Modern Trends in Imaging XII

Advanced methods in fluorescence microscopy

Luke Fritzky and David Lagunoff*
Core Imaging Facility, New Jersey Medical School, UM, NJ, USA

Abstract. It requires a good deal of will power to resist hyperbole in considering the advances that have been achieved in fluorescence microscopy in the last 25 years. Our effort has been to survey the modalities of microscopic fluorescence imaging available to cell biologists and perhaps useful for diagnostic pathologists. The gamut extends from established confocal laser scanning through multiphoton and TIRF to the emerging technologies of super-resolution microscopy that breech the Abbé limit of resolution. Also considered are the recent innovations in structured and light sheet illumination, the use of FRET and molecular beacons that exploit specific characteristics of designer fluorescent proteins, fluorescence speckles, and second harmonic generation for native anisometric structures like collagen, microtubules and sarcomeres.

Keywords: Fluorescence microscopy, confocal microscopy, multiphoton microscopy, total internal reflectance microscopy (TIRFM), lateral sheet illumination microscopy, deconvolution, stimulated emission depletion microscopy (STED), reversible saturable/switchable optically linear fluorescence transition microscopy (RESOLFT), photoactivation localization microscopy (PALM), stochastic optical restoration microscopy (STORM), structured illumination microscopy (SIM), super-resolution optical fluctuation imaging (SOFI)

1. Introduction

A reasonable claim can be made that fluorescence has enabled the most significant advances in light microscopy in the last 25 years. The advances have been made possible by a synergistic interaction among optical instrumentation, chemistry, molecular biology and nanotechnology. The result is an array of powerful methods for imaging and analyzing living and fixed cells and tissues. The principal impact of fluorescent microscopy has been on research with limited penetration in the realm of diagnostic pathology. The explanation for the difference lies in the constraints under which the diagnostic pathologist

typically works, utilizing wide field microscopy to examine fixed tissue. The future of advanced fluorescence microscopy in diagnostic pathology is likely to depend on the development of sample processing, including labeling techniques, that allow access to one or more new fluorescent modalities. Such an effort is complicated by the range of modalities of fluorescence imaging introduced in the last ten years, and the cost of purchasing and maintaining the specialized microcopes. However most research institutions have established well-equipped core facilities in which it is possible to explore the possibilities of applying fluorescence microscopy to diagnostic pathology. Endoscopists [1–4], ophthalmologists [5, 6] and dermatologists [7] appear to be moving rapidly in the use of confocal microscopy in the clinical realm.

Fluorescence is the property of molecules and ions of emitting light after absorbing photonic energy [8].

*Corresponding author: David Lagunoff, Adj. Professor, Department of Surgery, Director of Imaging Core Facility, Cancer Center, New Jersey Medical School, UM, NJ, USA. E-mail: lagunoda@umdnj.edu.

It is operationally distinguished from phosphorescence by the time interval between the excitation by absorbance and the emission, 10^{-9} to 10^{-6} seconds for fluorescence and 10^{-3} to 10^{2} minutes for phosphorescence. The preponderance of fluorescent molecules emit photons of lower energy than the exciting photons, shifting the emission spectra to longer wave lengths, the Stokes' shift. An important exception is exploited in multiphoton microscopy (vide infra). Fluorescence owes its usefulness principally to three characteristics: 1) sensitivity of detection, 2) selectivity arising from the characteristic excitation and emission spectra of differing fluorochromes, and 3) susceptibility to informative perturbation by conditions in the local environment of the fluorescent molecules. Sensitivity, defined as the number of molecules/unit volume that can be detected, is determined by the product of the absorbance of the molecule (molecular extinction coefficient) and the quantum yield (the ratio of photons emitted to photons absorbed). The operative correlate of sensitivity in microscopy is contrast, the distinction of the fluorescent object from the background. Contrast in the image of a microscopic object depends on multiple factors: the intrinsic sensitivity of the fluorophore; the wave length, bandwidth and intensity of the exciting light incident on the object; the time of exposure; features of the local environment, including oxygen tension, pH; the presence of other fluorescent and non-fluorescent molecules in the sample; and the optical characteristics of the microscope and the detection system.

The primary uses of fluorescence microscopy are the location of specific fluorescent molecules in 3-dimensional sample space, the distinction of two or more nearby foci of fluorescent molecules as separate, the identification of close molecular associations, and the tracking of cellular and molecular motion [9]. Location is dependent on contrast, and separation on resolution. In depth consideration of the determinants of contrast and resolution may be found in several recent reviews [10, 11]. Assessment of close molecular association is made possible by determination of Förster radiationless energy transfer (FRET). Cell motion can be followed by real time or time-lapse recording, and molecular motion by fluorescence recovery after photobleaching (FRAP), fluorescent loss in photobleaching (FLIP), fluorescent localization after photobleaching (FLAP), fluorescence correlation spectroscopy (FCS), photoactivation, or fluorescent speckle microscopy [12].

