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Basic Principles and Applications of Live Cell Microscopy Techniques: A Review

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ABSTRACT

Live cell imaging has provided great benefits in studying multiple processes and molecular interactions within and/or between cells. This review aimed to describe the common live cell microscopy techniques and briefly explain their principles and applications. A wide range of microscopic techniques, from conventional transmitted light to an array of fluorescence microscopy techniques, including advanced super-resolution techniques, can be applied for live-cell imaging. Transmitted light microscopy uses focused transmitted light that goes through a condenser to achieve a very high illumination on the specimen. On the other hand, fluorescence microscopy uses reflected light to capture images of cells or molecules that have been fluorescently dyed. Techniques for transmitted light microscopy are simple to use but have poor resolution. Although the resolution of fluorescent microscopy techniques is only approximately 200-300 nm, this is nevertheless an improvement over conventional transmitted methods. Conventional light microscopy's resolution was improved by the introduction of the super-resolution microscopy technology family. These methods "break" the diffraction limit, enabling fluorescence imaging with resolutions up to ten times higher than those possible with traditional methods. Each live cell imaging method has advantages and drawbacks. The primary deciding criteria for choosing the type of microscope are the study's objectives, previous experience, the researcher's interests, and financial viability. Hence, a thorough understanding of the technique and application of the various live-cell microscopy methods is paramount in life science studies.

Keywords: Application, Fluorescence, Imaging, Laser-Scanning, Live cell, Microscopy

INTRODUCTION

Nowadays, live cell imaging is a very common and essential tool that is involved in illuminating a great deal about cellular dynamics and function (Frigault et al., 2009). It is crucial for conducting life science research in the fields of biology and pharmacology (Jensen, 2013). During live cell imaging studies on the lives of cells, it is very important to avoid altering the physiological and biological processes under investigation (Sanderson et al., 2014). Direct observation of the processes within a cell provides a crucial additional dimension to researchers' understanding of cell physiology and biology (Murphy and Davidson, 2012).

Cells are not usually exposed to light during their growth. It is, therefore, crucial to minimize light exposure of cells during microscopy applications by employing microscope systems optimized to collect as much light as possible using superior-quality optical components and detectors (Frigault et al., 2009). Bright-field microscopy is a traditional technique used to examine cells and tissues that usually requires fixation and subsequent staining before visualization under the microscope. It is impossible to obtain information from a living cell using this method since the fixation kills the cell; hence only a static, snapshot view of cells can be obtained (Mokobi, 2022). Currently, there are a wide array of microscopy techniques for studying cellular dynamics and function, from cheap and simple to use to costly and highly advanced techniques available. These include dark field, phase contrast, polarized microscope, and differential contrast microscope are called transmitted light microscopy and a variety of fluorescence microscopy techniques (Stephens and Allan, 2003; Culley et al., 2018). Nowadays, no live cell microscopy technique is suitable for all possible investigations (Jensen, 2013). The primary factors for the type of microscope to be used are the study's objectives, the researcher's expertise, interest, and financial viability, as well as the specimen's thickness, vitality, and sensitivity to detection (Stephens and Allan, 2003; Sanderson et al., 2014).

Although the various types of microscopy techniques are described in the literature, most studies have suggested a few/single types of microscopies. Compiling the common live cell microscopy techniques fragmented in different kinds of literature is very important to access all the techniques easily and comprehensively (Stephens and Allan, 2003). Transmitted light microscopy techniques are easy to use; however, they cannot detect subcellular morphology, especially

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in thick tissue samples. Fluorescent microscopy can reveal subcellular structures, including in thick tissue samples (Schmolze et al., 2011). However, diffraction only allows the convectional fluorescence microscope's resolution to reach roughly 200-300 nm (Culley et al., 2018). Conventional light microscopy's resolution can be improved by developing super-resolution techniques (Stephens and Allan, 2003). These methods break the diffraction limit, enabling fluorescence imaging with up to ten times better resolutions than traditional methods (Culley et al., 2018). This review aimed to describe the common live cell microscopy techniques and briefly explain their principles and applications.

