Fluorescence Microscopy and its Applications

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Abstract

In this research paper, we provide an introduction to fluorescence microscopy techniques. We show how the development of these techniques has lead to significant advances in cellular physiology. As part of this discussion, we detail applications of optical imaging to a study of cell division and to a study of the nervous system. We will conclude by presenting some limitations and controversies of these techniques, and will also outline some exciting new developments for the future.

Introduction

The ultimate goal in microscopy is to view live cellular processes with a high spatial and temporal resolution, and for an indefinite duration of time. This challenge is difficult to face, but its accomplishment is of great benefit. Cellular processes can take place very rapidly, and the movement of cellular components can be complex and unintuitive. A great barrier to understanding such movements is the inability to view what is happening with high resolution. Some examples include cellular signaling, the contraction and relaxation of muscle cells, the movement of leukocytes, and the absorption and secretion of nutrients and metabolites by epithelial cells lining the gut [1]. The ability to track such movements enhances our knowledge of cellular physiology.

Fluorescence microscopy is the most popular method for studying the dynamic behavior exhibited in live cell imaging. This stems from its ability to isolate individual proteins with a high degree of specificity amidst non-fluorescing material. The sensitivity is high enough to detect as few as 50 molecules per cubic micrometer. Different molecules can now be stained with different colors, allowing multiple types of molecule to be tracked simultaneously. These factors combine to give fluorescence microscopy a clear advantage over other optical imaging techniques, for both in vitro and in vivo imaging.

This paper begins by presenting the background on the workings on fluorescence microscopy. We then demonstrate its usefulness by investigating some applications of fluorescent techniques. Finally, we discuss the remaining limitations of fluorescence microscopy, and some more advanced techniques and ideas for future research.

How Fluorescence Microscopy Works

Most cellular components are colorless and cannot be clearly distinguished under a microscope. The basic premise of fluorescence microscopy is to stain the components with dyes. Fluorescent dyes, also known as fluorophores or fluorochromes, are molecules that absorb excitation light at a given wavelength (generally UV), and after a short delay emit light at a longer wavelength. The delay between absorption and emission is negligible, generally on the order of nanoseconds. The emission light can then be filtered from the excitation light to reveal the location of the fluorophores.

The fundamental idea is to bind the fluorophores to molecules of interest, so that the spatial distribution of these molecules is revealed from the location of the fluorophores.

In the past, dyes were non-specific. This means that a dye might target all the DNA, or all of the proteins in a cell. Modern techniques are much more specific. Fluorescent labels can now be designed to target a specific protein (or other molecule) or interest. This was first achieved with the introduction of immunofluorescence. This can be used to locate any protein for which you have an antibody. A fluorescent dye is attached to the antibody to form a conjugate. The cells of interest are then fixed and permeablized, and stained with the fluorescent conjugate. The highly specific protein-antibody binding ensures that the fluorophores attach to the desired protein. Hence, illumination of the fluorophores under UV light will reveal the spatial distribution of the relevant protein.

The major disadvantage of immunofluorescence is that the cells must be fixed and permeablized before adding the fluorescently-labeled antibodies. This renders live-cell imaging impossible. It is difficult to discover the mechanisms of a complex cellular process when only snapshots are available. Licthman and Fraser compared this to the task of inferring the rules of American football based on 1000 photos of the game [2]. If each photo was obtained from a different game, and at a different time, it would be difficult to understand what was happening. The problem was to find a way to obtain the 1000 photos from the same game, and in temporal order. The solution came with the discovery of green fluorescent protein (GFP).

GFP is a fluorescent protein that was first found in the jellyfish Aequorea Victoria. It has the useful property that its formation is not species specific. This means that it can be fused to virtually any target protein by genetically encoding its cDNA as a fusion with the cDNA of the target protein. This can be done in a live cell, and hence the movement of individual cellular components can now be analyzed across time. There is no requirement to fix and permeablize the cells first. The discovery of GFP has made the imaging of real-time dynamic processes commonplace, and caused a revolution in optical imaging.

The GFP revolution goes even further with the devlopment of different colored GFP isoforms, such as yellow GFP and cyan GFP. This allows multiple proteins to be viewed simultaneously in a cell. This development also enables a new method for studying protein-protein interactions, called fluorescence resonance energy transfer (FRET). This will be discussed further in a later section.