2. Evolution of fluorescence microscopy

Early efforts at fluorescent microscopy of cells and tissues in the first half of the 20th century were thwarted by the low contrast of endogenous fluorophores (vitamin A, NAD(P)H, and most native proteins), inefficient microscopes and limited light sources. The introduction of fluorescent molecules capable of selective staining of cell components, such as acridine orange for staining of nucleic acids, conferred modest advantages over conventional absorbing stains. Entry into the modern era of fluorescence microscopy was initiated with the labeling of antibodies with fluorochromes [13, 14], followed by the introduction of epi-illumination reliant on efficient dichroic mirrors [15], the use of mercury and xenon vapor lamps, the introduction of laser light sources coupled with practical scanning devices, the employment of CCDs as photon sensors [16, 17], and the synthesis of fluorochrome labels more resistant to photobleaching than fluorescein [18, 19] (Fig. 2A). A major advance has been the cloning of intrinsically fluorescent proteins and the creation of mutants available for intracellular fusion with native proteins [20–24]. The development of quantum dots [25, 26] has provided a new class of fluorochromes. Not insignificant in the progress of fluorescent microscopy has been the availability of increasingly powerful and convenient computers.

The combination of novel instrument design and fluorophore development have contributed to the versatility of fluorescent measurements. With respect to instrument development, confocal laser scanning microscopy (CLSM), two-photon fluorescence microscopy, and total internal reflectance fluorescence (TIRF) microscopy represent the first wave of advanced fluorescent microscopy modalities still subject to lateral diffraction limits. Most recently, effort has been directed to designing microscopes utilizing structured illumination and super-resolution microscopes, capable of lateral (X-Y plane) resolution and axial (Z-axis) resolution better than 250 and 600 nm respectively, ingeniously exploiting special properties of fluorescent molecules. Lateral sheet illumination microscopy has been developed for rapid imaging of entire small object volumes the size of mouse embryos and zebrafish with reduced photon exposure.

Fluorophore synthesis has resulted in the availability of hundreds of small fluorescent molecules capable of specific binding to antibodies and cell proteins or to nucleic acids [18]. Yet other fluorochromes are
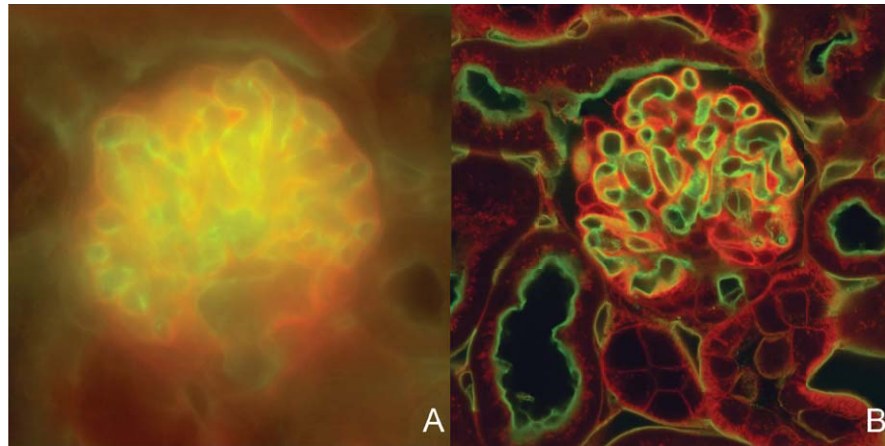
Fig. 1. Images of a 50 μm thick section of mouse kidney labeled with Isolectin B4 taken using a conventional widefield fluorescence microscope (A) and a confocal microscope (B). The out-of-focus light present in the widefield fluorescent image reduces contrast and obscures details that are clearly visible in the single optical section captured by the confocal microscope.

capable of sensing oxygen tension or pH and other ion concentrations [19]. A valuable feature of many of the new fluorochromes is their enhanced stability on exposure to light [17]; particularly notable in this regard are fluorescent quantum dots [25, 26]. The discovery and cloning of a naturally occurring fluorescent protein was a feat well worthy of the Nobel Prize in Chemistry in 2008. Deliberate mutation of such fluorescent proteins has created a range of probes with different properties that can be biosynthetically fused to native proteins of live cells. Super-resolution microscopy, is highly dependent on properties selected for in the mutant fluorescent proteins: photoactivation, photoconversion, photoswitching and excitation depletion. FLASH, an alternative to fluorescent proteins, incorporates a small peptide into a native protein; the peptide contains 4 cysteine residues appropriately spaced to bind a biarsenical derivative of fluorescein. The derivative only expresses fluorescence on binding to the tetracysteine domain [27]. Compared to the much larger fluorescent proteins, the addition of a tetracysteine motif is less likely to interfere with the function of its fused partner [28].

3. Confocal laser scanning microscopy

A major limitation of widefield epifluorescent microscopy is the loss of contrast and resolution caused by the presence of blurry out-of-focus fluorescent light within the imaging plane. Major sources of this light are fluorescence emitted by out-of-focus structures and scatter produced by refractive inhomogeneities in the specimen. Resolution is limited by diffraction which creates an expanded image of a subresolution point of light, the Airy disc, with a diameter inversely related to the numerical aperture (N.A.) of the objective lens. The diffraction pattern also extends in the axial dimension according to the point spread function (PSF) of the optical system as an hourglass shaped volume of light extending above and below the focal plane. This diffraction in the axial plane allows out-of-focus light from neighboring regions to reach the focal plane resulting in blur and loss of contrast in the captured fluorescent image. Furthermore, scattered light increases with the thickness of the specimen viewed; the thicker the sample, the greater the contribution of scattered light to the final image. As one attempts to view thicker specimens with bright 3-dimensional structures, the blurred and scattered light can obscure much or all of the detail in the captured image (Fig. 1). The presence of out-of-focus blur in widefield images places severe restrictions on the types of specimens that can be observed. In the case of living cells, only those flattened on the substrate can be imaged clearly, so that even cells rounded-up during mitosis may be too thick to discern significant details of the process [29]. The requirement for viewing thin sections restricts observations to those that have undergone processing (fixation, embedding, freezing etc.) and sectioning. Furthermore, since the depth of field of high numerical aperture lenses may be 1 micrometer or less, fixed sections as thin as 1

micron are required to limit loss of contrast with high N.A lenses [11].