DARKFIELD MICROSCOPY

In light microscopy, the objective lens collects two types of rays, namely diffracted rays and non-diffracted/undeviated rays. In bright field microscopy, the non-diffracted rays, also called zero-order rays, are essentially collected to form a bright background. These rays pass through the tissue but do not interact with the sample; therefore, they do not contain any information about the sample. In dark field conditions, the specimen is illuminated from the side such that those zero order/background/non-diffracted light rays are not collected by the objective lens. Therefore, the sample appears bright on dark background. To achieve this condition, dark field microscopy is equipped with a black disk or special dark-field aperture in the condenser (Murphy and Davidson, 2012).

Darkfield microscopy is used for many biological applications, including radioactive in situ hybridizations of mRNA expression, microbial detection, and diagnosis in clinical microbiology (Macnab, 1976; Chen et al., 2012).

PHASE CONTRAST MICROSCOPY

Biological specimens are usually transparent and do not absorb light but instead diffract it and cause phase shift. These specimens are called phase objects, meaning they can cause a phase shift when light passes through them; however, humans' eyes cannot detect these phase differences. Therefore, we need an instrument that converts phase shifts into amplitude differences. This activity is undertaken by a phase contrast microscope. For this purpose, a phase contrast microscope is equipped with two critical components named condenser annulus/phase annulus (a black plate positioned in front of the condenser) and a phase plate positioned at the objective lens. Phase contrast microscopy is widely used mainly for big structures (nuclei and other organelles) (Sanderson, 2001; Aryal, 2022). In addition, phase images are also often used as anatomical references for fluorescence signals (Sahu et al., 2006).

DIFFERENTIAL INTERFERENCE CONTRAST

Differential interference contrast (DIC) is one of the interference microscopy techniques (including phase contrast, and polarized microscopy) that generates contrast based on wave interference. It is important to visualize unstained, live, transparent cells. This method forms an image that has an impression of three dimensions. The DIC microscopy requires a polarized light source, two polarizers, and two special Wollaston prisms. When polarized light passes through the prism, which is called Wollaston prism 1, it will be divided into two components, pass through the sample, and will later be recombined at the second Wollaston prism. Superimposition of these two light components later by the analyzer results in images having contrast (Zernike, 1942; Murphy and Davidson, 2012). Differential interference contrast helps research live cells. In live, unstained tissue, it is incredibly helpful for resolving individual cells and cellular organelles. Slices of the brain, cell culture, and even whole organisms (such as embryos) can all be studied with DIC microscopy (Ziv and Schiller, 2007).

FLUORESCENCE MICROSCOPY

Transmitted light microscopy uses focused transmitted light that goes through a condenser to achieve a very high illumination on the specimen. In fluorescence microscopy, fluorescent dyes are used to label cells or molecules, and the light is reflected rather than transmitted to capture the images (Walker-DanielsandFaklaris, 2012). These molecules are capable of absorbance and emission of a specific range of wavelengths (Lichtman and Conchello, 2005). Fluorescence microscopy has a wide range of information-conveying "dimensions" because the light's spectra can be separated. This method might be used to carry out many simultaneous immune fluorescent labelings on a single slice. At least three colocalized antigens can be recognized simultaneously and individually if immunofluorescence is utilized. When using standard light microscopy, a reasonable resolution of no more than three to four colors, including any necessary counterstains, is possible (such as hematoxylin). However, colocalized antigens are difficult to separate in transmitted light microscopy (Schmolze et al., 2011).

Fluorescence microscopy became one of the most widely used and preferred modalities for live cell imaging (Wang and Lai, 2021). This is due to its very high contrast, sensitivity, specificity, and selectivity. Fluorescence microscopy provides a wide array of possible live cell studies, including motility, protein location, associations, and other phenomena such as ion transport and metabolism (Ettinger and Wittmann, 2014). There is a possibility of using different fluorescent dyes together to stain different components of the cell so that they can be detected simultaneously. This is rather barely possible with conventional transmitted light microscopy. Fluorescence microscopy has shown a

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great improvement over the last decade, which provides a wide array of research possibilities in live cell imaging (Fritzky and Lagunoff, 2013). This advancement is associated with increased use of fluorescent proteins, availability of numerous new fluorophores, development of different types of fluorescence microscopy techniques, including basic confocal microscope, and multi-photon microscopy for thick samples and better image contrast, and breaking of the resolution limit by the development of super-resolution microscopy (Shaner et al., 2005; Suzuki et al., 2007).

