stretched RAECs rhythmically, and used an FP that reveals cellular stress. They used another FP that reveals oxidation in the mitochondria. A third FP measured permeability.

With cyclical stretching, the FPs for cellular stress and the formation of ROS lit up in live cells in 10–60 minutes. Using different inhibitors, the researchers also showed that the NF-kB pathway and phosphorylation of ERK play roles in the permeability damage.

Beyond just revealing the process, Margulies and her colleagues studied some methods for blocking the problems. For example, adding an antioxidant—a scavenger that collects the ROS—reduces the permeability changes. So the authors concluded: "Using antioxidants such as tiron or NF-kB pathway–specific inhibitors may therefore be an effective strategy for preventing or treating ventilator-induced lung injury."

[*American Journal of Respiratory Cell and Molecular Biology*, 2013, **49**, 156–164 (DOI: 10.1165/rcmb.2012-0252OC)]

CASE STUDY THREE

For men with prostate cancer that has spread, the prognosis is poor: Only 30% of those men will be alive five years after being diagnosed, according to Cancer Research, UK. These odds might be improved with advanced gene therapies, which is one interest of Paul B. Fisher of Virginia Commonwealth University in the United States. In particular, Fisher studies genes that make people more susceptible to cancer or could help treat it.

For example, melanoma differentiation associated gene-7/interleukin-24 (mda-7/IL-24) destroys cancer cells but not healthy ones. In fact, the expression of this gene kills cancer effectively and safely enough that it has been tested in clinical studies. When delivered with an adenovirus, the expression of mda-7/IL-24 creates a protein that interacts with another called BiP/GRP78, which is related to the endoplasmic reticulum, and this process leads to cell death through autophagy. Specifically, this process arises from turning down the expression of antiapoptotic protein myeloid cell leukemia-1 (Mcl-1).

In a 2011 article, Fisher and his colleagues reported the discovery of several compounds, including one called BI-97C1, that bind to Mcl-1. Using live cells and FPs, they tested ways to induce autophagy in prostate cancer, which could lead to a medical treatment for prostate cancer.

[*Proceedings of the National Academy of Science*, 2011, **108**, 8785–8790 (DOI:10.1073/pnas.1100769108)]

CASE STUDY FOUR

The synapses that provide communication channels between neurons get created and eliminated over time. In development, synaptic pruning fine-tunes a brain's abilities; in neurodegenerative diseases, a reduction in synapses contributes to failing abilities. Some research showed that the protein caspase-3 plays a part in eliminating synapses, and Ali Ertürk and Morgan Sheng, both at Genentech in South San Francisco, California, wondered just how specific of a role this protein plays. In particular, they wanted to know if this protein can eliminate synapses without killing the entire cell.

To find out, they used an optogenetic approach in which Mito-KillerRed, a modified red fluorescent protein (RFP), triggered caspase-3 activity in very specific places on the dendrites of cultured neurons. The outcome of the optogenetic process was tracked through immunostaining and fluorogenic tracking of caspase. In writing about the fluorogenic caspase-3 monitor, the researchers pointed out: "This reporter has an advantage over caspase-3 immunostaining because it allowed ... monitoring of caspase-3 activation in the same neurons over time, before and after Mito-KillerRed/Mito-RFP photostimulation." They used a multiplex of the ROS reporter to monitor the ROS from KillerRed.

In studies where illumination of Mito-KillerRed activated caspase-3, the cells died; but if the researchers inhibited caspase-3, the cells survived. When they limited the illumination to very specific areas of a cellular dendrite, the synaptic spines disappeared in hours. In unstimulated dendrites of the same neuron, the spines survived. The authors noted: "Together, these data show that local activation of the mitochondrial apoptotic pathway can induce local pruning of dendrites and spines via a mechanism dependent on caspase-3 activity." This reveals a potential target for treating diseases, such as Alzheimer's, in which the elimination of synapses develops.

[*The Journal of Neuroscience*, 2014, **34**, 1672–1688 (DOI: 10.1523/JNEUROSCI.3121-13.2014)]

CASE STUDY FIVE

According to the National Parkinson Foundation, 4–6 million people around the world suffer from Parkinson's disease (PD), which is a neurodegenerative disease. One form of PD arises from mutations in a gene called PARK2*, which causes an autosomal recessive form of the disease. Conversely, this same gene—when not mutated—actually drives processes that protect the brain. Kalle Gehring of McGill University in Montreal, Canada, hopes that a better understanding of* PARK2 *could lead to ways to use it against PD.*

This turns out to be a complicated problem, because more than 120 mutations of this gene can cause autosomal recessive PD. Perhaps even worse, point mutations in virtually every part of the parkin protein can cause PD. Although parkin, the protein, participates in many processes, one of them is autophagy of damaged mitochondria.