The confocal laser scanning microscope (CLSM) has proved useful in remedying the limitations of widefield fluorescence microscopy. The first confocal scanning microscope failed to generate much interest in the biological community [30], so the realization of confocal microscopy as a viable research tool had to wait until the 1980's when advances in fluorescent labeling of biological specimens, the development of computers, the availability of less expensive lasers, and a method for scanning a laser beam across a sample, supported the development of convenient, commercially available confocal microscopes [31]. The present generation of confocal CLSMs, now virtually routine in most biology research, function as computer controlled, epifluorescent microscopes able to reject much of the out-of-focus fluorescent light. In a CLSM, the specimen is illuminated by laser light focused to a diffraction limited spot within the specimen. This significantly cuts down on the fluorescent light emitted by the sample in regions outside the focal plane of the objective lens. The fluorescent light emitted by the sample at each illuminated spot is separated from the incident exciting laser light with a dichroic mirror and then passed through a confocal pinhole, which rejects fluorescent light generated outside the focal plane as well as most of the light scatted by the specimen (Fig. 2B). An image of the specimen is created by scanning the diffraction limited spot in a raster pattern across the specimen using two mirrors mounted on electronically controlled galvanometer motors. The light passing through the confocal pinhole is detected with a photomultiplier tube, and the signal is processed by a computer to create a digital image.

The rejection of out-of-focus light by the CLSM permits higher contrast and somewhat better resolution imaging of biological specimens than widefield fluorescence microscopy. Specimens that would have appeared as an even, featureless haze under widefield illumination reveal significant structure in the CLSM. Furthermore, the rejection of out of focus light by the CLSM allows the creation of optical sections by collecting a series of images from stepped focal planes which can then be used to create three dimensional reconstructions of the specimen. The confocal microscope has allowed biologists to study structure in three dimensions and processes over time in living cells that could not have been addressed previously using either traditional light or electron microscopy.

4. Spinning disk confocal microscopy

A major disadvantage of the CLSM is the relatively slow acquisition of an image, caused by having to serially scan a point across the specimen. Resonant scanners in place of standard galvanometers can speed this acquisition time but the resulting images have a very low signal-to-noise ratio, due to the extremely short pixel dwell time coupled with the low quantum efficiency of the photomultiplier tubes used for detection [11]. In the spinning disk confocal microscope, the limited acquisition speed of the CLSM is remedied by scanning a series of points in parallel across the specimen, and the signal-to-noise ratio is improved through the use of high quantum efficiency EMCCD [32] and more recently scientific CMOS detectors. The spinning disk confocal microscope uses a Nipkow disk positioned at the conjugate focal plane. This disk is perforated by multiple arrays of pinholes arranged in spiral patterns, so that when the disk is spun, each hole scans a circular arc across the specimen [33]. Each of these confocal pinholes must be placed a certain distance apart to prevent crosstalk and loss of confocality, leading to very low illumination levels. If the pinholes are separated from one another by 10 times their diameter, then only 1% of the incident light will be able to pass through the spinning disk and illuminate the specimen [34]. This problem has been overcome in the Yokogawa version of the spinning disc with a series of microlenses that focus the excitation light onto the pinholes, allowing up to 70% of the excitation light to reach the specimen [33–35]. Spinning disk confocal microscopes with high quantum efficiency EMCCD sensors allow the detection of faint fluorescent signals undetectable with a CLSM. The high imaging speed coupled with sensitive detection makes spinning disk confocal microscopy an excellent choice for observing the dynamics within living cells. Even higher imaging speeds are possible with sCMOS cameras with little loss of sensitivity.

