microscopy is well suited for the study of such systems *in vivo*.

In the future a major application for single molecule detection techniques will be the molecular description of individual cells containing or expressing a distinctive collection of receptor molecules. SPT will have an application in all diagnostic situations, where diagnostic information may be derived from a few distinctive molecules on or within a small number of cells. Examples of this are the occurrence of graft *versus* host reactions or graft *versus* leukemia reactions after transplantation or the characterization of success after tumor therapy.

Therapeutic Consequences

In the ideal case, single particle tracking within living cells provides a view of cellular molecular dynamics by directly showing the pathways and activities of intracellular molecular components. It can provide information on molecular mobility thus giving insight into structural determinants of lipid or protein mobility. It reveals information on the assembly sites of protein complexes and ultimately even on interaction topology, loci and partners. A central topic in the current progress of medical research is the search for individual molecular patterns in a single patient's disease to find improved targets for individually fine-tuned therapeutic strategies. SPT provides the means to approach these questions.

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Fluorescence Microscopy: Spectral Imaging vs. Filter-based Imaging

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Definition

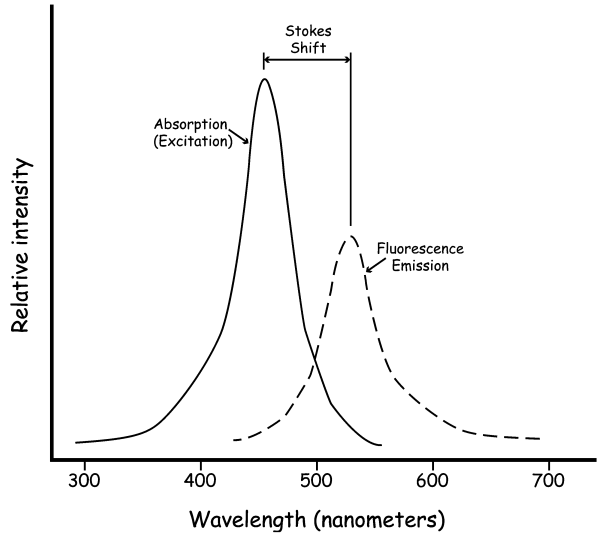
In spectral imaging, the entire spectrum of emitted light is utilized by positioning a prism or optical grating device in the emitted light path, which diffracts the light into its spectral components. These can be acquired in a sequential mode or, more efficiently, in parallel detectors. In contrast, optical filters positioned in the light path transmit a selected range of wavelengths with high efficiency while rejecting, through reflection and destructive interference, all other wavelengths.

Description

Light is an extremely versatile tool for studying biological systems and their functions in a non-invasive manner. When light shines upon an object, it can be reflected, transmitted, absorbed or re-emitted. Spectroscopy measures these phenomena to determine the physical properties of the substance being studied. The addition of imaging to spectroscopy aims to provide spatial information to the ability to determine what substances are contained within the object. Our own vision is a form of imaging spectroscopy. The combination of imaging and spectroscopy was pioneered on airborne satellite-based remote sensing, with the aim of determining from a distance the distribution of natural or man-made substances based on their spectral signatures.

Optical imaging combines a wide variety of light sources with non-invasiveness and high spatial and temporal resolution, allowing the investigation of structure-function from the subcellular level up to entire organisms and in real time. Fluorescence is the most rapidly expanding optical microscopy technique in both the medical and biological sciences, which has spurred the development of highly sophisticated fluorescence microscopes. In contrast to other modes of optical microscopy, fluorescence microscopy allows the detection of a single molecular species based on its light emission characteristics. ►Fluorescence is the process by which an atom or molecule absorbs light at a particular ►wavelength and subsequently emits light of a longer wavelength (the difference between excitation and emission wavelengths is termed the ►Stokes shift). Thus, using fluorescence microscopy one can determine with high spatial and temporal resolution, the precise subcellular localization of one or several fluorescently labeled molecules within a living cell. Furthermore, diffusion rates and interactions with other molecules as well as their changes in time and with pathological alterations can be determined. In addition, the fact that fluorochromes are susceptible to environmental influences (pH, oxygen content, ion concentrations, etc.) permits using them as a probe for local environmental characteristics.

The ever-expanding range of fluorescence microscopy applications requires the quantitative analysis of a wide number of fluorochromes at the same time and place within a sample. This requires methods that allow good discrimination of, often spectrally overlapping, fluorochromes. ►Fluorochromes (also called ►fluorophores) are substances, which fluoresce upon irradiation and are characterized by their excitation and emission spectra. The absorption or ►excitation spectrum is obtained by measuring the relative fluorescence intensity at a certain wavelength when the specimen is excited at varying wavelengths. The ►emission spectrum is red-shifted (Stokes shift) and results from excitation at a certain wavelength. Most excitation and emission curves overlap to a certain extent (Fig. 1).



Fluorescence Microscopy: Spectral Imaging vs. Filter-based Imaging. Figure 1 Excitation (solid line) and emission (dashed line) spectra of a fluorochrome.