Optical Imaging of Cell Division

We will now trace the historical developments of microscopy, and show their importance with reference to cell division. As this section will show, our understanding of mitosis has been closely linked with microscopy advances. Fluorescence microscopy and the discovery of GFP has led to an explosion of information on mitosis. This discussion will draw from a review in *Science* by Rieder and Khodjakov [3].

Cell division is fundamental to reproduction, and unrestrained reproduction causes cancer. Thus the study of cell division is an important topic. Up until the early 1950s, there were no techniques to view cells live under a microscope. This was because cellular components are not naturally contrasted under bright-field optics due to the high scattering of biological tissue.(see Figure 1A). Hence the cells were chemically preserved in a fixed lifelike state, and colored with dyes to generate contrast between their different components. As mentioned earlier, these dyes were non-specific and could not isolate individual proteins. The last 50 years have enabled live cell imaging, protein-specific dyes, and finally the combination of the two using GFP fluorescence microscopy.

Live cellular imaging first became viable in the 1950s with the introduction of phase contrast and differential interference contrast, seen in figures 1C and 1D. These techniques are both designed to increase image contrast without significant loss of resolution. They do not involve staining. Since mitosis is a very dynamic and visual process, live cell imaging began to reveal a galaxy of new information on the complex motions exhibited by chromosomes and other components during cell division. Further improvements came about thirty years later with the development of video technology. Since mitosis involves many concurrent events, it is advantageous to store videos on magnetic storage media for subsequent analysis. Furthermore, video cameras can capture at a higher resolution than the human eye.

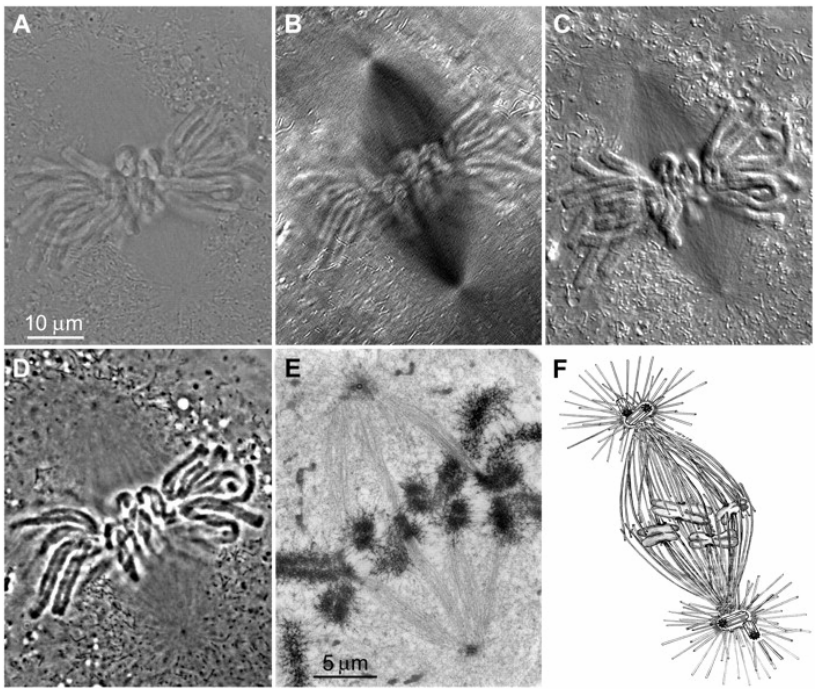

Figure 1: (A) Bright-field optics. (B) Polarized light. (C) Differential interference contrast. (D) Phase-contrast. (E) Electron microscopy. (F) Actual schematic. [3]

A limitation of these techniques is that they provide too much detail. Individual components cannot easily be isolated because everything is visible. For the case of static images, the development of immunofluorescence solved this problem. By

targeting individual proteins, more than 20 new proteins residing in the kinetochore have been identified since 1980. These proteins could be localized with high spatial resolution, and included structural proteins, proteins involved in attaching microtubules to the kinetochore and in moving the chromosomes. Others play a role in cytokinesis, and others control progression through mitosis [3].

To some extent, this method could be used for live cell imaging. Proteins such as tubulin can be isolated in bulk, fluorescently tagged, and then microinjected back into the cell. However, this method is limited to proteins that can be isolated in bulk, and to cells that can be microinjected. It was the discovery of GFP that truly allowed the spatial distribution of proteins to be followed in real time.