5. Multiphoton microscopy

During confocal microscopy, the laser beam is focused to a diffraction limited spot within the specimen so that most of the fluorescent light emitted by the specimen is within or near this spot, but as the laser beam passes through the specimen, fluorescence is generated from out-of-focus regions above
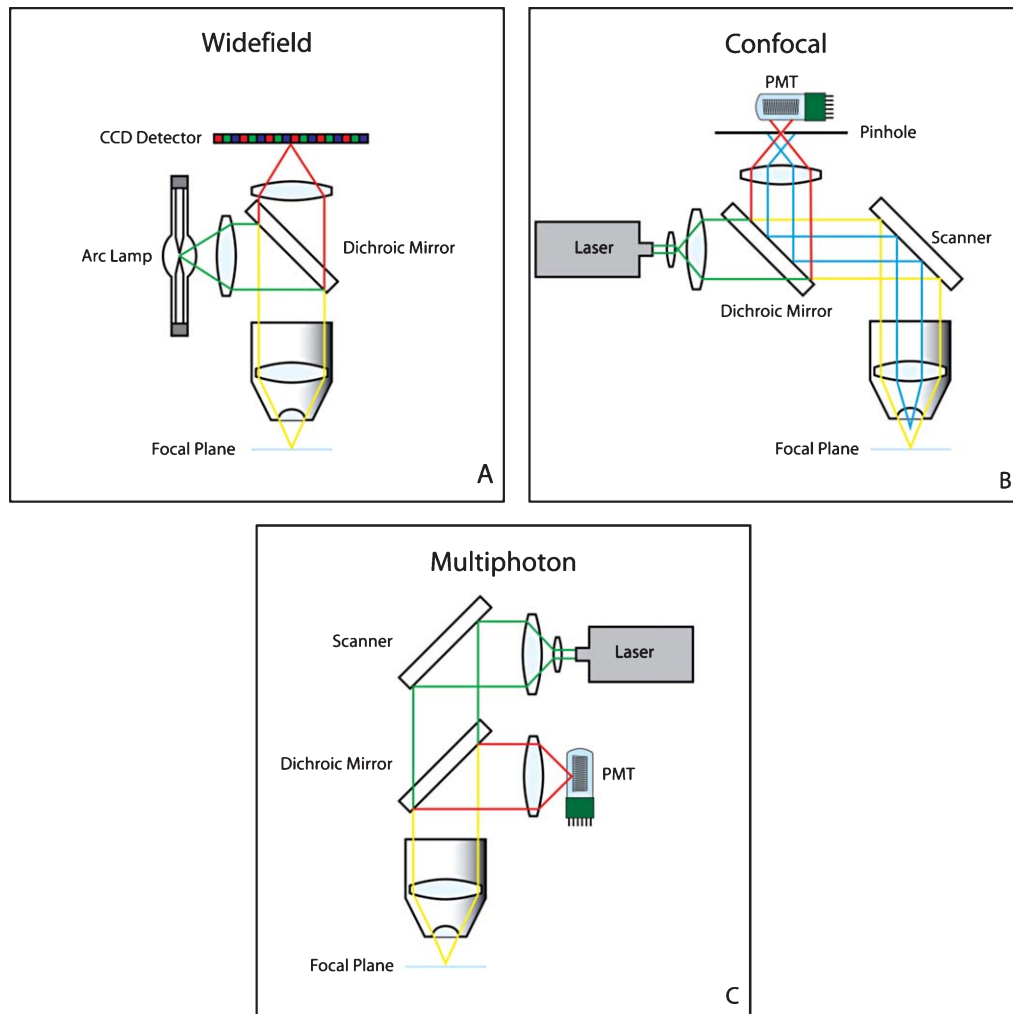
Fig. 2. Diagrams of the light paths of widefield(A), confocal(B) and multiphoton(C) microscopes. **(A)** In the widefield microscope, excitation light is produced by an arc lamp reflected towards the specimen through a dichroic mirror. The fluorescent light emitted by the specimen then passes through the dichroic mirror and is detected using a CCD camera. **(B)** In the confocal microscope, excitation light is produced by a laser beam and focused to a diffraction limited spot within the specimen. The fluorescent light emitted by the specimen is separated from the excitation light using a dichroic mirror and then detected using a photomultiplier tube (PMT). A confocal pinhole is placed before the PMT and allows light emitted from the focal plane to pass (dotted line) but blocks any fluorescent light from out-of-focus regions (solid line). The image is formed by tracing the laser spot across the specimen in a raster pattern using a scanner consisting of two mirrors attached to galvanometer motors. **(C)** In the multiphoton microscope, excitation light, produced by a pulsed infrared laser, is focused by the objective lens to a diffraction limited spot within the specimen. The laser light intensity is only high enough within the focal spot to induce multiphoton fluorescence excitation. Since there is no fluorescent light emitted from outside the focal spot, a confocal pinhole is not needed and all the fluorescent light emitted by the sample can be collected using a non-descanned PMT detector. As in the confocal microscope the image is formed by scanning the focused laser spot across the specimen in a raster pattern. (Modified from Helmchen and Denk [37], Franke and Rhode [93], Schmolze et al. [94] and Webb [95])

and below the focal plane, resulting in loss of contrast. The confocal pinhole removes much of this unwanted light but, in addition, also removes light originating at the focal point and subsequently scattered within the specimen, causing a reduction of detection sensitivity and limiting depth penetration. Multiphoton microscopy overcomes the loss of sensitivity and limited depth penetration of CLSM [36, 37]. In contrast to conventional fluorescence excitation in which a fluorochrome is excited by the absorption of a single photon of light, multiphoton fluorescence excitation is characterized by absorption of two or more photons

of light within a near instantaneous timeframe (∼0.5 fs), requiring high peak intensity of excitation light. When the fluorochrome returns to its ground state, it then emits near its usual wavelength. In multiphoton laser scanning microscopy, the laser beam is focused to a diffraction limited 3 dimensional volume within the specimen, and it is only within this volume that the excitation intensity is sufficiently high to induce two-photon excitation (Fig. 3). Since excitation and emission can only occur within this volume, a confocal pinhole is not required to remove out-of-focus light, and all light emitted at the focal plane, even scattered photons, can be detected. Multiphoton microscopes are thus able to use non-confocal, non-descanned detectors to provide greater sensitivity (Fig. 2c). The fact that two-photon excitation only occurs at the focal spot has the additional advantage that photobleaching is restricted to the focal spot, in contrast to confocal microscopy in which photobleaching occurs through the entire illuminated specimen, above and below the focal plane.