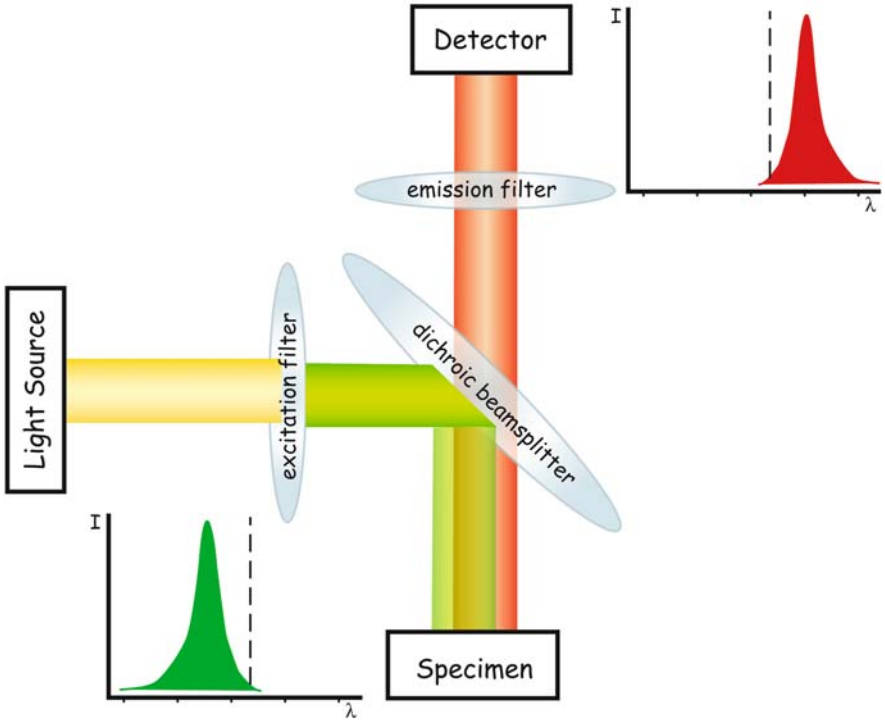
The essential feature of a fluorescence microscope is to provide a way of exciting the specimen with a selected wavelength followed by isolating the much weaker fluorescence emission (three to six orders of magnitude less than the excitation) coming from the specimen. The majority of commonly used light sources (natural and artificial) are polychromatic, i.e., emit a broad range of wavelengths that cover the entire visible light spectrum and often extend into the ultraviolet and infrared regions. In order to select the wavelength of excitation from a polychromatic light source, specialized filters are normally used. In addition, filters are also used to discriminate between excitation light and the light emitted by the specimen. This discrimination is possible since the emitted light is of longer wavelength than the excitation light. The choice of filters is of paramount importance in fluorescence microscopy and depends on the spectral characteristics of the fluorochromes used. Filters are constructed in a wide variety of shapes and physical dimensions and can be employed to remove or pass wavelength bands ranging in size from hundreds of nanometers down to a single wavelength. Many filters work by absorbing light, while others reflect unwanted light, but pass a selected range of wavelengths. The characteristics of a filter are seen by its transmission curve. The main types of filters for fluorescence microscopy are interference filters and ►dichromatic beamsplitters (3).

►Interference filters are glass substrates onto which thin layers of metal salts are deposited. There are different types, named band-pass and short and long-pass interference filters. They operate by transmitting a selected wavelength region with high efficiency while rejecting, through reflection and destructive interference, all other wavelengths. They can be positioned in front of the excitation light source (commonly a mercury or xenon lamp) to select the excitation wavelength or before the detection system (most often nowadays a CCD camera) to select a range of emitted wavelengths.

Dichromatic beamsplitters reflect light of wavelengths shorter than the specified wavelength and transmit light of longer wavelengths. The dichroic beamsplitter is positioned at a 45-degree angle to the optical axis of the illumination path and reflects the exciting rays into the objective in an epi-illumination setup where the objective serves as condenser. Light emitted by the sample is collected by the objective, traverses the dichroic filter and is further selected by the emission (interference) filter.

A typical filter set configuration for fluorescence microscopy consists of an excitation (interference) filter, a dichromatic beamsplitter and an emission (interference) filter mounted in a cube as shown in Fig. 2. The individual filters can also be positioned each separately into the light path as part of rotating filter wheels, which permit different combinations of the three basic components to be chosen in a more flexible way. The filters in a configuration should be chosen so that the wavelength range transmitted by the excitation interference filter matches the reflection range of the dichroic. In that way, the excitation light is effectively directed onto the sample. In addition the emitted light from the specimen must match a high transmission range from the dichroic in order to pass through to the detector. The emission filter, albeit less relevant, still imposes further constrains onto the light that reaches the detector.