Gehring and his colleagues compared how wild-type and mutant parkin go to the mitochondria, by labeling the protein with GFP and tracking it with time-lapse microscopy in HeLa cells. In addition, they used an RFP that reveals the mitochondria. They chemically stimulated the mitochondria to attract the protein and then timed the results—using colocalization of the GFP and RFP to indicate parkin in the mitochondria. The parkin from the mutant gene ended up in the mitochondria about three times faster than the wild-type.

The researchers went on to show that several steps activate parkin, and the mutations that lead to PD diminish or even completely remove parkin's neuroprotective capabilities. The authors noted: "Because parkin is neuroprotective in a number of PD models, the structure-based mechanism of activation presented here provides a potential framework to enhance parkin activity for therapeutics in PD." This combination of two FPs and making movies of molecules in live cells helped scientists better understand the basic biology of a PD-related gene and its protein, and the results might point to new pathways to explore as potential treatments.

[*Science Express*, 2013 (DOI: 10.1126/science.1237908)]

PROBLEMS AND SOLUTIONS

In studies that involve live-cell imaging, every decision in the process impacts the results. Selecting the best plates, for example, makes a difference. Not every plastic multi-well plate, for instance, provides the necessary optical characteristics for high-resolution imaging. Nonetheless, a variety of options are available, such as multi-well plates with glass bottoms. Conventional plastic plates can generate imaging problems, including birefringence and auto-fluorescence. In fact, vendors provide a wide range of products—from dishes to plates—made just for live-cell imaging.

The media in the dish or plate also makes a difference in live-cell imaging. Here again, vendors make media specifically for live-cell imaging. A researcher should look for a media that not only provides the physiological conditions necessary to keep the cells alive, but also a product that provides the clarity needed to produce the required image quality and the desired signal-to-noise ratio—that is, more of the desired signal and less background. Scientists can also purchase media that vendors optimize for live cell-fluorescence imaging. These forms of media can also come with buffering that extends the longevity of the cells relative to standard media.

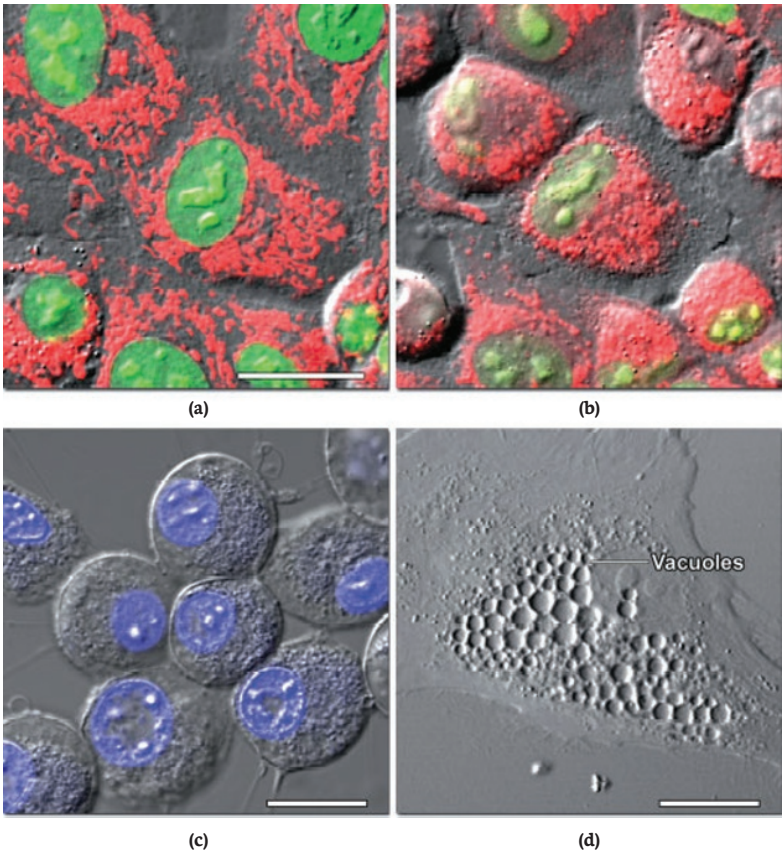
Beyond the right media, some cells may require more care to remain healthy, especially for temporal studies. In such cases, live-cell imaging might also require incubation, which can control temperature, humidity, gases and so on. Incubation can be used to keep cells in their most natural condition, and it can also be

used to study cells in modified conditions. The latter can be used in a variety of situations, such as exploring the impact of oxygen on cancer as mentioned above.