In order to excite fluorochromes that emit visible light, the multiphoton microscope uses a laser in the infrared range allowing deeper penetration within the specimen, since light scattering is inversely related to wavelength. Two-photon microscope designs standardly make use of a titanium sapphire mode locked laser emitting laser pulses in the required range of intensity and pulse length. The intensity at the peak of the laser pulse is high enough to induce two-photon excitation, but the overall exposure is significantly reduced compared to a continuous wave laser operating at the same power. The lasers used for multiphoton microcopy have the additional advantage that they can be tuned to specific wavelengths, allowing excitation of a modest range of fluorescent molecules with a single laser: unfortunately, the laser can cost nearly as much as all the other components of the microscope system combined. Other concerns with multiphoton microscopy are photodamage from the I/R illumination and three photon effects on UV absorbing entities in living cells.

The high intensity infrared lasers can also be used for second harmonic generation. SHG emission is not strictly speaking fluorescence since the emerging photons are produced instantaneously and have exactly half the wave length the energy of the incident light. Only molecular multimers that are anisotropic and polarizable yield second harmonic photons. Second harmonic generation is useful for imaging collagen,
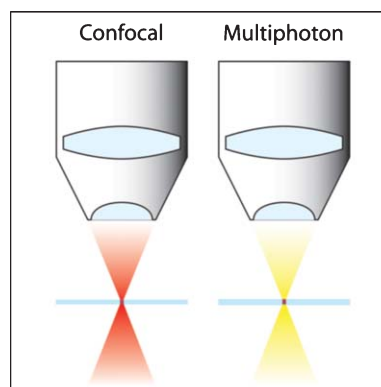

Fig. 3. Schematic diagrams of the illuminating light from a confocal and mutiphoton microscope. In the confocal microscope the excitation light is focused to a diffraction limited volume within the specimen. As the excitation light passes through the specimen, it can induce fluorescence from above and below the focal plane, thereby requiring the use of the confocal pinhole to remove this out-of-focus light from the final image. In the multiphoton microscope, the pulsed infrared laser is focused to a diffraction limited volume within the specimen and it is only within this volume that the intensity of the excitation light is high enough to induce multiphoton fluorescence excitation. (Modified from Helmchen and Denk [37])

microtubules and muscle in tissues in association with multiphoton microscopy [38, 39].

6. Total internal reflectance microscopy

TIRFM (total internal reflectance fluorescence microscopy) utilizes the property of light traveling in a medium of relatively high refractive index like glass or plastic to remain confined to the glass or plastic when incident on water with its lower refractive index at a critical angle from the normal [40]. Along the light path, an evanescent wave is generated within the denser medium at a right angle to the path of the incident beam. The wave propagates a very limited distance into the less dense aqueous medium and is able to excite fluorophores in a layer less than 200 nm into an adherent cell. The limited penetration of the evanescent wave substantially reduces out of focus light [41]. TIRF is thus useful for identifying molecules at or close to the cell membrane. The narrow layer of excitation allows assessment of recovery after photobleaching of the fluorophores in the layer for measurement of diffusion coefficients of fluorescent molecules in live cells, and the ability to excite fluorescence in limited volumes of the order of femtoliters permits fluorescent

correlation spectroscopy measurements that can yield information on the mean time of surface binding, surface diffusion coefficients and the mean number of fluorescent molecules per surface area. FRET and fluorescence life time measurements can also be carried out on molecules excited within the thin layer at the cell base. The narrow width of the evanescent wave penetration while a major benefit also represents a limitation of TIRFM. Several techniques have been used to image deeper into samples using TIRF. One of these is Highly Inclined Laminated Optical Sheet microscopy, (HILO) in which the laser beam, laminated as a thin sheet, is incident at a smaller angle than required for TIRF and passes through the center of the specimen plane allowing three dimensional imaging in a wider swath of the cell than is accessible with TIRFM [42, 43].

7. Lateral sheet illumination microscopy

Lateral sheet illumination microscopy (LSFM or SPIM, for single plane illumination microscopy) illuminates the sample with a laser beam that is projected on the sample as a thin sheet of light parallel to the focal plane of an objective [44, 45]. A CCD, EMCCD or, most recently, a sCMOS camera is used to acquire a wide field image. This configuration results in optical sectioning with low photobleaching and low phototoxicity for living tissue. Alternatively a focused beam can be swept through the sample, orthogonal to the objective, a plane at a time. For deeper penetration, two-photon excitation has been used [46]. Translation of the sample in the axial dimension allows the reconstruction of the sample volume in three dimensions. Large samples must be encased in a solid medium to maintain rigidity for translation in the Z-axis and any rotation required for increased depth of illumination. To avoid rotating the specimen and to record rapid events, multiview light-sheet has been developed using an arrangement of two objectives for illumination and two for detection allowing acquisition of four coordinated images per plane. Although LSFM was originally conceived and applied to microscopy of sample volumes the size of rodent embryos or zebrafish [47, 48], use of the flatter sheet provided by a Bessel beam, instead of the conventional Gaussian beam, has yielded good images of cultured live cells with high contrast and low photon exposure [49]. The use of wide field objectives of N.A. <1.2 in order to acquire complete optical sections does limit planar resolution.