The fluorophore absorbs light, which causes its electrons to be stimulated from their ground state (lowest energy level) to an excited state (higher energy level). Since some energy may be lost as heat or other forms of energy during the electrons' descent to the ground state, they emit light with a longer wavelength when they return to their lowest energy state. The emitted light is seen as fluorescence. The phenomenon is described by Jablonski's diagram (Coling and Kachar, 1997; Jin and Riedel-Kruse, 2018). The difference between the excitation and emission maximum of a fluorophore is called the Stokes shift. Each fluorophore has a property of absorption of a specific wavelength of light best (or better than other wavelengths of light) and a characteristic emission of a specific wavelength. This property of fluorophores is called absorption and emission spectra. As a result, the peak wavelength of excitation and emission is the same for all fluorophores. Individual fluorophores' variations in excitation and emission spectra can be used to distinguish distinct targets within the same sample (Lichtman and Conchello, 2005).

Specific parts of biological samples can be labeled using fluorophores or in some cases, fluorophores can be found naturally in the sample of interest (Lichtman and Conchello, 2005). Fluorophores can also be introduced into organisms through genetic modification to the organism's DNA to encode fluorescent molecules (Chudakov et al., 2010; Thorn, 2017). An alternative method is tagging antibodies with small molecule fluorophores, such as Texas Red, and then introducing them into the specimen (Lichtman and Conchello, 2005).

WIDEFIELD FLUORESCENCE MICROSCOPY

Wide-field fluorescence microscopy (WFFM) is an imaging technique where the whole specimen is shown with light of a specific spectrum in which the resulting image is either viewed by the observer or captured with a camera (Sanderson et al., 2014; Wilson, 2017). The fundamental components of WFFM are the excitation light source, beam splitter/mirror, dichroic and emission filters, the camera, and the objective lens. The excitation of the fluorophore by the polychromatic light source results in an emission of a longer wavelength of light that can be visualized through an eyepiece or via a digital camera. Widefield fluorescence microscopy has three filters that have different purposes. The excitation filter allows only the light spectrum passage that best excites the fluorophore. The dichroic mirror reflects the excitation light to the specimen and permits the passage of the emitted light from the specimen to the emission filter. On the other hand, it ensures that only the wavelength emitted from the fluorophore in the sample pass through (Jin and Riedel-Kruse, 2018). TheWFFM detects the resulting image via multi-point detector or digital camera (Lichtman and Conchello, 2005).

Wide-field fluorescence microscopy is sensitive, affordable, and versatile for live-cell imaging that can generate beautiful high-resolution images 20. It is simple and fast because all specimen parts are illuminated simultaneously, allowing for easier and faster imaging with a camera. However, as wide areas of the sample are illuminated, optical diffraction and out-of-focus lights can result in blurry images (with low contrast and spatial resolution, Lichtman and Conchello, 2005; Sanderson et al., 2014). This can be improved using deconvolution, an image restoration technique that uses different algorithms to send back the out-focus light to its original position and provide a high-contrast image (Swedlow and Platani, 2002).

Wide-field fluorescence microscopy is a significant technique for the observation and identification of cells, cellular components, and localization of the protein (Scientifica, 2022). It is very crucial to examine thin specimens like cells of the monolayer (Wang and Lai, 2021). Wide-field fluorescence microscopy can produce an image with better resolution compared to conventional transmitted microscopy (Vangindertael et al., 2018). It is an appropriate tool for long-standing and comparatively swift quantitative time-lapse imaging. Using this technique, it is possible to visualize the amount and localization of specific fluorescent molecules in a very short period (Scientifica, 2022). For instance, cellular processes that take place within cell-like neuronal signaling can be quantified in real-time (Scientifica, 2022). Other advantages of WFFM include its relatively cheaper price compared to other advanced techniques such as confocal laser-scanning microscopy (CLSM), its high resolution in the XY dimension and very fast temporal resolution, and it relatively requires the least amount of excitation light (Coling and Kachar, 1997; Vangindertael et al., 2018).