FPs also create some challenges. Fortunately, today's brighter and more stable FPs allow scientists to use them at lower concentrations, which reduces the likelihood of changing biological processes. Some FPs, especially in the early days of their use, tend to dimerization or aggregate in even bigger clumps. So scientists should select FPs that sidestep such tendencies. Overall, scientists should select reagents that provide the highest signal-to-noise ratio and use the minimal amount of reagent that provides the required signal.

New forms of genome editing technologies also give today's biologists more control in FP delivery. For example, the CRISPR (clustered regularly interspaced short palindromic repeats)/Cas9 and TAL (transcription activator-like) effector technologies make it much easier to engineer a targeted area of a genome, which can be tracked with an FP.

The imaging itself also raises issues. For instance, most cells can easily be damaged by illumination through phototoxicity. In particular, ultraviolet light or lasers can damage cells through photobleaching. So the light should be kept to a minimum and any unnecessary exposure should be reduced as much as possible. Using very efficient FPs helps. Also, some experiments require a balance between getting the best image and keeping the cells as healthy as possible.



Phototoxicity during live-cell imaging. (a) Rabbit kidney (RK13) cells expressing EYFP fused to a nuclear localization signal (green nucleus) were treated with the synthetic dye MitoTracker™ Red and imaged in fluorescence and DIC mode. (b) Same view field as panel a after time-lapse imaging for 2 hours at 15 second intervals. Note the fragmentation of mitochondria and rounding of the cells due to phototoxicity. (c) HeLa cells labeled with Hoechst 33342 imaged for 30 minutes at 10-second intervals with a 405 nm laser in a confocal microscope. Cells have detached from the coverslip and are rounding. (d) Vacuole formation in a fibroblast cell after imaging for 8 hours at 30-second intervals using tungsten halogen illumination and DIC optics. (From: Murphy DB, Davidson MW. 2012. Imaging living cells with the microscope. In *Fundamentals of Light Microscopy and Electronic Imaging, Second Edition.* Hoboken, NJ: John Wiley & Sons, Inc. doi: 10.1002/9781118382905.ch16.)

Before increasing the light exposure in an experiment, the scientist should improve the signal and maximize the sensitivity of the detector. To maintain the health of the cells as long as possible, only acquire the images that are needed.

In general, live-cell imaging depends on a consideration of several issues. As already pointed out, scientists need to balance the imaging and cell needs. Likewise, a biologist should consider the imaging requirements for the experiments at hand rather than every feature available. So even though high-level resolution and rapid speed are available, a scientist should focus on the parameters required for the experiments that need to be run. This also applies to the image processing required. Today's software offers many capabilities, including time-lapse imaging, tiling, stitching, multi-well plate scanning and so on. Some systems even provide automation, such as cell counting.

To some biologists, the complexity of platforms for live-cell imaging can be daunting. The need to select the optics—for microscopy and imaging—plus incubation if needed or some other special stage or plate for the cells can get overwhelming, especially once the need for FP and experimental modifications are included. But no one needs to start with a complicated system; even beginner systems now exist for live-cell imaging. These systems require less capital and experience to get started in this field. A biologist should first assess what experiments might be run, the conditions required and the desired level of imaging to answer the key questions. From the most basic perspective, a biologist only needs the spatial and temporal resolution that

meets a specific task. That is the first consideration, and other features can be added after that. In this way, a beginner in this field can get started easily and move to more advanced technology over time.

WHAT'S NEXT

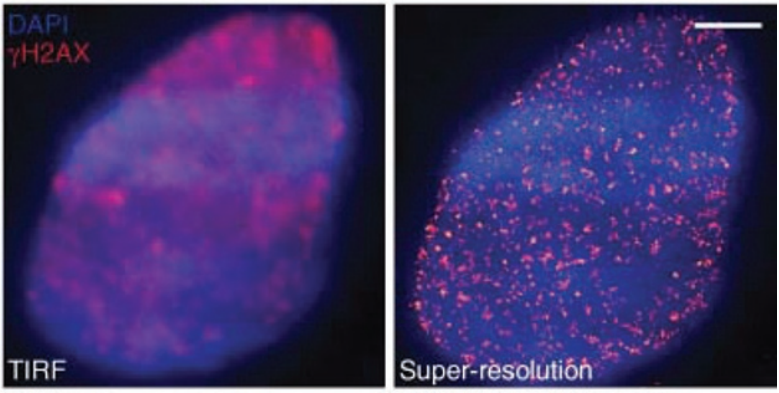
Although many advances in media, molecular probes and incubators as well as microscope optics, imaging devices and analytical software have completely changed the world of imaging since van Leeuwenhoek developed his microscope in the late 1600s, many more improvements lie ahead. Even with the long history of microscopy, we've seen less than 20 years of advanced live-cell imaging based on FPs and other technologies developed specifically for this field of study. Undoubtedly, this field will grow considerably more sophisticated but simultaneously more accessible. Already, new platforms make it easier for beginners to utilize this technology in their research. Consequently, more and more researchers are working with live-cell imaging. The ultimate breadth of this field in terms of applications and users remains to be seen, but it surely has not reached its peak.