The introduction of GFP allowed the study of proteins in organelles that cannot easily be isolated, such as centrosomes and kinetochores. The high sensitivity of fluorescence microscopy meant that proteins of very low concentration could still be detected. Centrosomes are normally invisible, but several stable cell lines were generated in which the chromosomes were clearly visible due to labeling with 7-tubulin-GFP, centrin 1-GFP, or centrin 2-GFP [3]. The ability to see and follow centrosomes in living cells rapidly led to several novel discoveries. For example, it was found that the centriole pair inherited after mitosis splits during or just after telephase [4].

There is an unfortunate side-effect of fluorescence microscopy: the high-intensity radiation used to excite the fluorophores can also kill the cells. UV, the most common frequency, is especially toxic. Hence great care must be taken to ensure that the cells remain viable during image collection. The effects of UV radiation include a lengthening of the cell cycle, delayed segregation, and the induction of strand exchanges which leads to the generation of subchromosomal domains in great-granddaughters and their descendants. Especially high doses arrests cells in G2 phase and prevents division altogether [5]. Hence, images of mitosis must use a low light intensity and short exposure time, thereby reducing image quality. A further discussion on limitations and issues associated with fluorescence microscopy is presented in a later section.

On a more positive note, attaching fluorescent tags to DNA has remarkably little effect on growth. The mechanisms for recognizing and repairing such unnatural DNA residues do not interfere with the cell cycle [5].

Optical Imagining of the Nervous System

Neural imaging is another area on which fluorescence microscopy has had a monumental impact. The two categories which we will discuss are the study of signal propagation in neurons, and the study of the actual formation of synapses. Although fluorescence microscopy should not yet be viewed as replacing the traditional methodologies, it provides a wealth of information that was not otherwise available, and holds great promise for the future.

Signal Propagation

The use of optical methods to study neural impulses is still in its infancy. The traditional technique is to use microelectrodes to measure electrical signals. This method is limited by the number of electrodes that can be placed. Fluorescence

microscopy uses molecular probes, which can potentially measure many more locations simultaneously. This provides a bigger picture of how the nervous system is organized, and on understanding the roles of the individual neurons in generating behavior [6]. There are two ways to detect neural impulses. The first is to use voltage-sensitive dyes that fluoresce at different wavelengths according to voltage changes (ie. membrane potential changes). The second is to use dyes that indicate calcium concentrations in a given area. Both of these are indicate an active synapse.

Although voltage-sensitive dyes are still in their infancy, they show great promise. The main requirement of such a dye is that it must respond to changes in the membrane potential in a time period that is rapid compared to the rise time of an action potential. Ideally we would like to view all action potentials in a given portion of the nervous system, and see how they travel and combine to initiate successive action potentials. This requires high spatial resolution, which can only be achieved using optical methods and voltage-sensitive dyes. Figure 2A shows a picture of an action potential taken with voltage-sensitive dyes, and 2B shows how the velocity and direction of propagation can be ascertained.