Even in perfect combinations of filters there is always some crossover or bleedthrough of light between the

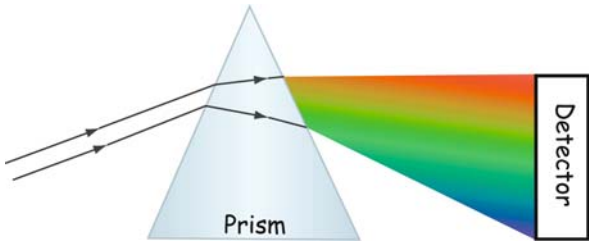


Fluorescence Microscopy: Spectral Imaging vs. Filter-based Imaging. Figure 2 Separation of excitation and emission using a classical filter set configuration. Polychromatic light (yellow light beam) is selected (green light) by the excitation interference filter and reflected onto the specimen by the dichroic beamsplitter. Fluorescence emission of longer wavelength from the sample (red light) is transmitted through the dichroic and further selected by the emission filter before it reaches the detector.

filters. Furthermore, the more discriminating a filter set is, the less light it passes and with increasing numbers of fluorochromes used in a single sample the more difficult it is to find filters which fit all the fluorochromes one needs. Moreover, the strongly overlapping emission spectra of commonly used fluorescent dyes and proteins severely limit their combined use in multicolor imaging using optical filters. A better solution is to have systems where the selection of wavelengths is flexible, in particular at the detection side or the entire range of emitted light (spectrum) is detected and resolved into the individual components. In recent years, several commercial manufacturers have started to implement both these solutions, in particular into their laser scanning confocal microscopes where the excitation light source is monochromatic.

Flexible selection of wavelength can be performed using tunable filters, in particular ►acousto-optical filters. These are electro-optical devices that allow the modulation of the light intensity as well as wavelength. Light penetrating the optical crystal is deflected depending on its wavelength and the wavelength of the ultrasonic field applied to the crystal. These filters do not have the mechanical limitations, speed constraints and vibrations associated with the rotating filter wheels. Furthermore, they offer far greater flexibility and durability as compared to interference filters upon exposure to heat and high intensity light.

In order to remove the constraints of filters on the detected range of wavelengths, spectral detectors can be used. The light emitted by the specimen consists of mixtures of wavelengths, due to autofluorescence and excitation crosstalk (excitation of multiple fluorochromes with overlapping absorption spectra) as well as the broad range of wavelengths emitted by single fluorochromes (represented in its emission spectrum). A prism or optical grating device positioned in the emitted light path diffracts this light into its spectral components, which can subsequently be acquired in a sequential mode or, more efficiently, in parallel detectors (Fig. 3). In order to select a particular range of the emission spectrum, a motorized slide aperture can be positioned before the detector, letting only a particular wavelength range of the spectrum pass. Alternatively, the entire spectrum can be detected and the contributions of the different fluorochromes calculated based on their spectral signatures. For this unmixing of the spectral signatures, the relative contributions of each of the fluorochromes into each



Fluorescence Microscopy: Spectral Imaging vs. Filter-based Imaging. Figure 3 Spectral detection of emitted light. Fluorescence emitted by the sample (arrows), consisting of a mixture of wavelengths, is dispersed *via* a prism and the entire emission spectrum gets projected onto the detection system.

detection channel are first recorded as reference spectra. The latter are used to build up a matrix of fluorophore specific weight for the calculation of an individual fluorophore's contribution to each pixel. By applying this matrix to the spectral image acquired, one can determine the contribution of the individual fluorophores at every pixel in the image and effectively separate their signals. This mathematical operation is called ►linear unmixing. In principle, any fluorescent contribution can be calculated back and this can even be used to subtract the contribution of background fluorescence from an image. The combination of spectral detection with linear unmixing analysis (1,4) allows the simultaneous use of highly overlapping fluorescent molecules, which has thus far been a major limitation in multicolor imaging. Moreover, since most of the emitted fluorescence is recorded, greatly increased sensitivity is attained as compared to classic filter-based systems.

The information summarized here focuses on commonly used fluorescence microscopy systems in biomedical research. Additional information on spectral imaging can be found elsewhere (2).

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Fluorescence Quantum Yield

Definition

Fluorescence quantum yield refers to the total number of photons emitted as fluorescence divided by the total number of photons absorbed by the molecule. The range of the fluorescence quantum yield is between 0 and 1.

►FRET

Fluorescence Recovery after Photobleaching

►FRAP and Other Photobleaching Methods
►Functional Imaging

Fluorescence Resonance Energy Transfer

►FRET

Fluorescence-Activated Cell Sorter

Definition

Fluorescence-activated cell sorter represents a highly specialized ►flow cytometer capable of detecting, separating and analyzing cells based on certain physical parameters, such as cell size, granularity, and the presence of cellular components, based on the presence of certain specifically bound fluorochromes.

►Flow Cytometry
►Immunochemical Methods, Localization

Fluorescent Proteins

Definition

Naturally occurring proteins with an intrinsic fluorophor generated by an autocyclization reaction of