8. Deconvolution

The image generated in fluorescent microscopy, or any microscopy for that matter, is not a quantitatively or even qualitatively perfect representation of the interaction of incident light with an object of interest; there is instrumental noise from the light source and sensor, scattering of emitted fluorescence by inhomogeneities in the volume of the sample, and the broadening of points of light by diffraction. Deconvolution is a computational process that utilizes the PSF of the imaging system to correct the fluorescent image for the convolution of photons resulting from diffraction during imaging [50, 51]. Three general approaches have been applied to obtain a PSF required for deconvolution: assumption of an appropriate theoretical PSF, determination of the PSF by imaging sub-resolution fluorescent beads, and derivation of the PSF directly from the data obtained imaging the sample, so-called blind deconvolution. Deconvolution procedures are conveniently divided into two categories: deblurring and image restoration algorithms. Deblurring is simpler and faster in execution and usually confined to two dimensions. Since deblurring algorithms remove out of focus blur rather than reassign it, they suffer from decreasing contrast and a higher likelihood of introducing artifacts. Image restoration is carried out in 3 dimensions and requires successive iterations, usually with constraints on the process at each step. Although computationally intensive, image restoration in most instances is the process of choice, as it can substantially restore the photons in the image to their point source origins, yielding improved resolution, greater signal to noise ratios (SNR) and extended dynamic range while retaining the quantitative information from the specimen in the image. Although the most dramatic effects of deconvolution are evident with widefield fluorescence microscopy, other modalities of fluorescence microscopy, CLSM, spinning disc confocal microscopy, multiphoton fluorescence microscopy and TIRF microscopy all benefit from its application. Numerous commercial and shareware deconvolution programs are available.

9. 4pi microscopy

In wide-field and confocal microscopy, the highest discernible resolution is limited to approximately 200–250 nm in the lateral dimension and 500–700 nm

in the axial dimension. The lower resolution in the axial plane is due to the fact that the objective lens in a standard microscope images the specimen from only one focal direction, resulting in a point spread function (PSF) that is elongated in the axial direction. 4pi microscopy achieves a three to seven fold increase in the axial resolution through the use of two opposed objective lenses focused within the same imaging volume [52–55]. This results in constructive interference of the spherical wavefronts of the two objective lenses and a PSF with a reduced central maximum and side lobes in each axial direction. The side lobes in the 4pi PSF result in the appearance of ghost images and steps must be taken to suppress these effects. 4pi microscopy is often used with multiphoton excitation, which can reduce the side lobes in the excitation PSF due to the decrease in the excitation efficiency outside the maximum region of the PSF. Additionally, the side lobes can be reduced by using a confocal pinhole that rejects light from out of focus regions. Any remaining ghost images can be removed using a linear deconvolution algorithm.

10. Super-resolution microscopy

Recently a set of ingenious approaches has been implemented for fluorescence microscopy that not so much break the Abbé limit as evade it [56–58]. The microscopes as a group are referred to as super-resolution instruments or sometimes nanomicroscopes. The most prominent are titled STED, for stimulated emission depletion [59, 60], RESOLFT for reversible saturable, optical, linear fluorescence transition [61, 62], PALM, for photoactivation localization microscopy [63], STORM for stochastic optical restoration microscopy [64, 65], SIM for structured illumination microscopy [66–68], and SOFI for super resolution optical fluctuation imaging. PALM, STORM and SOFI can be lumped together as blinking probe based methods or by some as pointillism microscopy, with a nod to the group of late 19th century French artists. Although these methods have probably not reached their full potential, they have already proved their value in producing high resolution images of cell structure in the nanometer range. Full details of the methods are beyond the purview of this presentation. The original literature and the numerous reviews should be consulted for more information [58, 69–71].

Suffice it to indicate here that STED uses two pulsed lasers to achieve its high resolution images. One laser excites the fluorophores and a second red laser beam is shaped to yield a concentric ring around the excited beam. The wave length of the second laser is selected to inactivate the fluorophores at the periphery of the excited region before they fluoresce by stimulating direct, non-radiative return to the ground state. The effect is to reduce the effective diameter of the exciting laser below the diffraction limit, with the effective diameter of the exciting beam inversely related to the power of the second laser. The improvement in resolution can be substantial in both the x-y plane and the z-axis without requiring any image processing. Choice of fluorophores compatible with STED is limited, and multichannel imaging is challenging.

RESOLFT was developed in an effort to overcome the requirement for high intensity of laser pulses required for emission depletion in STED and improving the speed of imaging [61, 62]. The most recent incarnation of RESOLFT utilizes a reversibly photoswitchable fluorophore with long-lived dark and fluorescent states and rapid off switching. The point scanning microscope employs four separate laser beams each applied in the focal region of the objective, a circular "on" spot at 405 nm, two doughnut-shaped "off" beams at 491 nm from separate lasers (one in the x-y plane and the other along the z-axis), and a circular excitation beam at 491 nm. The beams are rapidly cycled pixel by pixel, effecting a decrease in imaging time of 50 fold or more compared to STED. The average power of illumination is decreased by at least a factor of 10, and these innovations resulted in no reduction in resolution or contrast in the images obtained when compared to STED.