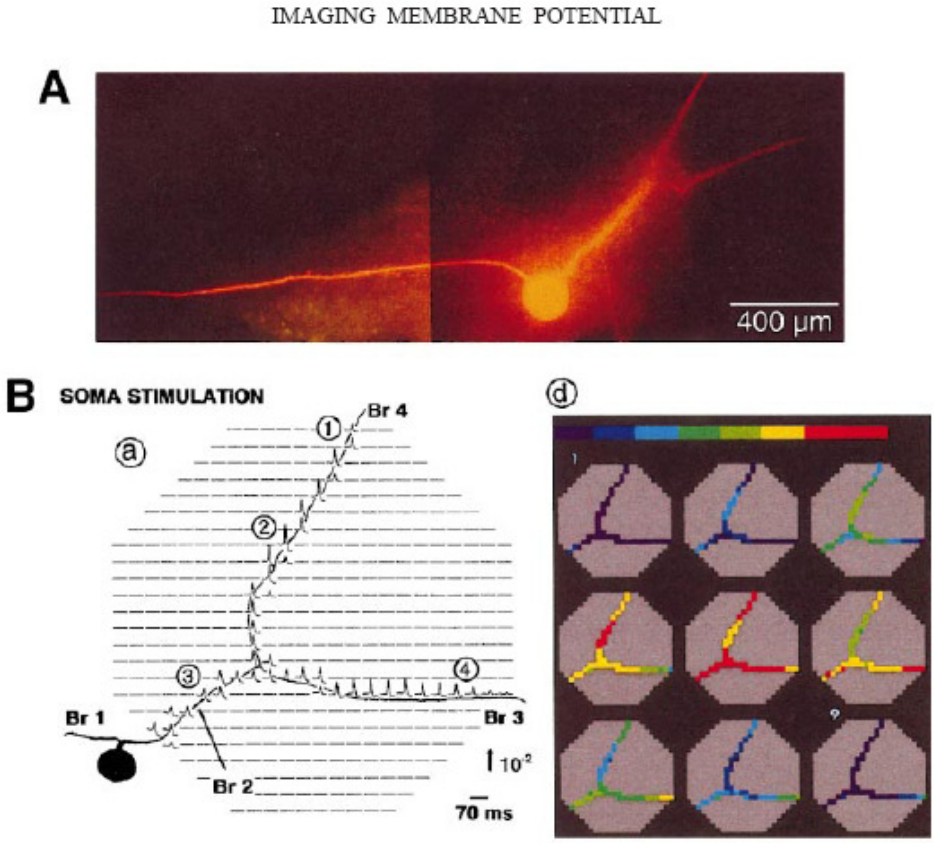

Figure 2: (A) shows an image of a giant metacerebral neuron after injection with the fluorescent voltage-sensitive dye JPW1114. (B) shows recording of the voltage at points over the axonal branches during an action potential. The diagram on the left depicts a series of small graphs that show the voltage rising and falling. The diagram on the right gives a color-coded representation of this data, red being the peak and violet being the minimum, as shown in the scale. [6]

Unfortunately, many voltage-sensitive dyes are toxic, and often only work at low temperatures. However, this avenue of research presents exciting possibilities for the future.

Synapse Formation

A study of synapse formation is of great interest in determining the development of the brain. Prior to optical methods, such experiments had to study brain tissue that was cultured in a petri dish. There was no way to observe synapse formation in an undisturbed real-life environment. This is now changing. As an example, Joshua Sanes at Washington University developed mice that contained a fluorescent protein in some cerebral cortex neurons. Subsequent teams used these mice to image individual neurons in the cerebral cortex over a period of weeks [7]. Although traditional fluorescence microscopy can only penetrate a few millimeters into the tissue, two-photon microscopy uses infrared light, enabling it to penetrate much further into the tissue. This allowed the groups to image cortical neurons without completely removing the skull, leaving a paper-thin layer intact. Two-photon microscopy, which will be discussed later, also does far less damage to living tissue.

Our first case study is a study by Smith at Stanford university. The most popular animal of choice, the zebrafish, was used in an analysis of synapse formation. Traditionally the zebrafish would first need to be anesthetized, which clearly alters behavior. On the basis of such studies, scientists had assumed that the process of neuron connection came about when one neuron extended an axon and felt around for a dendrite. When the two met, a bond was formed [2]. However, GFP allows staining of the proteins found on the dendrites and axons. By immobilizing the zebrafish (but without anesthetic), two-photon microscopy allows the process of synapse formation to be viewed in detail without damaging the living tissue. Researchers discovered that the dendrite actually plays an active role in synapse formation, and reaches out and grabs an axon. For example, when studying the retina, it was found that optic nerve axons form passively in the visual region. They then wait for the dendrites from the brain to grow into this region and begin the formation of synapses [2].

A second case study takes advantage of the different colored fluorophores that are now available. As mentioned earlier, a strain of mouse has been created with a fluorescent protein in certain neurons. Four strains of such mice were created in which the neurons glow in four different colors. These mice were then mixed and mated, resulting in offspring that contain neurons of multiple colors. This was used to study the procedure by which axons connect to muscle. Previously, it had been thought that the muscles would choose the axon to which they connect. However, fluorescence microscopy showed the different colored axons all competing with each other to bind to the muscle. The dynamism of the process was surprising, and the complexity was such that scientists cannot currently predict which axon will connect to the muscle until it actually happens [2].

Hence, optical methods hold great promise for neural imaging. Eventually, scientists may be able to view individual neurons with high resolution, watching the synapse

formation and the signals being propagated between them. Techniques in the pipeline make this reality not so far off – for live unanesthetized rats and mice – whose brains are organized much like our own.

Limitations of Fluorescence Microscopy

We have already alluded to some of the detrimental effects that fluorescence microscopy can have on living tissue. We will now discuss these effects more formally. Toxic effects generally arise for one of three reasons, and we discuss each in turn.

