Fluorescence Microscopy – A Short Introduction

Fluorescence microscopy is the use of a microscope to excite fluorescent molecules and use the emission wavelengths to form an image. This method allows for the precise identification and localization of specific structures, as well as the investigation of processes within cells. It is a powerful method that allows observing features beyond the optical resolving power of the compound microscope, although the observed size of such tiny features or sub-resolution particles is given by the smallest separation distance that can be optically resolved (see **Fig. 1**).

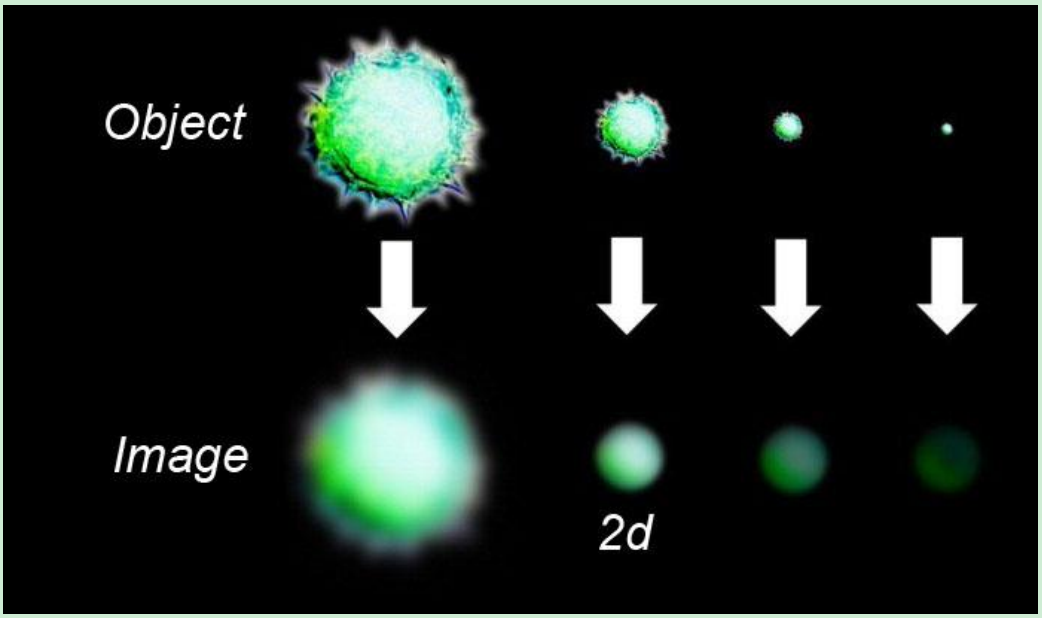


Fig. 1*: If a fluorescent particle is smaller than the optical setup's minimum resolvable size (diameter = 2d), its image size does not decrease further, but its contribution to image contrast diminishes as the particle gets smaller.*

Since 2004, numerous studies have been published in various journals discussing methods that can surpass the Abbe limit. Although we believe that describing these new methods, known as super-resolution techniques, as "breaking" the Abbe limit is inaccurate, we acknowledge the novel insights they offer into the capabilities of light. To achieve resolution beyond the diffraction limit imposed by a given wavelength, it is essential to appropriately manipulate and control the light.

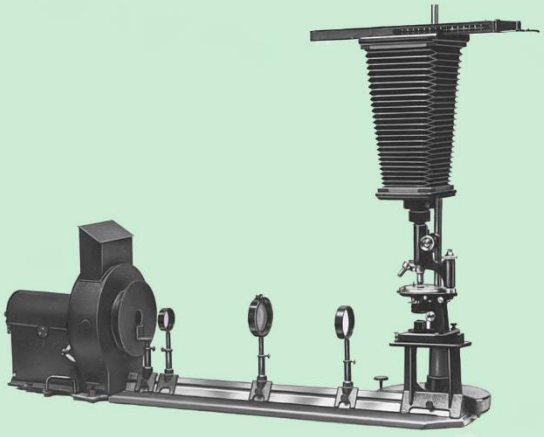
The following super-resolution techniques enable the capture of image details with a resolution beyond the diffraction limit in either axial and/or lateral directions: 4Pi, structured illumination (SIM), stimulated emission depletion (STED), single-molecule localization and composition (PALM/STORM), and near field (SNOM and TIRFM). For instance, total internal reflection fluorescence microscopy (TIRFM) utilizes evanescent waves, providing a very shallow penetration depth of approximately 200 nm. Although TIRFM enhances axial (vertical) resolution, it does not improve lateral resolution. Several of the aforementioned techniques can be combined, such as 4Pi-STED. Comprehensive overview articles on these advanced techniques were published in 2006 and 2010 [**1**].

