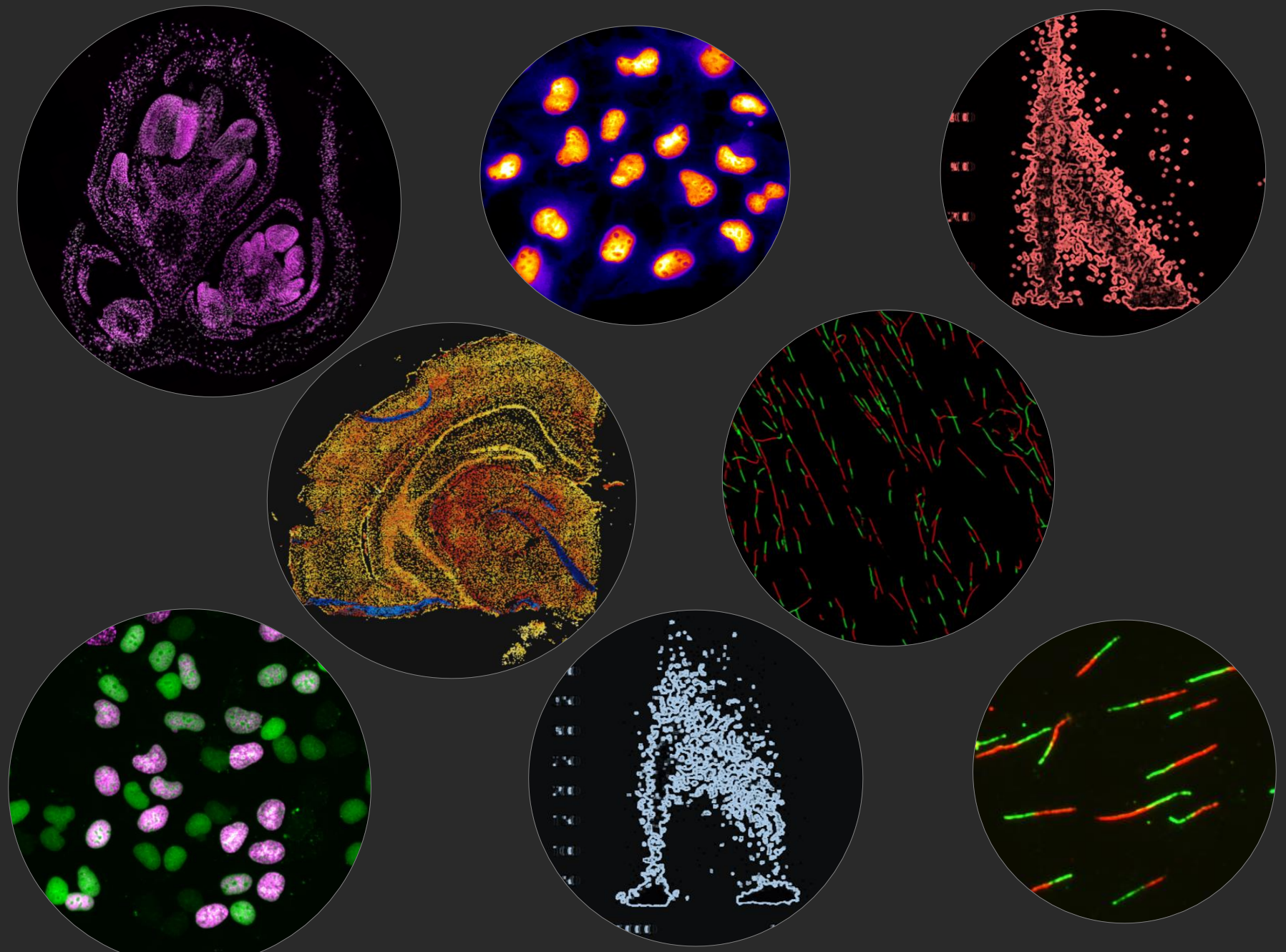
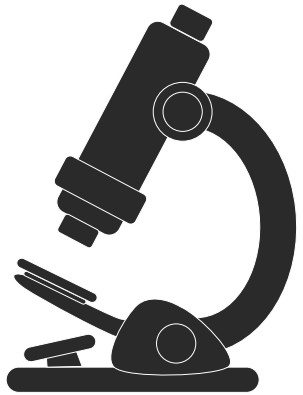


Image courtesy of Vaclav Bacovsky, Jana Krejci & Hana Polasek-Sedlackova

Bi9393:
Analytical cytometry

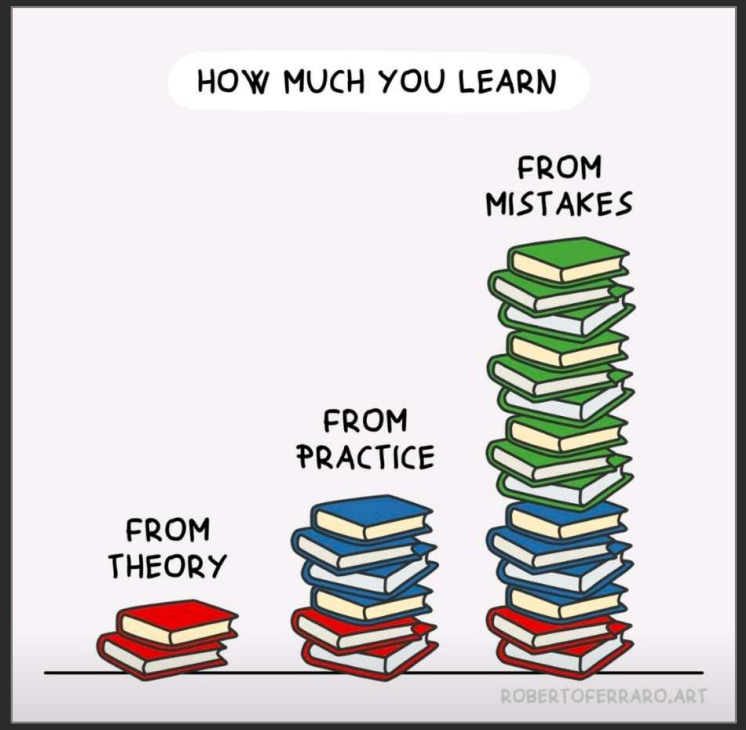
Microscopy techniques



Hana Polasek-Sedlackova
&
Simran Negi
(teaching assistance)