STORM/PALM and SOFI [imaging require fluorophores capable of being switched between on and off states. In STORM and PALM, using low laser energy, small numbers of fluorescent molecules are randomly switched on and off across the sample and numerous images of single fluorescent molecules recorded [64, 65]. The precise origins of the point sources of fluorescence are calculated, and the final image built up from thousands or hundreds of thousands of images. This approach tends to be slow and fluorophore limited. It is more sensitive than STED but slower in acquisition and dependent on extensive data processing. As with STED the high resolution images obtained, even in living cells, are impressive and enlightening. SOFI also uses blinking fluorescent proteins, but instead

of calculating locations it collects small numbers of images and depends on calculation of the fluctuations pixel by pixel to obtain a distribution of fluorescent point sources [72, 73]. The resolution achieved with STORM/PALM is somewhat better than that possible with SOFI, but SOFI is considerably faster and simpler for both 3dimensional reconstructions and dual color imaging. It is also somewhat more robust under adverse conditions. STORM, PALM and SOFI are susceptible to breakdown if the fluorophore density is too great.

11. Structured illumination microscopy

Structured illumination microscopy (SIM) falls somewhere between diffraction limited and super-resolution technologies in resolution and complexity [68]. In its simplest version in the Zeiss "Apotome' and Quioptiq "Optigrid" a 3 line grid is projected onto the focal plane; one image is taken at each of three positions and the underlying image of the sample is calculated. No improvement in resolution is produced, but the removal of out of focus light improves the contrast and allows optical sectioning. SIM achieves its increase in resolution by imposing a pattern with high spatial frequency on the illuminating light. When the pattern, created by a grid, a grating or two interfering laser beams is projected onto the focal plane, the pattern interacts with the fine detail in the specimen to produce a set of moiré fringes. The location of the pattern is varied over three angles and several positions across the specimen, and an image recorded at each of the positions with a sensitive camera. The spatial frequencies in the specimen captured in the fringes can be extracted and used to reconstruct an image of the specimen at twice the Abbe limit of lateral resolution. The 3dimensional SI microscope uses 3 mutually coherent illuminating beams to generate an interferometrically patterned excitation in both lateral and axial dimensions [66, 67]. Although the requirement for multiple images slows the acquisition, implementations of SIM are able to record the required nine images in 600 milliseconds, fast enough for following many cellular proceses.

12. Useful properties of fluorescent proteins

The many fluorophores available and their specific excitation and emission spectra allow simultaneous tracing of two, three and more molecules in space and time. This ability may be limited by emission spectral overlaps as the spectrum becomes increasingly crowded with fluorophores. Fortunately a combination of hardware and software solutions are available. Using dispersive elements, prisms or gratings, in front of the PMT, a pixel by pixel spectral scan of the emission spectra can be collected as a lambda stack, generating a spectrum scan down to 2 nm resolution [74]. Using standard spectral data, overlapping spectra can be resolved algorithmically into their component spectra. This technique also permits removal of autofluorescence from the image.

Beyond their high intrinsic sensitivity and spectral fingerprints, fluorescence molecules possess additional properties exploitable in microscopy [75]; notable are switchability, essential for the super resolution techniques [69, 71], Förster radiationless energy transfer (FRET) [76], photobleaching [77], photoactivation and fluorescence lifetimes [78]. FRET occurs when two fluorescent molecules, a donor and an acceptor, with dipoles aligned and within 1–10 nm of one another, transfer energy from donor to acceptor without photon emission. The fluorescence emission intensity of the excited donor is decreased and that of the acceptor molecule increased. The energy transfer decreases with the 6th power of the distance between dipoles on the same or adjacent molecules and is a function as well of the overlap of the emission spectrum of the donor with the excitation spectrum of the acceptor. FRET allows an estimation of close contact between two fluorescent molecules, and can be assessed by determining the increase in fluorescence of the acceptor when the donor is excited or the decrease in fluorescence of the donor in the presence of the acceptor; alternatively an increase in fluorescence of the donor on photobleaching of the acceptor can be used. Measurement of the decrease in fluorescent lifetime of the donor has emerged as a particularly reliable measure of FRET [79, 80]. These several measurements all require careful attention to detail in order to obtain valid results, but when carried out with all the necessary controls, FRET measurements can be far more useful than simple co-localization of two fluorophores.

Bimolecular fluorescence complementation provides an alternative means for measuring protein-protein association in living cells [81]. Two fragments of a fluorescent protein, each selected to be non-fluorescent alone, but fluorescent when brought together, are fused each to one of a pair of interacting

proteins. When these constructs are expressed in a single cell under conditions in which the proteins associate, the complementary fragments are brought together and as a consequence fluoresce. Bimolecular fluorescence complementation requires smaller numbers of associations for useful signals and does not have as stringent requirements with respect to distance as FRET. On the negative side, the formation of a fluorescent product with bimolecular fluorescence complementation is not instantaneous with protein-protein association.

Molecular beacons exploit the ability of certain molecules to efficiently quench the fluorescence of fluorophores situated on the some macromolecule [82, 83]. Most molecular beacons consist of synthesized DNA sequences comprising a loop-stem sequence in which the stem is a hairpin held together by complementary base pairing; one end of the hairpin has a fluorophore attached and the other a quencher. In the unbound state fluorescence is quenched; on binding of the loop to a specific complementary sequence of mRNA, the loop opens, forcing the arms of the hairpin apart, allowing the fluorophore to escape from the quenched state. The same type of constructs can be formed using PNA instead of DNA with some reported advantages.

