Three basic types of fluorescence microscopy and recent improvement

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Abstract. Fluorescence microscopy is one of the most used imaging modalities in molecular biology and living specimens. To increase image contrast and spatial resolution, different type of fluorescence microscopy has been developed. This review introduces three main types of fluorescence microscopy: wild-field microscopy, confocal microscopy, and total internal reflection fluorescence microscopy. The basic principles are similar but with different modifications, which also indicates their attributes and limitation. The recent improvement on these microscopies is also discussed. Some most recent techniques show advance in overcoming common fluorescence microscopy's weakness, and future perspectives are also discussed.

1 Introduction

Sir George Gabriel Stokes first described fluorescence in 1852. It is a phenomenon that organic and inorganic specimens absorb light and subsequently re-radiate light. Because some energy is lost during fluorescence, the emitted light has less energy than absorbed. Light with a longer wavelength shows less energy than light with a shorter wavelength, so the emitted light usually has a longer wavelength than absorbed, called Stokes shift. The emission and absorption are simultaneous and usually about 10^{-9} to 10^{-8} seconds. Molecules that show fluorescence are called fluorophores or fluorochromes, which have a characteristic absorbance and emission spectrum. Fluorochromes were introduced in biological investigations in the 1930s and stimulated the development of fluorescence microscopy (FM). They are considered stains that attach to visible or sub-visible structures with high specificity in their attachment targeting, such as proteins, lipids, or ions [1, 2].

Due to the different energy between emitted light and excitation light, the goal of the FM is to separate these 2 lights and only allow emitted light to reach the detector (Fig. 1). The basic function of FM is to irradiate the specimen with its characteristic band of wavelengths and then separate its characteristic emitted light from the excitation light. Because the excitation light has higher energy, it is necessary to filter out this very bright excitation light to view the relatively weak fluorescence. Optical filters generally achieve this separation. Dichroic mirror serves as the primary optical element to reflect and separate the light, but it is not perfect. To enhance the selection, excitation and emission filters are commonly used. The excitation filter ensures only the

characteristic wavelength of excitation light is transmitted. The emission filter is used to block excitation light, pass emitted light, and make sure only the emission light can reach the detector. However, to create a useful image, FMy needs to obtain sufficient light; filters always cause loss of light intensity. Using a long-pass filter, it is possible to collect all the light from the emission spectrum [3]. The emitted fluorescence can be viewed by the eyes or captured electronically.

Fig. 1. The basic setup of a fluorescence microscope.

The source is filtered by an excitation filter and then reflected off the dichroic mirror toward the specimen. A dichroic mirror and emission filter fluorescence. Only the emitted light reaches the detector.

Due to not being available in traditional optical microscopy, fluorescence microscopy has become an essential tool in biology, biomedical sciences, and material science. In the fields of botany, petrology, and the semiconductor industry, autofluorescence has been thoroughly exploited. However, in biology and biomedical sciences, the situation is often complicated

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due to the extremely faint or bright, nonspecific autofluorescence in animal tissues and pathogens. Fluorochromes are often considered in the latter studies. By introducing fluorochromes in non-fluorescing material, identify cells and sub-microscopic cellular components have been achieved. The specificity of fluorochromes makes it possible to identify several target molecules simultaneously within the same sample by using multiple fluorescence labeling simultaneously. Some fluorescence microscopes can show the 3-dimensional interior of cells and organisms [4].

The major weakness is the limitation of the resolution. FM cannot provide spatial resolution below the specimen's diffraction limit [5]. The diffraction limit restricts optical resolution in about half the wavelength of the emitted light [6]. Therefore, the resolution of FM is fundamentally limited by the wavelength of light. In addition, the wavelength of the excitation light and the microscope optics also limits spatial resolution [3]. Its dependence on probes also limits FM. For molecular structures that are incapable of autofluorescence, suitable fluorochromes are required. Some fluorochromes show cytotoxicity and damage DNA [7]. It spends time and money to design and select suitable fluorochromes. In addition, the simultaneous use of multiple fluorescences may lead to a poorer signal-to-noise ratio (SNR). This is because the induced interactions between proximate probes can detract from the effectiveness [8]. In some cases, sample cells can be damaged by exposure to light called phototoxicity [9]. The lower wavelengths of light used for excitation are particularly damaging to cells due to their higher energy. In other cases, fluorochromes may lose their capacity to fluoresce, called photobleaching [10]. This is due to the accumulated chemical damage from the electrons excited during fluorescence. Despite this, FM is still a popular technique in live-cell observation and structure elucidation of biomolecules in tissues and cells in situ.