SEDLACKOVA LAB

Don't be afraid to ask questions during the lecture.

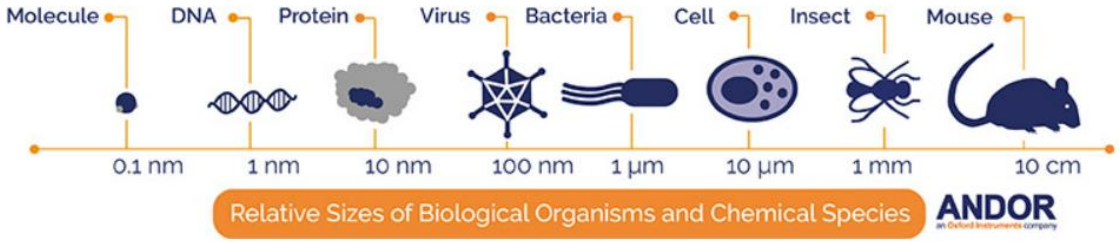


During this lecture Mistakes are:

Expected
Respected
Inspected
Corrected

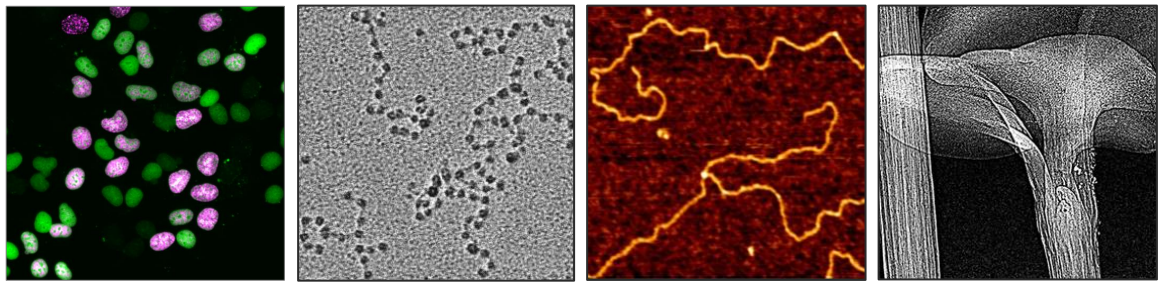
Introduction to Microscopy

Microscopy is the technical field that involves using microscopes **to see objects** and areas of objects that are **not visible to the naked eye** (objects that are outside the resolution range of the normal eye).

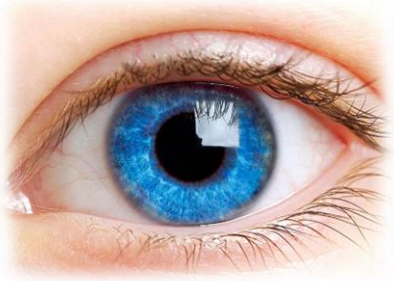


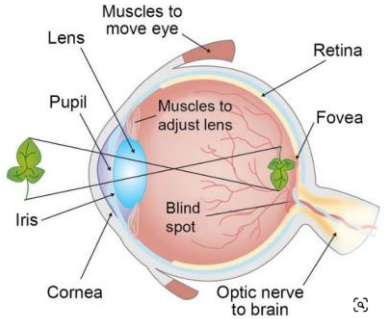
Microscopy can be divided into **three branches**:

1) **optical microscopy**
2) electron microscopy
3) scanning probe microscopy
4) X-ray microscopy



How can we see objects?





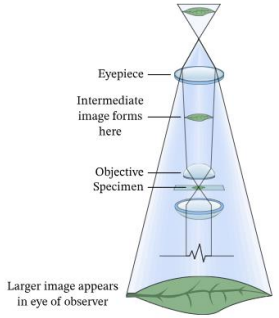
The **eye** as the visual organ in combination **with the brain** behind it – is the most efficient image-processing system available to date.

What if the object is too small?

Magnifying glass as the first aid to see small objects.

Limitation: a magnification of more than 8-fold or 10-fold is not possible.

What if one lens is not sufficient?