Firstly, the fluorophore itself may interfere with the signaling pathway, or alter cellular function in some way. GFP and its variants are 25 to 27 kD proteins, often larger than the protein of interest. This large size can potentially with the distribution, function, and fate of recombinant proteins [8]. It has already been mentioned that many voltage-sensitive dyes were found to be toxic.

Secondly, the excitation light itself may damage the living tissue, which may affect the behavior of the sample or even cause its death. Several examples of this were mentioned earlier, such as the well-documented knowledge that laser illumination can lead to arrest of the cell cycle.

The third effect arises as a result of the combination of fluorophores and excitation light. UV light causes the fluorophore to move into an excited triplet state, and while in this state the fluorophore can undergo a reaction with molecular oxygen to release a free radical. Such free radicals can also cause cell damage and eventually cell death. This may sometimes be avoidable by placing the specimen in an oxygen-deprived environment. That is clearly not an option with live cell imaging.

An example of this phototoxicity occurs with fluroescently labeled leukocytes. It has been found that leukocyte rolling, and adhesion to endothelial cells, can be provoked by photoexcitation of these fluorescent dye molecules [9]. This alters the leukocyte-endothelial interaction. Such side-effects can only be avoided using conditions with lower light or shorter exposure. This will be discussed further in the next section.

The other well-known limitation of fluorescence microscopy is that of photobleaching. While in the excited triplet state, fluorophores can undergo a covalent modification that destroy their ability to fluoresce. This does not damage the cell as such, but it destroys our ability to view the fluorescent signal, thereby limiting the length of experiments. Photobleaching is a huge problem and usually takes place prior to any of the photo-toxic effects described above. Figure 3 shows the impact of photobleaching on a sample stained with multiple colored fluorophores. These images were taken over a period of ten minutes, and the intensity of all the fluorophores has dropped over this time.

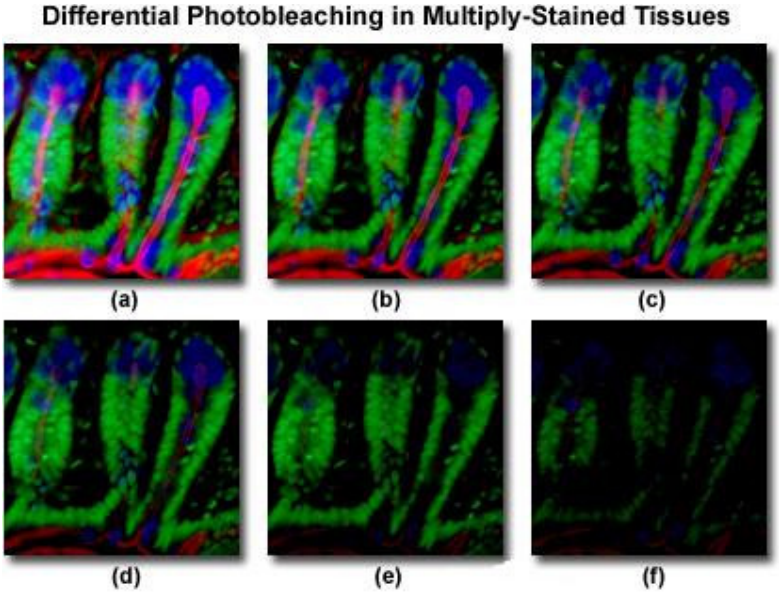

Figure 3: These images show a multiply-stained cryostat thin section of mouse intestine, with images taken at two minute intervals. Three fluorophores - red, green and blue - were used to stain different cellular components. The red and blue intensities drop rapidly at two minutes and are nearly gone at six minutes. The green intensities are more resistant to photobleaching, but drop steadily over the course of the ten minutes [10].

Mitigating these Effects

The first approach to reducing the photobleaching and phototoxic effects is to simply reduce the intensity of the light. The impact of this is a reduction in the signal-to-nosie ratio (SNR). This occurs because the lower light intensity will not excite as many fluorophores, and hence the signal is weaker. A similar effect will occur if exposure times are reduced. A long exposure time is necessary to get a clear and accurate signal, but will result in a longer exposure to UV light, hence increasing phototoxicity and photobleaching.