However, we do not include super-resolution in this introduction to fluorescence microscopy. This discussion exclusively focuses on traditional **widefield fluorescence microscopy**, which adheres to the resolution limit dictated by Abbe's theory of image formation.

Several algorithms exist for deblurring and restoring images obtained from a fluorescence microscope. These include linear and nonlinear methods, maximum-likelihood restoration, blind deconvolution, and the nearest-neighbor method. Discussion on this topic can be found in the book by Qiang Wu *et al.*, Microscope Image Processing, Academic Press, Elsevier Inc., London (2008).

Molecules with conjugated double bonds (alternating single and double bonds) are suitable for fluorescence microscopy. In these molecules, electrons are distributed over several atoms, making them easier to excite by incoming radiation (photons) of a suitable wavelength.

The phenomenon of fluorescence has been known since 1577 [**3**, page 183]. The British mathematician and physicist **Sir George Gabriel Stokes (1819 – 1903)** described fluorescence in 1852. Stokes coined the term when he observed that the mineral fluorspar emitted red light upon illumination with ultraviolet excitation.



In 1908, **August Köhler (1866 – 1948)** and **Henry Wilhelm Siedentopf (1872 – 1940)** introduced the first fluorescence microscope. Before this invention, ultraviolet radiation was used to increase resolution but not to intentionally excite fluorophores. Köhler and Siedentopf's first UV microscope used a cadmium spark light source with a wavelength of 275 nm. In 1910, the brighter light of the carbon arc with appropriate excitation filters was used.

Fig. 2 shows the spectral distribution for wavelengths (λ) in nanometers (nm) with 1 nm = 10^{-6} mm. Blacklight is UV light at wavelengths around 315 to 400 nm. Infrared starts just above red light from 700 nm to 1 mm. The visible spectrum that can be perceived by the human eye is between blacklight and infrared.

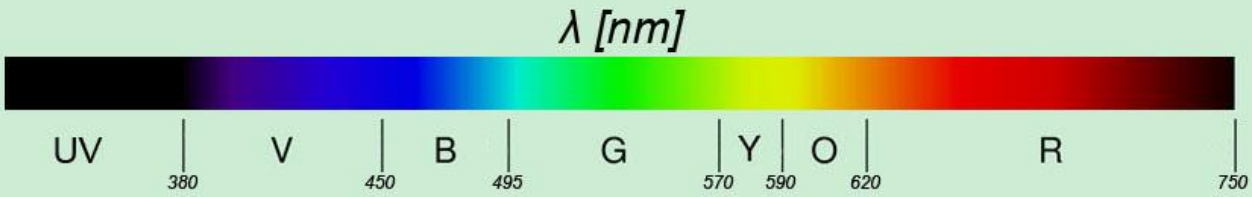


***Fig. 2**: Spectrum showing different wavelengths.*

When certain substances are irradiated with light of a specific wavelength (λ) or frequency (ν), they emit radiation at a wavelength longer than that of the exciting light. This phenomenon is known as Stoke's law. The shift to longer wavelengths (or shorter frequencies) occurs because energy is converted to activate

vibration states within the sample molecules, also referred to as heat production. Let S_0 represent the ground state (lowest energy level of a quantum system that is measured in electron volts) of the fluorophore (fluorescent molecule) and S_1 its first electronically excited state. The fluorescence excitation/emission process can be described by:

$$S_0 + h\nu_{excitation} \rightarrow S_1(\Delta t) \rightarrow S_0 + h\nu_{emission} + \text{Heat}, \qquad \text{with} \quad \nu_{excitation} \geq \nu_{emission}$$

The quantum state S_1 has a lifetime Δt, known as the optical transition time. The optical transition period of fluorescence (time spent in S_1) lasts approximately 0.5 to 20 nanoseconds, meaning the excited radiation persists only while the specimen is exposed to exciting light.

Fig. 3 shows a schematic of an incident-light fluorescence microscope used to irradiate a specimen with light and capture the resulting emitted radiation.