CONFOCAL LASER-SCANNING MICROSCOPY

Confocal laser-scanning microscopy (CLSM) is a type of microscopy characterized by a pinhole opening that serves as a barrier to light originating from other focal planes in the sample to get rid of out-of-focus light from the image, which leads to better contrast with low noise and high resolution (Webb et al., 2004; Cole, 2014; Sanderson et al., 2014). A laser is used in place of a lamp in all CLSM systems, sensitive photomultiplier tube detectors (PMTs) are used

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to measure the intensity of light emitted, and computers are used to operate the scanning mirrors and assist in image collection and display (Ulrich, 2015).

In CLSM, a pinhole aperture on a confocal plane with a scanning point on the specimen and a second pinhole aperture in front of the detector allows consistent light, which is an excitation source, from the laser system to pass through. The secondary fluorescence emitted from points on the specimen (in the same focal plane) passes through the back side of the dichromatic mirror and is focused as a confocal point at the detector pinhole opening while the laser is reflected by a dichromatic mirror and scanned across the specimen in a defined focal plane. Out-of-focus light rays are the significant amounts of fluorescence emission that do not confocal with the pinhole and occur at sites above and below the objective focal plane (Hashimoto et al., 2007). As a result, the photomultiplier detects only fluorescence originating from the in-focus sample plane (Ulrich, 2015).

It is an essential technique with a broad range of applications in biomedical research (López-Macay et al., 2016). Confocal laser-scanning microscopy is required when working with thick specimens, such as tissue slices and small organisms/live animals like Drosophila or zebrafish, to eliminate out-of-focus light. Due to the ability to produce serially thin optical sections (Webb et al., 2004; Cole, 2014). Confocal microscopy is particularly useful for acquiring and analyzing images from thick specimens, such as tissue samples containing structures like cells, and for observing subcellular structures, which are either autofluorescent or have been targeted with fluorescent molecules (López-Macay et al., 2016). Because of how much light is rejected by the pinholes and the low quantum efficiency of the photomultipliers used to create the image, the photon dose needed for imaging in CLSM is significant (Cole, 2014). Nevertheless, CLSM scans point by point with the so-called raster imaging, which is a very slow process. Slowness in microscopy may increase the vulnerability to Photobleaching (Webb et al., 2004).

MULTI-PHOTON CONFOCAL LASER-SCANNING MICROSCOPY

Multi-photon confocal laser-scanning microscopy (MP-CLSM) is equipped with a spinning Nipkow disk comprising an array of pinholes and microlenses and uses arc-discharge lamps for illumination instead of lasers and multiple-beam microscopes to readily capture images with an array detector (Ulrich, 2015). A single fluorophore molecule's ability to simultaneously absorb two or more low-energy photons of light is the foundation of multi-photon confocal laser-scanning imaging (Cole, 2014).

Multi-photon confocal laser-scanning microscopy is a compelling technique for examining thick tissues and uses more than one longer wavelength of light (infrared and near-infrared) wave instead of one with high energy/short wavelength (Andresen et al., 2009). Hence, the out-of-focus light produced is quite faint and does not excite anything to an appreciable level, the only area where excitation comes from is at the focal point. Therefore, light is obtained only from the in-focus spot, as a result, and there is no requirement for a pinhole. As a result, MP-CLSM is more efficient for collecting light as it does not require a pinhole. High light penetration into the living body is obtained while infrared and near-infrared rays are used. This makes MP-CLSM a preferable technique for visualization and examination of thick samples (usually for thicker than 100 µm samples) (Mülter, 2019; Schneckenburger and Richter, 2021). Confocal laser-scanning microscopy/confocal microscopy can be utilized for samples up to 100 micrometers thick, but for thicker specimens, it is not possible to eliminate all of the focus light via the pinhole in CLSM (Dunn and Young, 2006; Schneckenburger and Richter, 2021).