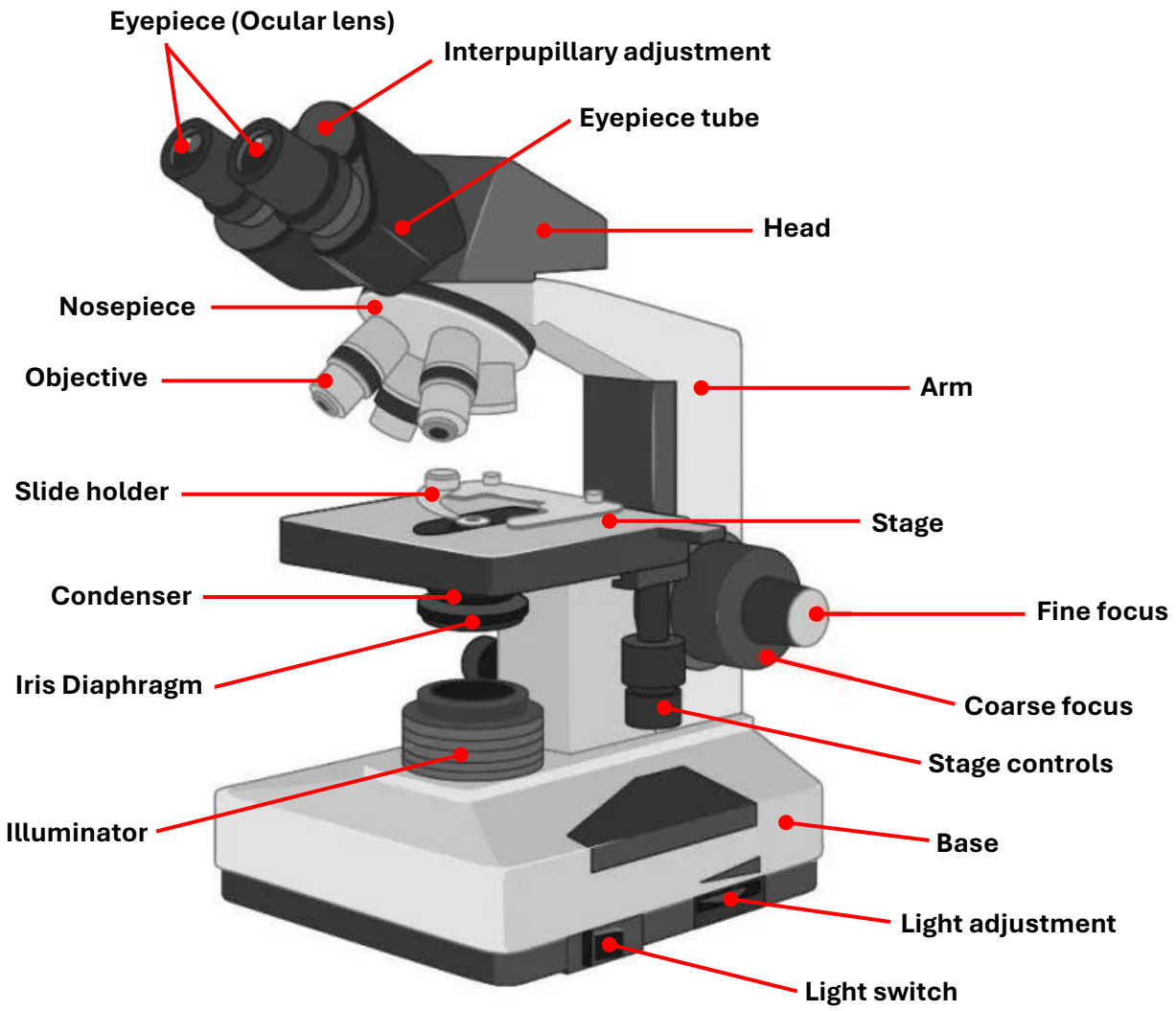


If one lens is not sufficient, several lenses can be arranged one behind the other. Lenses in **objective & eyepiece (ocular)** cause multiplication of magnifying effect = **principle of microscope**.

History of Microscopy

Courtesy of Sona Legartova & Simran Negi

Do you know microscope parts?



Optical components of a microscope

The **eyepiece** (or **ocular lens**) is the lens at the top of a microscope that the viewer looks through. Magnification of eyepiece ranges from 5x – 30x (10x standard eyepiece).

The **eyepiece tube** carries the eyepiece lens and holds it in the right place that aligns perfectly with the objective lenses.

Objective lenses are the primary optical lenses for specimen visualization on a microscope. Objective lenses collect the light passing through the specimen and focus the light beam to form a magnified image.

Nosepiece is also known as the revolving turret. Nosepiece is a circular structure housing the objective lenses. There are holes where the different objective lenses are screwed in.

The **stage** is a flat platform that supports the slides. The stage has a **hole (called aperture)** for the illuminating beam of light to pass through. The stage clips hold the slides in place.

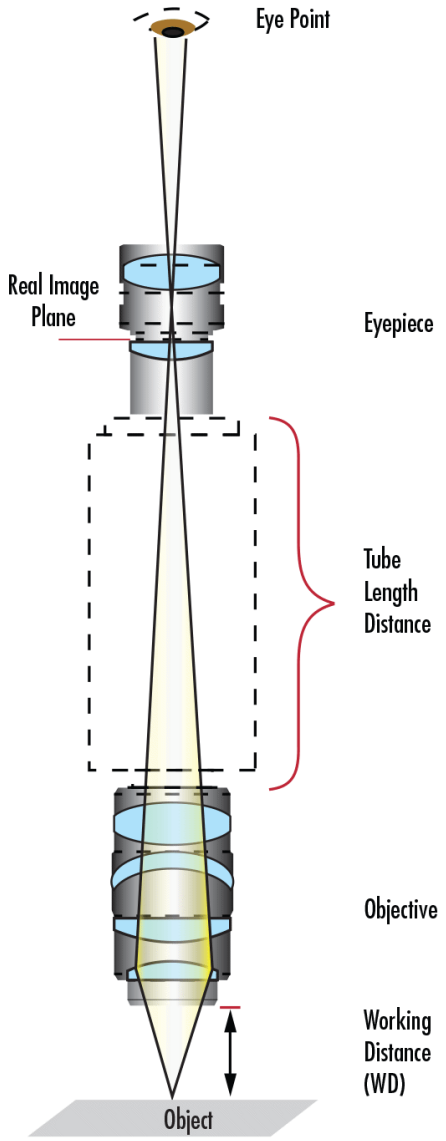
Condensers are lenses that are used to collect and focus light from the illuminator into the specimen. Condensers with an iris diaphragm are major components to achieve **Köhler illumination** (uniform illumination of the sample) reducing image artifacts and providing high sample contrast.