The basic FM is wide-field (WF) fluorescence microscopy, which is excellent for 2D images of specimens, and the entire field can be captured at once. Confocal microscopy is an advanced FM that can show 3D images with higher SNR than WF fluorescence microscopy. Total internal reflection fluorescence microscopy (TIRFM). This report will introduce these 3 major types of fluorescence microscopies and discuss their attributes and shortages how scientists try to overcome these shortages and improve their properties. The report will also discuss the most recent technique improvement in FM.

2 Wide-field fluorescence microscopy

2.1 Principle of WF fluorescence microscopy

Wide-field (WF) Fluorescence Microscopy is a basic fluorescence microscope that cell biologists commonly use. For this microscope, a parallel beam of light illuminates the whole specimen at once to excite the fluorophore. All the resulting fluorescence of specimens can be viewed simultaneously, allowing simple and fast

imaging. For multiple-prob specimen, all the Fluorescence can be viewed at once. Due to all parts of the specimen can be viewed at once, it allows a quick selection of fluorescent cells to image.

2.2 Limitation

The wide-field illumination and view not only focus information from the corresponding section of the specimen but also allows out-of-focus light to arrive camera, leading to low contrast and spatial resolution [4]. For thick samples, light from sample planes above and below the focal plane will also be detected. The proximity of fluorochrome also contributes to low SNR. Due to optical diffraction, each fluorochrome shows a broad fluorescence zone that can interact with nearby fluorescence and cannot be resolved. Therefore, if fluorochromes are closely associated, it will result in a bright blur rather than discrete objects. Another shortage is its limitation in 3D imaging. The WF microscope doesn't gather sufficiently complete information to allow 3D imaging due to its non-specific data collection, including out-of-focus blur [11]. The resolution of the z-axis is about 1 μm .

2.3 Recent improvement

However, Wide-field Fluorescence Microscopy is well suited in thin specimen imaging, like single-cell layer imaging and macroscopic structure. These specimens closely adhered to a glass surface can limit out-of-focus light arriving. By using spatially structured illumination, the resolution of WF microscopy can be increase. Gustafsson et al. [4] applied structured illumination microscopy in 3D and doubled the 3D resolution in WF microscope. Xue et al. applied Computational Miniature Mesoscope (CM2) to the wide-field microscope and successfully presented 3D imaging [12]. By using computational algorithms, they augmented the optics and expanded imaging capability. This combination enlarges miniscope systems imaging areas from lower than 1 mm^2 to 56 mm^2 and wide-field measurements depth of field to 2.5-mm and resolution to $\sim 7\text{-}\mu\text{m}$ lateral and better than 200- μm axial.

3 Confocal Fluorescence Microscopy

3.1 Principle of confocal microscopy

Thin specimens limit WF microscope. As specimen thickness adds, out-of-focus light increases and hinders imaging. Confocal microscopy can eliminate out-of-focus light from images and allow thick specimens to be viewed with high resolution [13]. The best resolution that a confocal microscope can attain is about 200nm [14]. The major difference of confocal microscopy in the region of illumination and detection. A confocal microscope only illuminated a diffraction-limited region of the specimen at one time and only accepted a signal from that region [15]. The confocal microscope has been

widely used in immunofluorescence and greatly benefits in overcoming background glare [15].

3.2 Laser scanning confocal microscopy

Laser scanning confocal microscopy (LSCM) place a pinhole conjugated to the focal plane (Fig. 2). By closing the pinhole to various extents, the out-of-focus light is blocked, and only the light from the focused point in the specimen can reach the detector. By a sequential scanning method of the excitation and detection point, fluorescence intensity across the specimen can be collected, and images can be sequentially generated [3]. The excitation light and pinhole are stationary, but the region they focus on is optically moved by an orthogonal pair of oscillating mirrors, x-axis mirror, and y-axis mirror. Therefore, out-of-focus elements cannot affect the background because they receive little illumination, and a pinhole rejects any out-of-focus light.

In addition, confocal microscopy also provides more resolution in depth. Closing down the pinhole increases the resolution in Z-axis. By assembling a series of thin specimens along the z-axis, it is possible to build a 3D image [16]. Comparing with a WF microscope, the confocal microscope can provide a 3D structure with a higher resolution, and the z-axis resolution can attain 700nm.