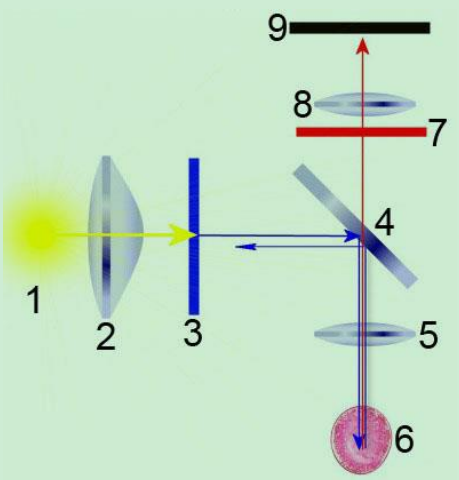


***Fig. 3**: Schematic of incident-light fluorescence setup. Excitation (blue arrow), emission (red arrow). Light source (1), collector lens (2), exciter filter (3), dichroic mirror (4), objective (5), specimen (6), barrier filter (7), ocular (8), sensor (9).*

In contrast to fluorescence, a phosphorescent material (which should not be confused with chemiluminescence, where an excited state is created via a chemical reaction) does not immediately re-emit the absorbed radiation. The emission during phosphorescence can range from a few milliseconds to several minutes

Quantum yield, an important measure of fluorescence, is defined as the ratio of emitted photons to absorbed photons, indicating the efficiency of the fluorescence process.

A fluorescent specimen is **self-luminous**. Each point source acts independently, so the emitted light phases are incoherent. Incoherent light cannot generate interference, while coherent light can. A single fluorescent point creates an **Airy disk**, a focused light spot resulting from diffraction at a lens's aperture with a circular shape. **Rayleigh's criterion** determines the resolution of two adjacent points as

$$d = 0.61\,\lambda \,/\, \mathrm{NA}_{\mathrm{Obj}},$$

where $\mathrm{NA}_{\mathrm{Obj}}$ is the numerical aperture of the objective, with a practical upper limit of 1.40 for oil immersion objectives.

Autofluorescence

Autofluorescence, or intrinsic fluorescence, occurs when certain specimens naturally emit light after being illuminated by a specific wavelength. Examples include:

- Chlorophyll in plants (Blue -> Red)
- Collagen fibers in animal tissue (UV -> Blue-green)
- Elastic fibers (UV -> Blue-green)
- Protein-bound NADH (UV -> Blue)

This phenomenon in plant and animal tissues is mainly due to *aromatic molecules* found in many structures, such as proteins and coenzymes. For example, chlorophyll contains a porphyrin ring with double bonds and fluoresces strong red under green or blue excitation.

Plant tissue is generally more autofluorescent than animal tissue. With UV excitation and a colorless UV barrier filter, higher plant sections display various colors (see table below).

Chloroplasts	Deep red
Cell walls	Bluish-white or blue
Cuticle (protective, hydrophobic, waxy coverings)	Blue

Mouth of Dermacentor variabilis (American dog tick). Used Ortholux II with Ploemopak 2.2. The fluorescence response has been recorded with filter cube I2 (blue → green).

Autofluorescence differs from induced fluorescence, which is caused by fixation with aldehydes such as formaldehyde.

Fluorescent Staining

After staining a specimen with a fluorescent dye (also referred to as fluorochromes or fluorophores), the stained components can be selectively identified when illuminated by the appropriate wavelength. This process is also known as secondary fluorescence. In 1933, **Max Haitinger (1868 – 1946)** introduced the use of fluorescent dyes for staining histological specimens.

Fluorescence staining is suitable for in-vivo analysis due to its high sensitivity and the low concentrations of fluorochromes required. Eosin is a commonly used fluorescent stain, produced by the action of bromine on fluorescein. Eosin stains cytoplasm, collagen, and muscle fibers. It is often used with Hematoxylin (which is not fluorescent) for staining animal tissues for brightfield observation, but its fluorescent properties can also be utilized with a fluorescence microscope. For certain applications, Eosin Y ($C_{20}H_8Br_4O_5$) is used, as it exhibits strong fluorescence with yellow excitation (514 nm). Blue and green excitations are also effective.