Iris Diaphragm is located below the condenser and above the light source. This apparatus can be adjusted to change the intensity and size of the cone of light projected through the slide.

The **illuminator** is the light source for a microscope, typically located at the base of the microscope. Halogen bulbs are commonly used to provide a steady light source. Currently, LED lights become more and more popular.

Open and Closed Iris Diaphragm

Principles of Optical Microscopy: Magnification vs. Resolution



What is overall system magnification?

Objective and **eyepiece** increase overal system magnification.

Total magnification = objective magnification x eyepiece magnification

Magnification alone is not enough: The resolution matters..

Light diffraction: If we look at the object through the microscope light will be diffracted. The smaller the object, the more pronounced the diffraction of incident light rays will be.

Sharp images of small structure: microscope must collect as much possible diffracted light (objective covers a large solid angle).

Numerical aperture (N.A.) of the objective: is a measure of the solid (opening) angle covered by objective.

N.A. = n x sinα

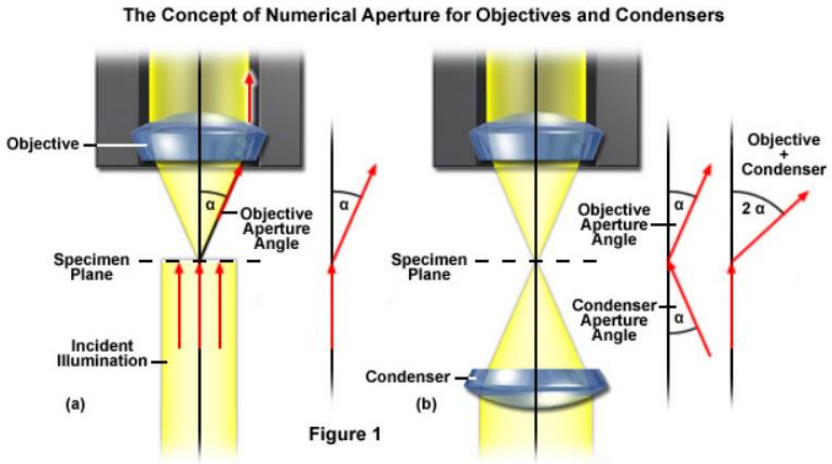
n is refractive index of the immersion medium used between the objective and object (n = 1 for air; n = 1.51 for oil glass)

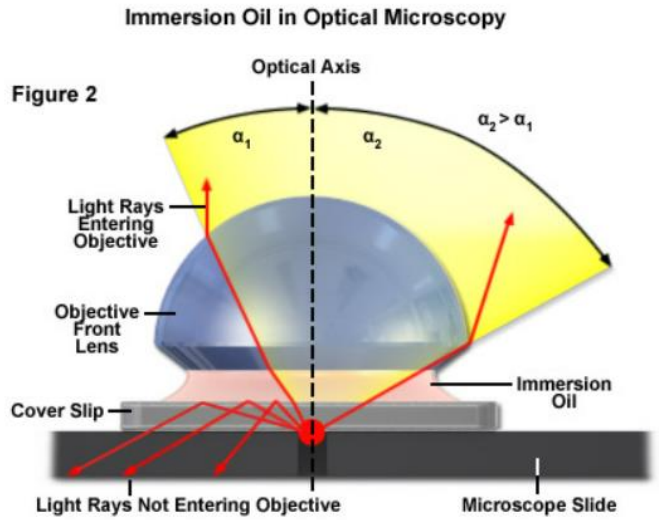
What does resolution actually mean?

Resolution of microscope (d): is a measure of the limit up to which two small objects are still seen separately.

d = λ/2N.A.

λ is wavelength of light, e.g. 550 nm (green),
2N.A. = (N.A.objective + N.A. condensor)





Principles of Optical Microscopy: Parameters of objective

Figure 1

More information on objective abbreviations can be found here:

Parameters of objective

Mechanical tube length is defined as the distance from the nosepiece opening, where the objective is mounted, to the top edge of the observation tubes where the eyepieces (oculars) are inserted. Tube length has been standardized to the Royal Microscopical Society (RMS) suggestion of **160 millimeters for finite-corrected** transmitted light microscopes. **Infinity-corrected objectives** project an image of the specimen to infinity, but the body tube of the microscope must contain **a tube lens**.