Moreover, by using a laser scanning system, dwell time decreases, which reduces the damage of photobleaching and photodamage. Dwell time is the time that excitation light remains in one location in the specimen. For LSCM, the laser beam continually scans across the sample, and the image is generated from discrete pixels that follow the laser scan proceeds. The dwell time is related to the image's recorded speed and pixel resolution. The example indicated by Sanderson, Smith, Parker & Bootman explains this relationship clearly: if the detector records 30 images per second, and each image has 512 horizontal pixels and 521 vertical lines, the dwell time would be $33\text{ms}/512 \times 512 = 127\text{ns}$. In this case, each location of the sample only needs to be illuminated for a very short time for LSCM, while, for WF microscopy, the whole sample needs to be exposed for 33ms [3]. Therefore, confocal microscopy decreases the exposure time of specimens to light and reduces the damage of photobleaching and phototoxicity.

Fig. 2. The basic setup of laser scanning confocal microscopy.

Light from the laser is scanned across the specimen by the scanning mirrors. The emitted light passes through a pinhole to the detector. Size of the pinhole limits out-of-focus light to the detector.

3.3 Spinning disk confocal microscope

LSCM uses a single-point scanning system which needs much time to scan the whole specimen. To capture image at high speed, spinning disk confocal microscopy are developed [17]. Spinning disk confocal microscopy allows a multiple-point scanning system. In this instrument, the sample is both illuminated and viewed through a spinning disk with rows of pinholes arranged. As the disk spins, each pinhole on the spinning disk serves as a point source of light to scan across specimen; similarly, the emitted light also passes through a corresponding pinhole before separated by DM. Spinning disk microscopy indicates a faster image acquisition rate and lowers light requirement than LSCM. Drawbacks include the loss of resolution due to cross-talk between multiple fluorescence points and fixed pinhole size, which is optimized for a specific objective. One recent technique is ribbon scanning confocal microscope [18]. By employing resonant scanners and high-precision x, y-axis scan mirrors continuously capture data across samples, reducing time to data collection.

3.4 Limitation

However, the attributes of confocal microscope also connote shortages. Confocal microscope blocks out-of-focus light by keep pinhole small, but much in-focus light is also discarded. There is a tradeoff between light-collection efficiency and resolution. Considering the weak fluorescence of biological samples, the loss of in-focus light usually outweighs resolution benefits. The best resolution is $0.2\mu\text{m}$ on the x-axis and $0.6\mu\text{m}$ on the y-axis. The x-axis resolution in confocal microscopy is worse than the WF microscope. In practice, confocal microscopy often uses wider pinholes to generate a similar x-axis resolution as the WF microscope [4]. Similarly, the high resolution of the Z-axis is achieved by closing the pinhole, which also sacrifices the level of light reaching the detector. In addition, similar to WF microscopy, confocal microscopy is also limited diffraction effects. Another consideration is the image speed. In the WF microscope, image capture speed is mainly determined by the technology of image acquisition and brightness of the image. However, with confocal microscopy, an additional factor can be the speed of the laser raster scan of the specimen. Due to inertia, the speed of scan mirrors is limited; thus, the most confocal system can only capture 1 to 10 images per second, and dwell time also increases substantially [19]. In fact, LSCM performs worse in photobleaching and phototoxicity than WF microscopy. The higher price is another shortage. Generally, confocal microscopy costs 2-7 times more than WF microscopy.

3.5 Recent improvement

To increase the resolution of confocal microscopy, Airyscan technology is applied [20]. Airyscan has a 32-channel detector array that can reassign pixels and summate images from all detectors. This system provides 1.7 times higher resolution in the x-, y- and z-axis. A re-scan confocal microscope includes an additional pair of scanning mirrors (re-scanner) which improves x-axis resolution by 1.4, but y-axis resolution shows no improvement [20].

4 Total Internal Refraction Microscopy

4.1 Principles of TIRFM

Total Internal Refraction Microscopy (TIRFM) is based on the excitation of fluorophores by evanescent wave or field when the laser beam internally refracts. Total internal refraction (TIRF) is an optical phenomenon that the light will reflect instead of refracting when the light travels from a medium of high refractive index to that of low refractive index, and the incident angle should be greater than the critical angle. This phenomenon follows Snell's Law.

In the case of TIRF, reflected light turns into an electromagnetic field at the interface, and an evanescent field forms through the media of low refractive index, which amplitude decays exponentially (Fig. 3). Therefore, the penetration depth of the evanescent field can only be around 100 nm. In a typical experimental setup, the fluorophores near the interface having the capacity of electronic transition with or close to the wavelength of the laser beam can be excited by the evanescent field.

Fig. 3. Objective-based TIRFM.

The incident laser produces an evanescent field, which excites the fluorophores close to the interface and generates high SNR images.