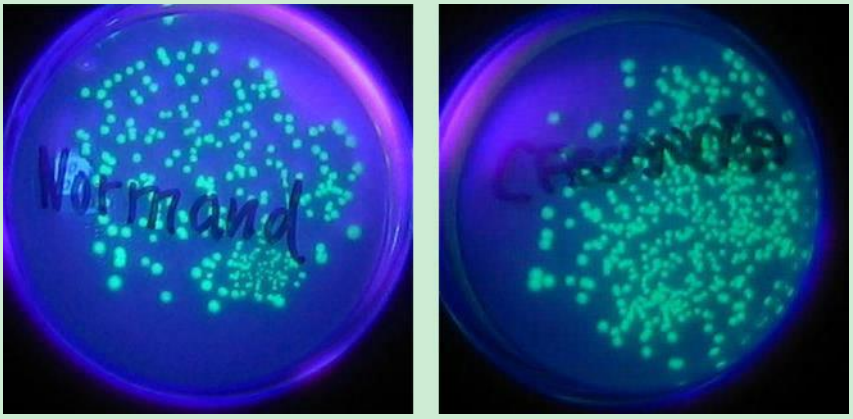
Immunofluorescence and Fluorescent Proteins

In 1941, **Albert Hewett Coons (1912 – 1978)** invented immunofluorescence, turning fluorescence microscopy into a key tool for biological research. In response to foreign molecules, the immune system produces antibodies (immunoglobulins). Each type of antibody binds to a very specific target molecule (antigen) in order to either inactivate its target or label it for destruction. First, a fluorescent dye couples to a specific antibody (also referred to as labeling of antibody). Then this fluorescent antibody binds to its antigen, which could be another antibody in case of *indirect* immunofluorescence. Finally, the fluorescent antibody is detected by fluorescence in the light microscope. Most commonly, two sets of antibodies are used, a primary antibody against the antigen of interest and a subsequent, secondary antibody that recognizes the primary antibody. The secondary (also called “indirect”) antibody is labeled with a fluorescent marker.

Immunofluorescence is highly specific because of its genuine serological (antibody-antigen) reactions. Examples of suitable fluorescent labeling substances are *fluorescein isothiocyanate* (FITC) and *tetramethylrhodamine isothiocyanate* (TRITC). – In order to insert antigens through the cell membrane, the membrane must be made transparent for these molecules to enter. This can only be accomplished with fixed and, consequently, dead cells, which makes this method unsuitable for studying dynamic processes in life cells.

A method for studying dynamic processes *in-vivo* is the fluorescence of green-fluorescent protein (GFP). Developed by **Osamu Shimomura (1928 – 2018)** in 1961, this technique involves inserting the GFP gene into a cell's genome, causing the cell to produce GFP. The insertion is typically achieved by cooling the cell to open its pores. This experiment details inserting the jellyfish gene from the crystal jelly (*Aequorea victoria*) into *Escherichia coli* bacteria. The procedure is:

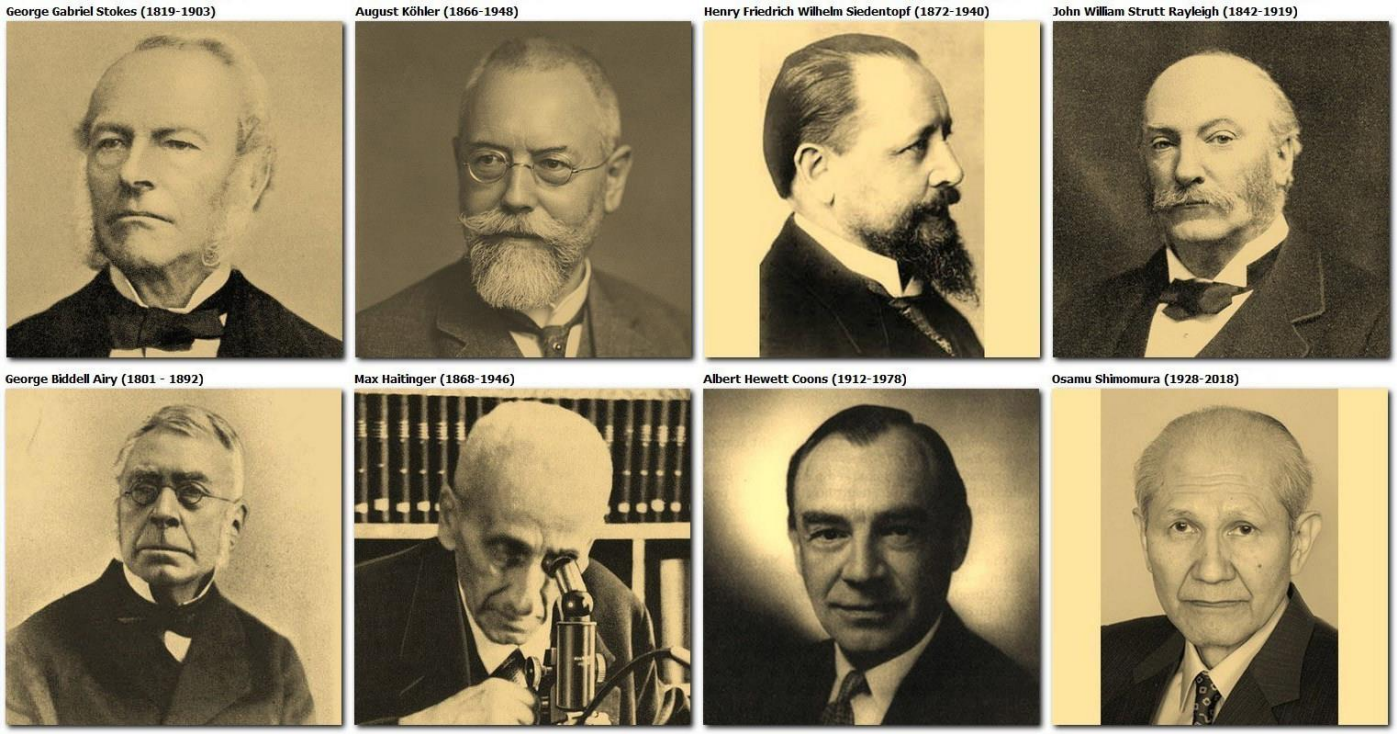
1. Add GFP-gene to a vial of E. coli bacteria.
2. Cool down the vial containing the bacteria and GFP-gene for 30 to 45 seconds. The GFP-gene enters the bacteria through its pores that are now open.
3. Warm up the vial for 30 seconds to close the pores. This allows the GFP-gene to be inserted into the bacteria's genome (actually, it is inserted into the pGLO plasmid).
4. Spread the bacteria from vial onto Petri dish containing nutrient agar.
5. Put Petri dish inside incubator for 24 hours.
6. With an excitation wavelength of 395nm, observe emission at 509nm (green). The bacteria have produced the GFP (see images below).



Experiment of inserting jellyfish gene from the crystal jelly (Aequorea victoria) into Escherichia coli bacteria.

Several different variations of the original GFP exist. Some of them fluoresce also in different colors, such as BFP, CFP and YFP.

Famous people working on Fluorescence Microscopy:



Additional Information

More information about fluorescence microscopy can be found in [**2** - **6**]. Over the last years, there were numerous articles published in the Micscape Magazine [**7** – **13**]

In a subsequent section "*Fluorescence Microscopy – Instrumentation*" [**14**], we are discussing the details of the necessary instrumentation used for widefield fluorescence microscopy. This type of instrumentation culminated with the introduction of the famous episcopic design by Ploem.

References

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*[**2**] F.W.D. Rost, Fluorescence microscopy, Volume 1, Cambridge University Press (1992).*

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*[**4**] D. B. Murphy, Fundamentals of Light Microscopy and Electronic Imaging, Wiley-Liss, Inc. (2001).*

*[**5**] B. Herman, Fluorescence Microscopy, 2nd edition, Taylor & Francis Group, New York (1998).*

*[**6**] Maksymilian Pluta, Advanced Light Microscopy, Volume 2, Specialized Methods, Elsevier, Amsterdam (1989).*

*[**7**] Laurent Delvoye, Fluorescence microscopy with super LEDs, Micscape Magazine, UK (2009).*

*[**8**] David Walker, Forays into Fluorescence, Simple transmitted blue light autofluorescence of mosses and algae imaged with a digital SLR, Micscape Magazine, UK (2009).*

*[**9**] David Walker, Forays into Fluorescence – 2, A selection of prepared unstained slides studied in visible light fluorescence, Micscape Magazine, UK (2009).*

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*[**11**] David Walker, Forays into Fluorescence – 4, Notes on the autofluorescence of old dry mounts of snail radulae, Micscape Magazine, UK (2010).*

*[**12**] David Walker, Forays into Fluorescence – 5, Notes on adapting a Zeiss Photomicroscope (or Universal) to LED sourced epi-fluorescence**,** Micscape Magazine, UK (2011).*

*[**13**] David Walker, Forays into Fluorescence – 6, Trials of studying autofluorescence with a stereo microscope using blue or near UV LED excitation, Micscape Magazine, UK (2011).*

*[**14**] Gregor Overney, Fluorescence Microscopy – Instrumentation, Micscape Magazine, UK (2025).*