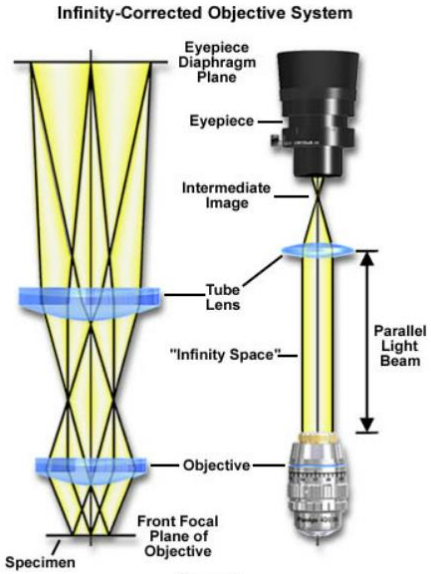


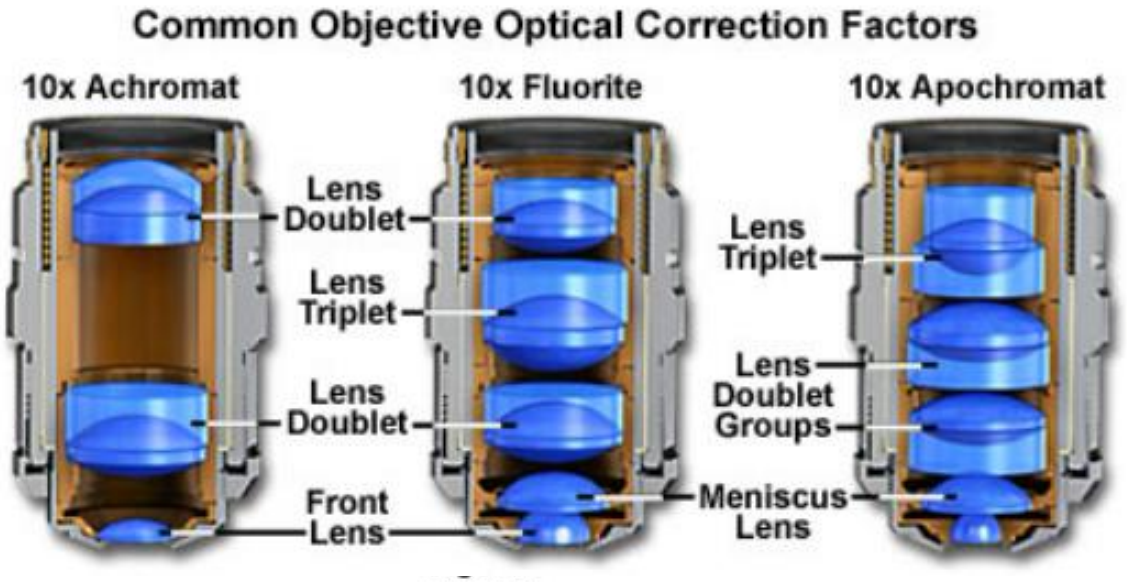
Figure 3

Cover Glass Thickness: Most transmitted light objectives are designed to image specimens that are covered by a cover glass (or cover slip). The thickness of these small glass plates is now standardized at **0.17 mm for most applications**.

Working Distance is the distance between the objective front lens and the top of the cover glass when the specimen is in focus. In most instances, the working distance of an objective decreases as magnification increases.

Coverslip #	Thickness
0	0.085 - 0.13 mm
1	0.13 - 0.16 mm
1.5	0.16 - 0.19 mm
2	0.19 - 0.23 mm
3	0.25 - 0.35 mm
4	0.43 - 0.64 mm

Optical Abberations & Objective Optical Correction

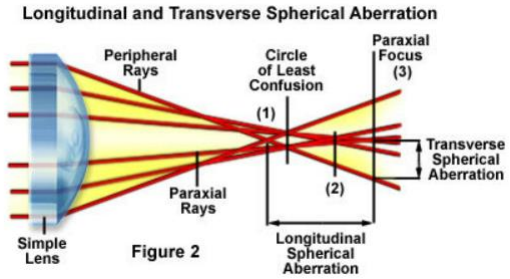


Objective Type	Spherical Aberration	Chromatic Aberration	Field Curvature
Achromat	1 Color	2 Colors	No
Plan Achromat	1 Color	2 Colors	Yes
Fluorite	2-3 Colors	2-3 Colors	No
Plan Fluorite	3-4 Colors	2-4 Colors	Yes
Plan Apochromat	3-4 Colors	4-5 Colors	Yes

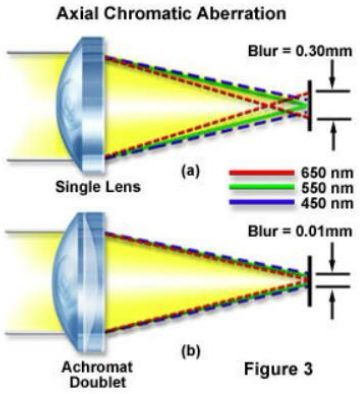
ACH, Achromat; PL FL, Plan Fluorite; PL APO, Plan Apochromat

Optical abberations

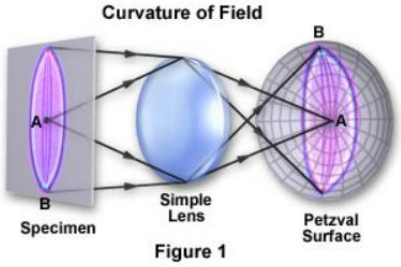
Spherical Aberration: occur when light waves passing through the periphery of a lens are not brought into focus with those passing through the center. Waves passing near the center of the lens are refracted only slightly, whereas waves passing near the periphery are refracted to a greater degree resulting in the production of different focal points along the optical axis.



Chromatic Aberration: is optical defect which results from the fact that white light is composed of numerous wavelengths. When white light passes through a convex lens, the component wavelengths **are refracted according to their frequency**. Blue light is refracted to the greatest extent followed by green and red light, a phenomenon commonly referred to as **dispersion**.



Field Curvature: is the natural result of using lenses that have curved surfaces. When visible light is focused **through a curved lens, the image plane produced by the lens will be curved**. When the image is viewed in the eyepieces (oculars) of a microscope, it either appears sharp and crisp in the center or on the edges of the viewfield but not both.