Having a lower photobleaching effect is another advantage of MP-CLSM over CLSM since MP-CLSM uses longer wavelengths. On the contrary, the image of MP-CLSM has a lower resolution, compared to CLSM because the resolution is the function of the excitation light wavelength (Rocheleau and Piston, 2003). In MP-CLSM, a single laser excitation is used for more than one fluorophore instead of multiple lasers for different fluorophores. This results in limited chromatic aberrations. Hence, MP-CLSM is ideal for co-localization studies or co-dynamic measurements such as Förster resonance energy transfer (FRET), or two-color image-correlation microscopy (Piston, 2006; Kawano et al., 2008). Multi-photon confocal laser-scanning microscopy can also be used for longitudinal studies of cortical structures of the mouse brain at high optical resolution (Grutzendler et al., 2011).

SPINNING-DISK CONFOCAL MICROSCOPY

Unlike confocal microscopy, spinning-disk confocal microscopy (SD-CM) possesses many pinholes. Hence, spinning-disk confocal microscopy is equipped with multi-point detectors, such as complementary metal oxide semiconductors and charge-coupled device cameras (Bai et al., 2020). As mentioned before, cameras have higher quantum efficiency than single-point detectors such as photomultipliers. Therefore, SD-CM is a preferred microscopy method for dim samples. In addition, the architecture of multiple pinholes provides SD-CM with the associated great speed. Hence SD-CM is better than confocal microscopy in time-lapse live cell studies because it is associated with shorter exposure time and minimization of photobleaching. Nevertheless, confocal microscopy is still a method of choice for thick samples; SD-CM and WFFM are rather preferred for thin samples (Schneckenburger and Richter, 2021). With spinning disc confocal microscopy, there is a slight loss of confocality due to the possibility of some out-of-focus light

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leaking through many pinholes; however, this is made up for by a superior signal-to-noise ratio and a reduced risk of photobleaching (Stehbens et al., 2012).

STIMULATED EMISSION DEPLETION MICROSCOPY

The resolution of a light microscope is limited by diffraction to about 200-300 nm (Culley et al., 2018). Therefore, objects residing at a closer distance than 200 nm, cannot be distinguished. This is why electron microscopy was invented, and its higher spatial resolution has allowed us to make great discoveries in life sciences. However, it is also evident that it is impossible to use an electron microscope to examine a cell in three dimensions, especially inside a living cell or living tissue. Therefore, using focus visible light is required. Hence, an invention of the light microscope that would overcome the diffraction barrier and provides an image with a very good spatial resolution comparable to the electron microscope would be very important (Hell and Wichmann, 1994; Meyer et al., 2008).

The primary component of a light microscope, the objective lens, is used to concentrate light onto a single point. However, because light travels as a wave, the lens is unable to focus all of the light into a single spot. This will cause the light to spread out and create a blob that is at least 200 nm across the focal plane. This blob is termed a point spread function. All the features falling within this spot are flooded at the same time with light. In fluorescence microscopy, excitation light is utilized, and for the same reason explained above, all the features will give of signal and be collected by the lens, and impossible to tell these features apart. To separate two objects by a light microscope, they have to be apart by the distance "D", which can be calculated by dividing the wavelength of light twice by the numerical aperture of the objective lens as given in the following Formula 1 (Bagnell, 2012; Diaspro and Bianchini, 2020). Where D is the distance between the objects, Lambda is the wavelength of light, and NA is the numerical aperture D = lambda / 2 NA (Formula 1).