4.2 Prism-based TIRFM and objective-type TIRFM

The prism-based method employs a prism to generate evanescent waves and collects fluorescence images with an objective lens [21]. But because of its structure, this method has a deficiency in complex operations of

specimens, such as adding drugs and medium. On the other hand, the objective-type approach depends on a large numerical aperture (NA) objective to produce an evanescent field and to image [22]. Compared with a prism-based microscope, objective-type TIRFM is more prevalent in the market, for it is much easier to operate and has higher precision.

4.3 Advantages

Because of the low penetration depth of evanescent wave (usually less than 100nm), there is almost no background fluorescence, causing super z-axial resolution and high SNR. In addition, since only a small portion of the specimen is exposed to evanescent waves, the effect of toxic gas and phototoxicity drastically weakened, extending experiment time. Meanwhile, TIRF is generally accepted and only restricted by available laser beams, so it is relatively easier to perform live-cell imaging techniques like FRET. TIRF microscopy was widely adapted to the situation where specimen contains many fluorophores, and the research needs imaging, such as the investigation of cell membrane and endocytosis.

The research about protein to single sites of endocytosis was always hindered due to the lack of direct images of this process. In a study about Plant Science on clathrinid, TIRF microscopy spatially and temporally probes the process of endocytosis and helps to find out the protein resulting in endocytosis in plants [23]. In another research to inhibit the infection of HIV, combined with cryo-electron tomography (cryoET), TIRFM can image the fusion of blebs from native cell membranes as targets and pseudo virion of HIV envelope glycoprotein with or without Serinc host restriction factors. According to the images, researchers can investigate the fusion behavior and thus recognize which Serinc can inhibit HIV fusion [24].

4.4 Limitation

Because the energy of the evanescent wave decreases exponentially from the interface, the fluorescence signal is not only strictly limited near the total reflection interface (generally the interface between the glass slide and the sample, in the range around 100 nm) but also too weak to detect.

Also, the low penetration depth of the evanescent field contributes to the limitation of specimens. Specimens can only be applied to specific cell types and lie flat on the coverslip, such as epidermal cells [23]. At the same time, TIRFM relies on the specimen's operation, such as fluorescent labeling of proteins, which may change the structure and function of specimens.

4.5 Recent improvement

TIRF is used in combination with many other microscopes to compensate for its shortcomings and achieve better results.

In recent research, fast two-dimensional total internal reflection fluorescence, high-resolved structured illumination microscopy (SIM), and traction force microscopy (TFM) are combined for higher spatial and temporal imaging. 2D TIRF-SIM-TFM microscopy supplies information of cellular structure and magnitude and the direction of force produced by cells. Thus, the microscopy has >2-fold spatially and >10-fold temporally resolution of planar cellular force probing [24].

Also, the combination of fast high-resolution spinning disk and total internal reflection fluorescence microscopy using a new light path can fast produce higher temporal resolution images about the dynamic cellular processes between membrane and cytoplasm [25].

5 Conclusion

WF, confocal microscopy, and TIRFM are 3 basic types of fluorescence microscopy. Each of them shows different attributes and limits. WF is a quick and simple technique with an advantage in single-cell layer imaging, but it has relatively low SNR and a shortage in 3D imaging. By using spatially structured illumination, recent research has shown great success in WF 3D imaging. Confocal microscopy shows an advantage in 3D imaging but with long image speed. Spinning disk confocal microscopy shows reduction of time of data collection. TIRFM is an advanced FM with high SNR and relatively low photobleaching and phototoxicity but shows specimen limits. New techniques have been combined with the basic type of fluorescence microscopies and show great improvement in their drawbacks. However, some shortages still limit the imaging in all three basic FM.

The main shortage of all these 3 fluorescence microscopies is the diffraction limit which limits the resolution. New fluorescence microscopy has been developed to overcome this weakness. Based on WF, TIRF, or confocal microscope setups, super-resolution microscopy (SRM) greatly improves optical resolution. By changing the way of excitation and detection, SRM bypasses the diffraction limit to nanometre scales and allows imaging of subcellular organization. SRM makes it possible to view cellular structure in detail of electron microscopy level and keep sample activity. SRM can also view 3D structure details and show single-molecule localization, for example, how proteins are distributed and organized in cells.

Although extensively adopted for a long time in cellular biology, FM still has challenges. High cost, type of fluorochrome, and high noise level, and other limitations are worth exploring. Because different specimens need specific fluorochrome, we propose investigating how to get or design proper fluorochrome to specimen efficiently. Meanwhile, noise level always deteriorates results of algorithms, which is shown in the combination of CMOS and SMLM. In past years, the most adopted way to overcome limitations is to combine different microscopes or cameras to achieve better

results. In this way, prospective research can try different combinations for greater images. For example, researchers can combine low-cost with other microscopes, producing low-cost and high-resolution images.

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