A trade-off can be presented in fluorescence microscopy in terms of spatial resolution, temporal resolution, SNR, and the duration of the sample. Fluorescence microscopy is usually done with a confocal microscope. In confocal microscopy, each pixel must be read individually. This takes a finite amount of time, and hence acquiring a large number of fine-resolution pixels reduces the speed at which successive images can be obtained. One way to acquire both a high number of pixels/image and a high number of images/second would be to reduce the exposure time of each pixel. However, as mentioned earlier, this reduces the SNR. We could offset this SNR reduction by increasing the excitation light intensity, but this will reduce the duration of the sample due to photobleaching and phototoxicity. Thus a trade-off exists in which we can achieve some desirable qualities but at the expense of others.

Hence, a biologist must arrange the system so that this trade-off does not cause detrimental effects. For example, the role of calcium concentrations in cellular signaling is characterized by calcium sparks. These are a poorly understood phenomena that take place over the space of milliseconds. If we wished to investigate this, then we would need a very high temporal resolution. As a compromise, we

might be willing to use a high light intensity because photobleaching and phototoxic effects are unlikely to occur during the short space of time which we will be imaging over. On the other hand, we cannot use a high laser intensity for a study of mitosis because this would arrest the cell cycle. Hence, a study of mitosis might have to settle for a lower spatial resolution.

A possible area for research is to use mathematical modeling techniques to enhance the resolution of images. This would allow high spatial and temporal resolution but without excessive photo-damage. Another technique that has been mentioned several times is that of multi-photon excitation. This is discussed in the next section, along with some other advanced fluorescence microscopy technologies.

Advanced Technologies

Fluorescence microscopy has many offshoots that provide promising information in themselves. Some examples of these are multi-photon imaging, fluroescence resonance energy transfer (FRET), fluorescence lifetime imaging microscopy (FLIM), and quantum dot technology. We will conclude with a brief summary of these modern technologies.

Multi-photon excitation is primarily designed to reduce damage resulting from the excitation light. In this technique, a fluorophore is not just excited by one photon, but by two (or more), coming from different directions. The fluorophore will fluoresce when it simultaneously absorbs both photons. The advantage of this technique is that the photons can be low-intensity infrared rays, rather than high intensity UV rays. This limits photodamage is limited to the focal region where the two rays intersect, rather than the entire upper and lower regions of the excitation cone. Another advantage is that this lower frequency light can penetrate much deeper into the tissue, and is less vulnerable to scattering [11].

FRET can provide high resolution information about the spatial location of proteins. It occurs when the energy is transferred from a blue donor fluorophore to a red acceptor fluorophore. This can only occur when the fluorophores are very close together (less than 50 Angstroms), and hence the proteins only undergo FRET when direct binding is taking place. Consequently, this technique provides information about the locality of the proteins that is much higher than the resolution of the microscope.

FLIM is a technique in which the lifetime of the fluorescent signal is measured, rather than its actual intensity. The lifetime refers to the time in which the fluorophore remains in an excited state before returning to the ground state. This time is measured by observing how long it takes for the fluorescent signal to drop to 1/e of its original intensity. The advantage of FLIM is that it is independent of the fluorophore concentration, the excitation light intensity and photobleaching. The factors that do affect fluorescence lifetime include ion intensity, hydrophobic properties, oxygen concentration and molecular binding [12]. Hence FLIM can be used to study these properties with high accuracy.

Quantum dots are very small fluorescent nanocrystals that are gaining popularity as fluorophores. This is for two major reasons. Firstly, they are more stable and less

vulnerable to photobleaching, allowing imaging for longer durations. Secondly, their small size potentially allows imaging at a higher resolution, since the resolution of an image can never improve upon the width of the fluorophore itself. It is likely that quantum dot technology, or perhaps a newer technology, will eventually replace organic fluorophores such as GFP.

Conclusion

Fluorescence microscopy has been shown to be a groundbreaking and effective technique that has transformed our understanding of cellular physiology. The ability to view activity in a cell is crucial to advancing our knowledge. Examples of this have been provided with case studies of mitosis and of neural imaging. These are not isolated examples. Fluorescence microscopy has influenced almost every aspect of physiology.

As with any technique, fluorescent microscopy is not without its drawbacks. Limitations such as toxic effects and photobleaching have been discussed, along with some partial solutions. The field is rapidly expanding, and the final section has introduced some of the new cutting edge techniques. As advances continue to occur in both microscopy technologies and in molecular probe technologies, our ability to non-invasively image can only improve. Perhaps one day we can achieve the ultimate goal of viewing all activity in a cell, live.

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