



Green Fluorescent Proteins Improve Myofibril Research

by Joseph W. Sanger and Jean M. Sanger

SUMMARY

The ability to express fluorescent proteins in muscle cells through transfection with plasmids encoding green fluorescent proteins has expanded the way light microscopes can follow dynamic processes in live myocytes.

Muscle cells produce force through the interaction of filaments of actin and myosin. In cardiac and skeletal muscle cells, the arrangement of these filaments and the proteins with which they interact produce a highly organized cytoskeleton of myofibrils that extend the length of the cells. The muscle fibers, in turn, are composed of linearly connected contractile units called sarcomeres. One of the challenges of muscle research is to determine how the component proteins of the myofibrils assemble into regular sarcomeric arrays.

The study of muscle cells, or myocytes, is critical to many areas of medical research, because many skeletal and cardiac disorders involve mutations in the myofibrillar proteins, leading to impaired contractions and the disorganization of myofibrils. Understanding precisely how the myofibril is assembled could contribute to developing cures for some of these disorders.

A decade ago, the real-time visualization of proteins in muscle cells depended on purifying the protein from a muscle source, coupling it to a fluorescent dye and microinjecting it into cells. If the fluorescent protein retained its native properties and the cells survived the injection process, the injected protein would assemble with its endogenous counterpart

in the myocytes and serve as a tracer for the protein of interest and for the cellular substructure in which the protein was localized.

The process left much to be desired. It could take researchers weeks to isolate, purify and couple the protein to a fluorescent dye and microinject it back into a living cell.

The coming of GFP

One of the most important advances in cell biology during the 1990s was the introduction of cDNA expression plasmids with sequences for fluorescent proteins. The original green fluorescent protein (GFP) expression plasmid used a sequence from the jellyfish *Aequorea victoria*. The protein yielded only a weak fluorescent response; however, through mutagenesis, researchers produced enhanced GFPs that are 35 times as bright as the original or that fluoresce in different colors, such as blue, cyan and yellow.

Because GFP contains a stable fluorophore and the GFP-linked proteins are continuously synthesized in transfected cells, the fluorescent signal can be followed over many hours or days as long as overexpression of the protein does not adversely affect the cells. Researchers also can transfect cells with members of the GFP family that fluoresce at different

wavelengths to follow the assembly of different proteins into myofibrils. The available imaging hardware — including cameras, filters and digital storage devices — and state-of-the-art image processing software make it feasible to localize and measure fluorescently tagged molecules and study their dynamics with time-lapse observations of living cells.

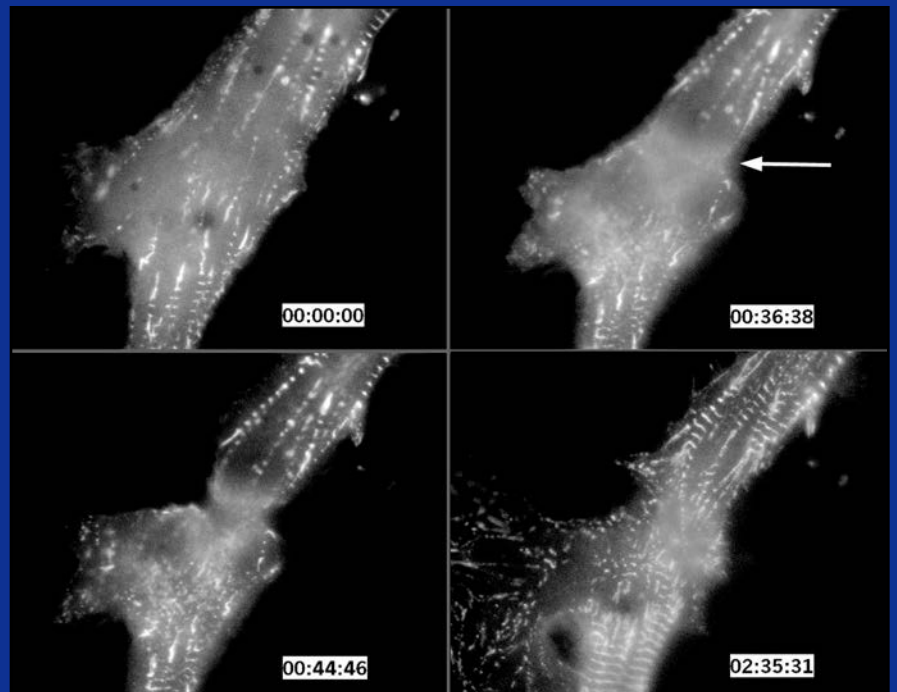
Cell preparation

Avian embryonic muscle cells offer an excellent platform to study myofibril formation in tissue cultures by phase-contrast and epifluorescence microscopy. Fertilized eggs are the starting points for cultures of cardiac and skeletal muscle cells. Researchers dissect the embryos to remove the hearts and breast skeletal muscle, and they incubate the tissues in a trypsin solution and plate the myocytes on glass coverslips. Over the next week in culture, the skeletal muscle cells fuse and assemble myofibrils, and the cardiomyocytes spread, assemble myofibrils and, in some cases, undergo cell division.

For each 35-mm dish of cells to be transfected, 1 to 2 μg of plasmid DNA is added to 100 μl of serum-free culture medium in one tube, and a lipid-based reagent such as LipofectAmine is added to a serum-free medium in a second tube. The contents of the tubes are incubated according to the manufacturer's protocol. The combined mixture is added to a dish of cells and incubated for five to six hours.

The researchers usually can detect GFP fluorescence after a day. The assembly of myofibrils and fusion of skeletal muscle cells, and the contraction and division of cardiac muscle cells, occur as often in the transfected cells as they do in nontransfected cells (Figure 1).