Some of the future advances will image living cells in increasingly natural conditions. Instead of looking at two-dimensional sheets of cells, for instance, biologists will explore cultures growing in three dimensions. Biologists already know that going from 2D to 3D can make a difference. For example, one study showed that breast cancer cells behaved differently when examined in low-attachment plates that allowed cells to make 3D formations, relative to 2D sheets of cells. Certainly, many other cellular systems—possibly all of them—perform differently as flat sheets versus three-dimensional shapes. Biologists just need easy and efficient ways to image cells in these more natural conditions.

Along a similar pathway, biologists would like to study live cells in bigger groups. This will move imaging from sheets of cells

and 3D groups to tissues, organs and even entire organisms. Some systems already provide capabilities of imaging fluorescently labeled cells in living animals, typically rodents, but the depth and resolution are limited.

In systems that image larger samples and for live-cell imaging in general, resolution will continue to improve. Even now, super-resolution imaging allows live cells to be viewed at resolutions better than expected with light microscopy. These systems started as custom platforms built by experts, but increasingly commercial versions are available, which require less expertise and maintenance. As these systems and others that offer improved resolution become more widely available, biologists will explore even finer details in the healthy and disease processes going on in cells.



Super-resolution microscopy by localization. Nucleus of a cell stained for γH2AX. The TIRF image on the left is diffraction-limited resolution, whereas the super-resolved reconstruction on the right shows enhanced spatial features. Scale bar is 5 µm. (From: Reid DA, Rothenberg E. 2000. Single-molecule fluorescence imaging techniques. *Encyclopedia of Analytical Chemistry*. doi: 10.1002/9780470027318.a9494.

Even with past and future improvements in mind, live-cell imaging, like most approaches to scientific research, will always require scientists to make compromises. The most beautiful images, for example, might not be required for the desired data. Also, the question at hand in combination with the sample will determine the necessary signal-to-noise ratio. Likewise, not every experiment will demand the highest temporal and spatial resolution available. In the end, every biologist interested in live-cell imaging will need to make many choices. As those choices get made and questions answered, however, we will learn far more about the behavior of cells—the fundamental unit of life—and what we can do to live longer and healthier lives.

FURTHER READING

1. Day RN, Davidson MW, 2009. The fluorescent protein palette: tools for cellular imaging. *Chemical Society Reviews* 38:2887–2921. doi: 10.1039/b901966a.
2. EVOS™ Cell Imaging Systems, https://www.lifetechnologies.com/evos.
3. Frigault M, *et al.* 2009. Live-cell microscopy – tips and tools. *Journal of Cell Science* 122:753–767. doi:10.1242/jcs.033837.
4. Kilgore JA, Dolman NJ, Davidson MW. 2013. A review of reagents for fluorescence microscopy of cellular compartments and structures, part II: reagents for non-vesicular organelles. *Current Protocols in Cytometry* 66: 12.31:12.31.1–12.31.24. doi: 10.1002/0471142956.cy1231s66.
5. Kilgore JA, Dolman NJ, Davidson MW. 2014. A review of reagents for fluorescence microscopy of cellular compartments and structures, part III: reagents for actin, tubulin, cellular membranes, and whole cell and cytoplasm. *Current Protocols in Cytometry* 67:12.32:12.32.1–12.32.17. doi: 10.1002/0471142956.cy1232s67.
6. Miyawaki A, 2011, Proteins on the move: insights gained from fluorescent protein technologies. *Nature* 12:656–668. doi:10.1038/nrm3199.
7. Molecular Probes™ Handbook—A Guide to Fluorescent Probes and Labeling, 11th Edition, https://www.lifetechnologies.com/handbook.
8. Schnell U, 2012. Immunolabeling artifacts and the need for live-cell imaging. *Nature Methods* 9:152–158. doi:10.1038/nmeth.1855.

9. Shaner NC, *et al.,* 2008. Improving the photostability of bright monomeric orange and red fluorescent proteins. *Nature Methods* 5:545–551. doi:10.1038/NMETH.1209.
10. Waters J, 2009. Accuracy and precision in quantitative fluorescence microscopy. *Journal of Cell Biology* 185: 1135–1148. doi: 10.1083/jcb.200903097.

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