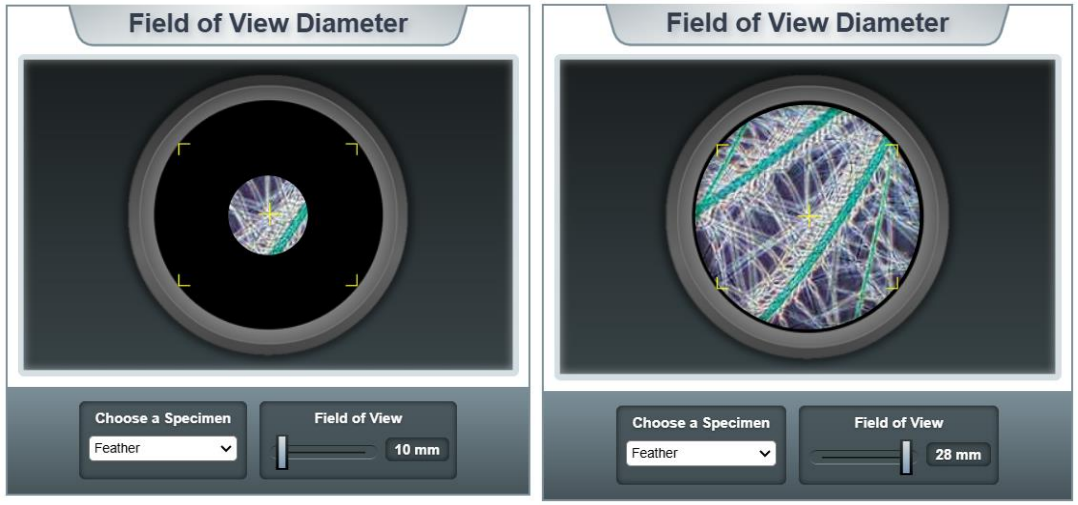
Field of View: *another parameter to consider when selecting objective*

Field-of-view number, or simply the **field number**, is the diameter of the view field in millimeters measured at the intermediate image plane.

Field Size = Field Number (fn) / Objective Magnification (Mo)

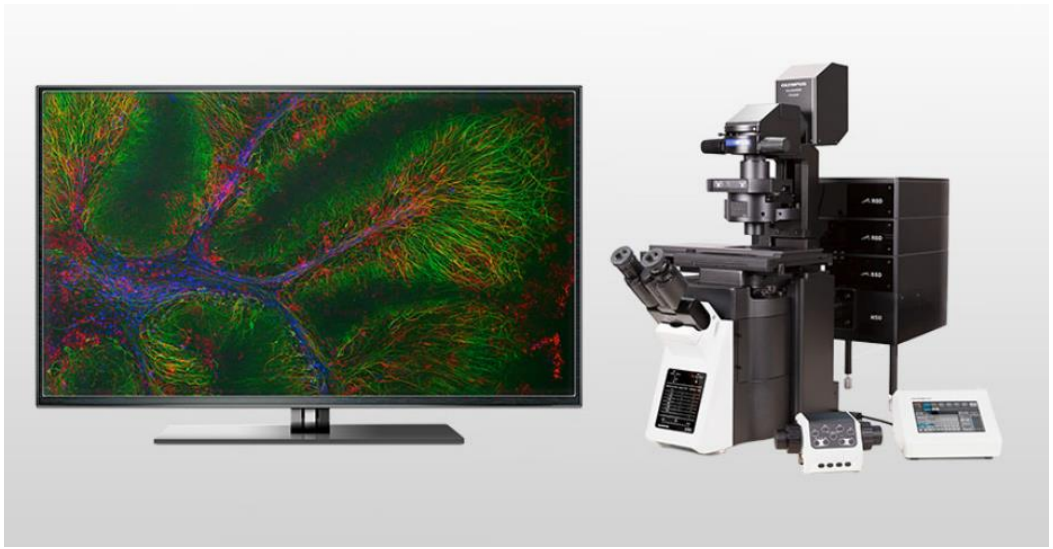
Higher magnification means smaller field size.