Figure 1. Four time points from a time-lapse sequence of a dividing cardiomyocyte transfected with GFP- α -actinin reveal that the myofibrils that are absent from the center of the metaphase and anaphase cell (images at top) re-form when the daughter cells spread out after cytokinesis (bottom). The arrow in the image at top right identifies the cleavage furrow.



The advantage of transfecting cells with GFP is the ability to analyze the distribution of the molecules in living cells. We use an inverted microscope with a stage that is maintained at 37 °C with a heat curtain. We have constructed a homemade chamber to maintain an atmosphere of 5 percent CO₂ around the culture dish. Observation of the transfected cells is achieved with phase-contrast and epifluorescence optics, and a cooled CCD camera controlled by imaging software captures sequential pairs of images.

To visualize all the myofibrils in thick muscle cells, the images must be acquired in several focal planes. When fluorescent images of several planes have been collected for one point in time, they can be combined digitally to reveal all of the fibrils. Changes in the intensity of the GFP signal can be measured over time and correlated with changes in structure.

After evaluating the various software

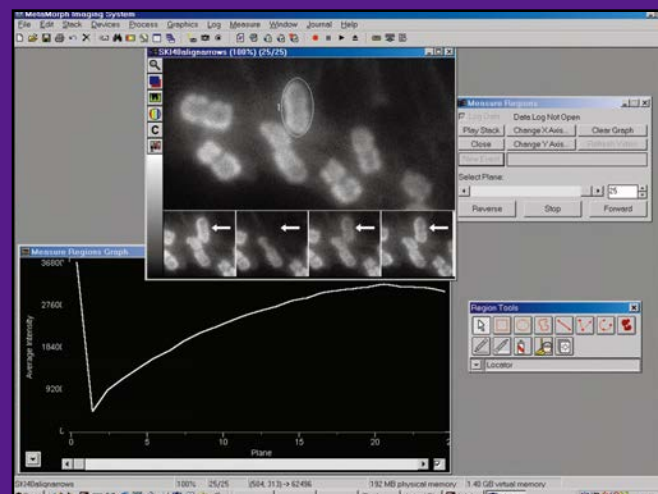


Figure 2. The MetaMorph imaging software enables researchers to measure fluorescence recovery after the photobleaching of actin-GFP in the attachment site of *E. coli* bound to PtK₂ cells.

packages that automate the acquisition and processing of microscope images, we selected the MetaMorph imaging system from Universal Imaging Corp. of Downingtown, Pa. The software enables us to automate most microscopes, improving the speed and accuracy of our

experiments, as well as allowing us to perform other functions.

The application of a pseudocolor improves the display of information such as the colocalization of two probes or the differences in the fluorescent intensity in images acquired with a monochrome camera.

We can manipulate groups of images as stacks to maintain the organization of images recorded over time from different focal planes and locations and at different wavelengths. The use of multiple stacks makes it possible to produce movies or montages and to process

images in a parallel manner.

We collect time-lapse images to measure cell morphology and migration and any changes in the cytoskeleton, and to create an animated series of images to display cytoskeletal dynamics during myofibrillogenesis, cell division and bacteria

interactions with host cells. Recently, we used these methods to test aspects of several theories of myofibril assembly that had been proposed based on the study of fixed cells.

We discovered that the fine fibrils called premyofibrils, which form at the spreading edges of cardiomyocytes, contained punctate concentrations of alpha-actinin, called Z-bodies. The punctate Z-bodies grow and align with Z-bodies in adjacent fibrils.

Mature myofibrils

Over time, adjacent fibrils and Z-bodies fuse and form mature myofibrils and Z-bands in cytoplasmic regions where linear arrays of Z-bodies are located. These new myofibrils align with those at their Z-bands to form myofibrils that span the length of the spread cell. Results are consistent with a model that postulates that the fibrils near the cell membrane are premyofibrils, precursors of mature myofibrils.

In our studies on live cells undergoing division, the fluorescently labeled actin and myosin molecules were recruited to

the future cleavage furrow before there were visible signs of contraction in the region. The contraction of the region was accompanied by the loss of these two proteins from the furrow. We measured the intensity of the fluorescence with MetaMorph's area measurement function and plotted these changing intensities against time as the cell progressed from prophase and metaphase through cell division. Previous work using the immunofluorescence of fixed cells indicated that actin and myosin proteins were concentrated in the furrow region only in cells undergoing furrowing.

In other research, such as the study of the interaction of infectious bacteria with host cells, movement is an important factor. Besides bacterial movement, the dynamics of the actin cytoskeleton induced by the bacteria in the host cells can be studied by photobleaching actin-GFP in transfected host cells. In these cases, we used MetaMorph's built-in capabilities for calculating distance and fluorescence over time. We calculated the changes in the fluorescence intensity after photo-

bleaching using the software's stack functions, which allow each image in a time series to be aligned. We subtracted the background and put in graph form the recovery of fluorescence in the bleached zone as a function of time (Figure 2).

Fluorescent futures

Before researchers could express fluorescent proteins in muscle cells through transfection with plasmids encoding GFP, the real-time visualization of processes in these cells was a complex, time-consuming and daunting feat. With the introduction of enhanced GFP, the investigation of myofibrillar proteins may lead to a better understanding of skeletal and cardiac disorders and, eventually, to the development of new cures. □

Meet the authors

Joseph W. Sanger and Jean M. Sanger are professors in the department of cell and developmental biology at the University of Pennsylvania School of Medicine in Philadelphia. They have co-authored several books and articles on cell research.