Speckle microscopy [12] was a serendipitous discovery observed with microinjected fluorophore-labeled tubulin monomers. Under conditions of low intracellular concentrations of microinjected labeled monomers or limited expression of protein subunits fused with a fluorescent protein, small foci of fluorescence, the speckles, are seen distributed along the length of the corresponding multimeric protein in diffraction limited images. Detailed analysis reveals that small numbers of the labeled monomers randomly insert into the multimer as it is assembled, and if the monomers remain for a period of time equal to or greater than the time required to obtain a single frame, the speckles appear. Recording the speckles over time allows both evaluation of protein turnover and of the movement of the multimeric platform on which the speckles reside. Speckles, although initially studied with widefield fluorescence, have made the transition to confocal, both point scanning and spinning disk, as well as to TIRF microscopy. Cytoskeletal elements, and cytoskeletal associated proteins have been profitably studied with the technique.

Photobleaching of a fluorescent protein offers a means of acquiring information on intracellular molec-

ular associations and diffusibility [9, 84]. In FRAP (fluorescence recovery after photobleaching) a small volume of a cell is bleached, and the recovery of fluorescence in that volume, is dependent on molecules from the unbleached region moving into the bleached region [85]. In FLIP (fluorescence loss in photobleaching), a substantial volume of the cell is repetitively bleached and the redistribution of diffusible molecules from the unbleached region of the cell exposes the fluorescence of non-diffusible fluorescent molecules in the unbleached region [84]. Fluorescence correlation spectroscopy is made possible by measuring fluorescence intensity in femtoliter volumes of a living cell [86, 87]. Under conditions in which an appropriately small number of fluorescence molecules, $<10^3$, are measured in the volume, the fluctuation of the fluorescent signal caused by molecules entering and leaving the volume is measured over time. The data acquired is subjected to autocorrelation and can be analyzed to determine diffusion coefficients, molecular concentration and molecular associations. Photoactivation occurs when a non-fluorescent molecule on exposure to photonic energy is converted by to a stable fluorescent state. In the instance of caged fluorescence molecules, the fluorophore is coupled to a chemical that masks the fluorescence; when the masking agent is removed on exposure to light, usually UV, the fluorophore reverts to its fluorescent state. Fluorescent proteins have been developed that express low fluorescence until exposed to UV light [88]. Photoactivation permits accurate timing of the subsequent movement of the fluorescing molecule.

13. The future

For the researcher contemplating using advanced fluorescence microscopy, the choice may seem daunting, and the multiplicity of acronyms invoked by the developers of new methodologies can be an initial barrier Table 1. Murray [11] in his excellent presentation suggest some rough guidelines which we offer here with some modifications: for samples <3 μm in thickness, wide-field with deconvolution; for samples between 5 and 30 μm, scanning or spinning disc confocals; for samples >30 μm, multiphoton, 3D structured illumination or light sheet illumination. Of course considerations of the speed of acquisition, the resolution, the range of fluorophores and details of the character of the sample must also be considered. Fortunately many

Table 1

Comparison of the several types of advanced fluorescence microscopy. (Modified from Fischer et al. [92] and Toomre and Brewersdorf [70])

Fluorescent Microscope	X-Y Resolution	Z Resolution	Depth of Imaging	Contrast	Speed	Limitations
CLSM	250 nm	>600 nm	50 μm	Moderate	Moderate	Bleaching Toxicity
Spinning disc	250 nm	>600 nm	150 μm	High	Fast	No low mag Fixed pinhole Photodamage
Multiphoton	250 nm	>600 nm	800 μm	Moderate	Moderate	Photodamage Expensive laser
TIRF	250 nm	100 nm	200 nm	Very high	Moderate	Restricted depth
Structured Illumination	100 nm	250 nm	20 μm	Moderate	Slow	Complex instrument
STED	50 nm	150 nm	20 μm	Moderate	Moderate	Limited fluorophores Limited multichannel
STORM/PALM	20–40 nm	40–80 nm	10 μm	High	Slow	Limited fluorophores
Light sheet	500 nm	1–3 μm 300 nm with Bessel Beam	500 μm	High	Fast	Low N.A.objectives

research institutions have core facilities with a range of microscopes available and knowledgeable personnel to help with the choice.

Although not as much used as enzymatic IHC, immunofluorescence is not unknown to surgical pathologists, and FISH (fluorescent in situ hybridization) is the method of choice in many pathology laboratories for in situ hybridization. Whether or not any of the advanced fluorescence microscopy techniques will be incorporated into diagnostic pathology remains to be seen. If staining methods can be developed, light sheet fluorescence microscopy could prove useful for initial "grossing" of small biopsies, providing 3 dimensional images. Imaging of thick 20–50 micron vibratome sections with multiphoton microscopy combined with second harmonic generation could offer new insights into relationships of neoplastic cells to blood vessels and lymphatics. Efforts promoting such imaging have been reported [89–91], and as confocal imaging by endoscopists and dermatologists becomes more prevalent, correlations of their findings with high resolution imaging in the pathology suite may prove valuable if not essential. The introduction of new hardware like LED light sources and high resolution, high sensitivity, high speed sCMOS cameras may also spur modern departments of pathology to greater use of data-rich, digital fluorescent images for detailed quantitative analysis.

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