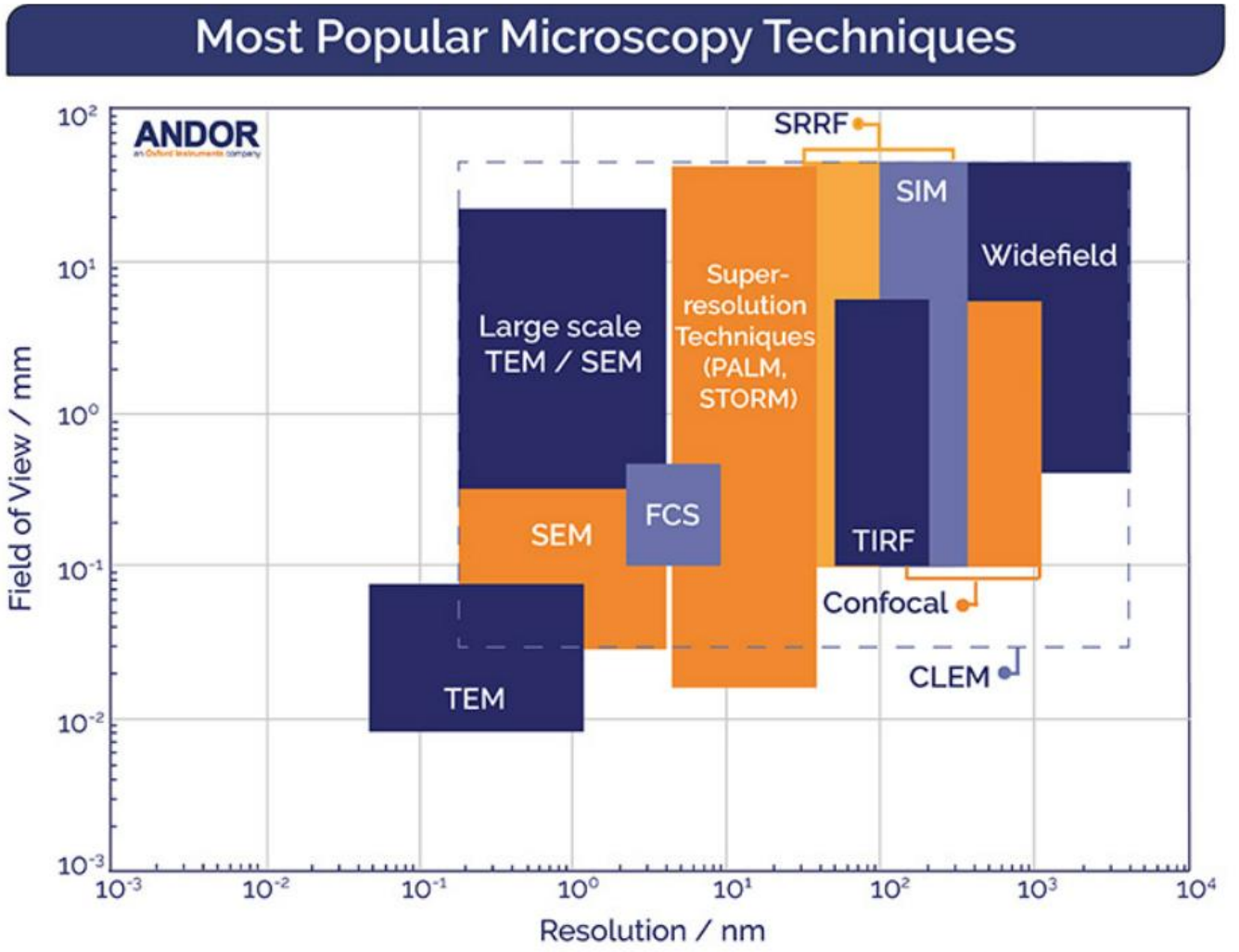
In early microscope objectives, the maximum usable field diameter tended to be about **18 millimeters** or considerably less, but with modern plan apochromats and other specialized flat-field objectives, the maximum usable field can sometimes **exceed 28 millimeters**.



How to choose corrrect objective?

Microscopy techniques: *How to select rights scope?*





Brightfield, Darkfield, Phase contrast, DIC Microscopy

Brightfield Microscopy

Brightfield microscopy employs a basic optical system where light is gathered by the condenser, transmitted through the specimen and into the objective lens.

Applications: examine cells, tissues, microorganisms, and structure of materials

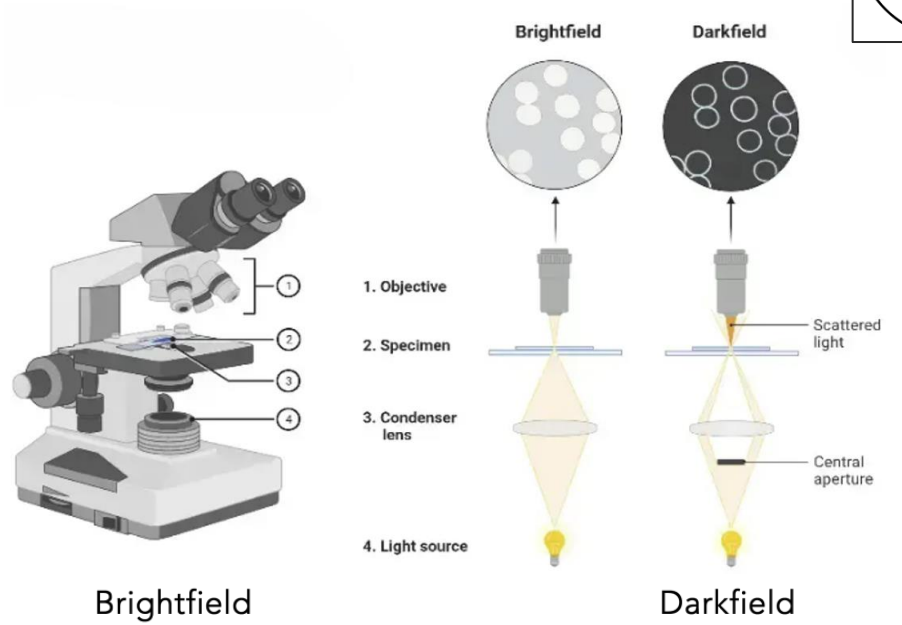
Limitations: While effective for samples with sufficient contrast, brightfield microscopy is **challenged by transparent specimens**.

Darkfield Microscopy

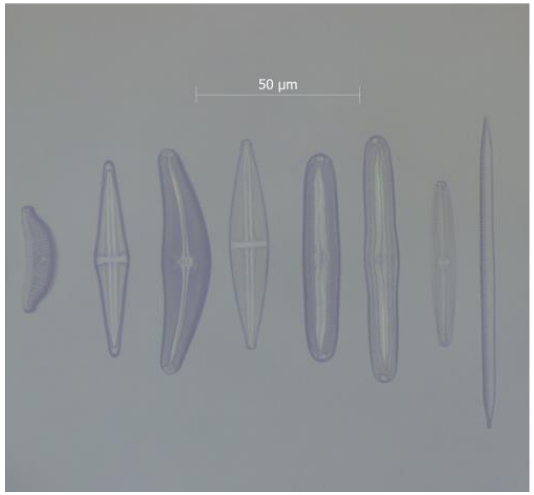
Darkfield microscopy achieves high contrast by **blocking direct light from reaching the specimen**. With a special condenser, light is directed at the sample from the sides, creating a hollow cone of illumination. When a specimen is present, it scatters the light, causing it to enter the objective lens. This produces a bright image of the specimen against a dark background, enhancing visibility, especially for unstained and transparent samples.