The least possible value accounts for at least about 200 nm in XY and up to 500 nm in the Z dimension. In stimulated emission depletion (STED) microscopy, it is not only one beam for exciting molecules that is focused into the 200 nm range used, but also a beam of light that has a typical shaped-donut is also used (Culley et al., 2018). As it is not required to shut off all molecules, the STED beam has been shaped into a donut pattern. It is needed to keep an area where the molecules are still capable of emitting. Only a portion of the molecules is permitted to emit, although all of them are colored by excitation light, and the remainder is silent. This is achieved by using photons that do not have an energy that is high enough to excite molecules. If the photon energy fits the fluorescence data, the energy between the fluorescent state and the ground state, those photons are capable of sending molecules back down to the ground state instantly by taking away the majority of the energy in the red-shifted beam. The beam here is red-shifted because it has lower photon energy, and its role is simply to silence the molecules. The right wavelength should be utilized to silence the molecules. However, there is a need to make sure that there are enough red photons in the red-shifted beam. This is because if there are enough red photons, it is possible to be sure that once a molecule gets excited, there is always a red photon out there that will instantly kick the molecule down to the ground state (Hell and Wichmann, 1994; Meyer et al., 2008; Wildanger et al., 2009).

LOOP MICROSCOPE

A Loop microscope is a remarkable method that enables us to observe and quantify surface structure with unprecedented precision and detail (West and Eaton, 2010). With its ability to manipulate objects with nanometer-scale features, an image in a vacuum, air, or liquids with sub-nanometer resolution, and measure forces with greater than pico-Newton resolution, the loop microscope has become a beneficial instrument in a wide range of disciplines (Abramovitch et al., 2007). It can be used to photograph samples that are compliant, such as biologically important materials. This ability has been utilized to examine mechanical dynamics and characteristics at individual cells' level down to individual molecules. The *in-situ* analysis of drug-induced alterations in cell shape, membrane stability, and receptor contact forces, as well as the investigation of cell motility, are a few effective uses at the cellular level (Abramovitch et al., 2007; Hessenauer et al., 2021). The activity of RNA polymerase, the motion of molecular motors, including proton-powered turbines and myosin V, the transcription process, and the structure of a wide range of viruses have all been studied using single molecules (West and Eaton, 2010). To examine the mechanical characteristics of biological structures and the forces of molecular interactions, this microscope has also been extensively employed as a force transducer (Abramovitch et al., 2007).

The loop microscope is a helpful actuator for nanotechnology and can manipulate material as well. Employing customized tips makes it possible to apply a range of forces, including contact, magnetic, thermal, and electrical forces. It has been applied to nano assembly, nano manipulation, and lithography (West and Eaton, 2010). The loop microscope will remain a crucial tool in the researcher's toolkit due to the ongoing interest in understanding materials and biological systems at the nanoscale and the promise of nanotechnology (Abramovitch et al., 2007; Hessenauer et al., 2021). Unlike most microscopes, the loop microscope performs differently, and the probe physically interacts with the sample (West and Eaton, 2010). The loop microscope loop starts with a sample to image. The sample is usually on a raster-scanned

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surface back and forth. When a cantilever's sharp tip is brought in close contact with a surface, the cantilever deflects due to the interaction between the tip and the surface. The resulting deflection is measured by reflecting a laser beam off the back of the cantilever onto an optical detector. An optical detector's deflection signal is compared to a nominal deflection value that represents the imaging force, and the difference is reduced by employing a feedback controller. The surface profile is often estimated from the control signal itself (Meyer, 1992; Abramovitch et al., 2007; West and Eaton, 2010).

CONCLUSION

Different types of microscopy techniques can be used to study live cells and tissues. All live cell microscopy techniques discussed have their pros and cons. Decisions on the choice of the microscopy technique should be made based on the objective of the research and the capability of the technique to provide the required information without damaging the tissue. For this purpose, optical light paths should be optimized for sensitivity, speed, and resolution based on the objective of the research. Hence, a thorough understanding of the technique and application of the various live-cell microscopy methods is paramount.

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Authors' contribution

Tewodros Abere Mekuria drafted the manuscript. Mebrie Zemene Kinde edited the draft of the manuscript, and prepared it for publication. The authors read and approved the final version of the manuscript for publishing in the present journal.

Competing interests

The authors declare that they have no competing interests.

Ethical consideration

All authors have reviewed their work for ethical problems, such as plagiarism, consent to publish, misconduct, data fabrication and/or falsification, duplicate publishing and/or submission, and redundancy.

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