Applications: live, unstained specimens, such as bacteria, protozoa, and spirochetes (in materials science to detect small particles and defects)

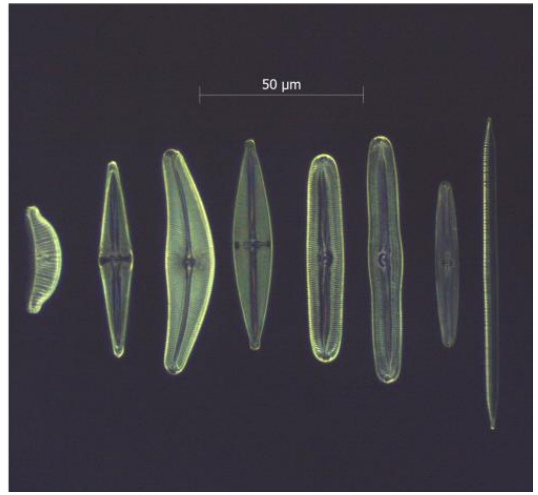
Limitations: The dark background can make it challenging to observe **fine details within the specimen**, while dust and debris can appear as bright objects, potentially obscuring the sample. In addition, the **high light intensity** required for darkfield can potentially damage sensitive specimens.



Brightfield



Darkfield



Brightfield, Darkfield, Phase contrast, DIC Microscopy

Phase Contrast Microscopy

A special **condenser with annular ring** and objective lens are used to convert phase differences (invisible to the human eye) into amplitude differences (visible as variations in brightness). This enhances the contrast of the specimen without the need for staining.

Applications: widely used in cell biology to study living cells, observe intracellular structures, and monitor dynamic processes

Limitations: often exhibit **halo artifacts** around the edges of structures, the phase rings in the objective lens can slightly **decrease the overall resolution,** requirement of **specialised phase contrast objectives and condensers** than standard brightfield optics.

Differential Interference Contrast (DIC) Microscopy

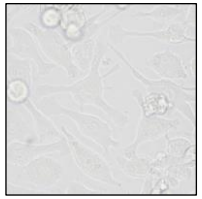
DIC microscopy, also known as Nomarski interference contrast, **employs polarised light** and a series of optical elements to create **two slightly offset beams** of light that pass through the specimen. The differences in optical path length between these beams, caused by variations in the specimen's refractive index, result in interference patterns that generate the image.

Applications: studying unstained biological specimens, such as living cells, embryos, and microorganisms

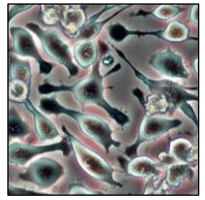
Limitations: suited for **thin, transparent samples** with subtle refractive index variations; requires specialized optical components, it increases the overall **cost of the microscope system**

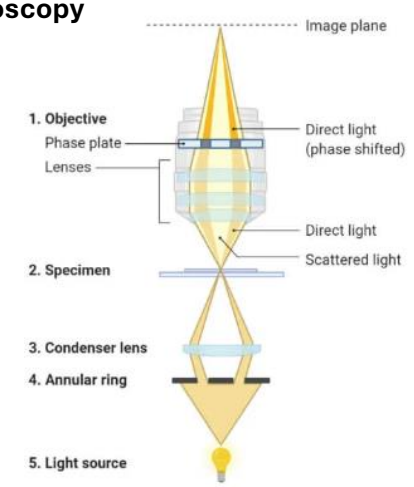
Phase Contrast Microscopy

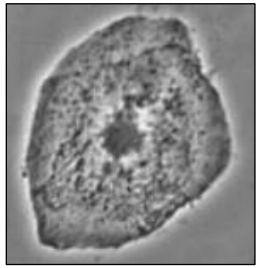
Brightfield

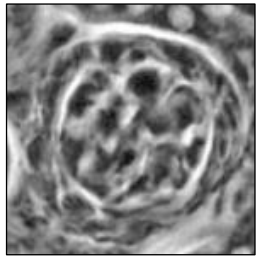


Phase contrast

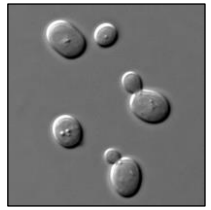


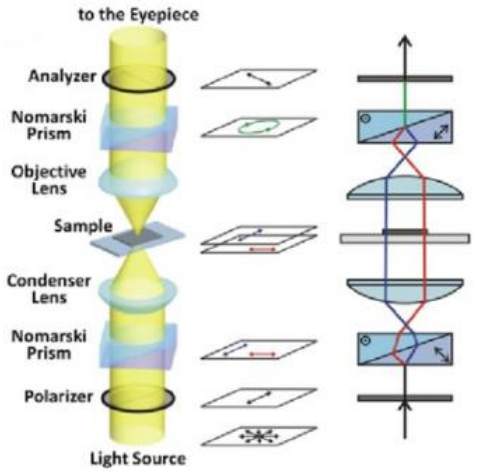


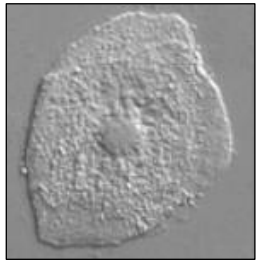




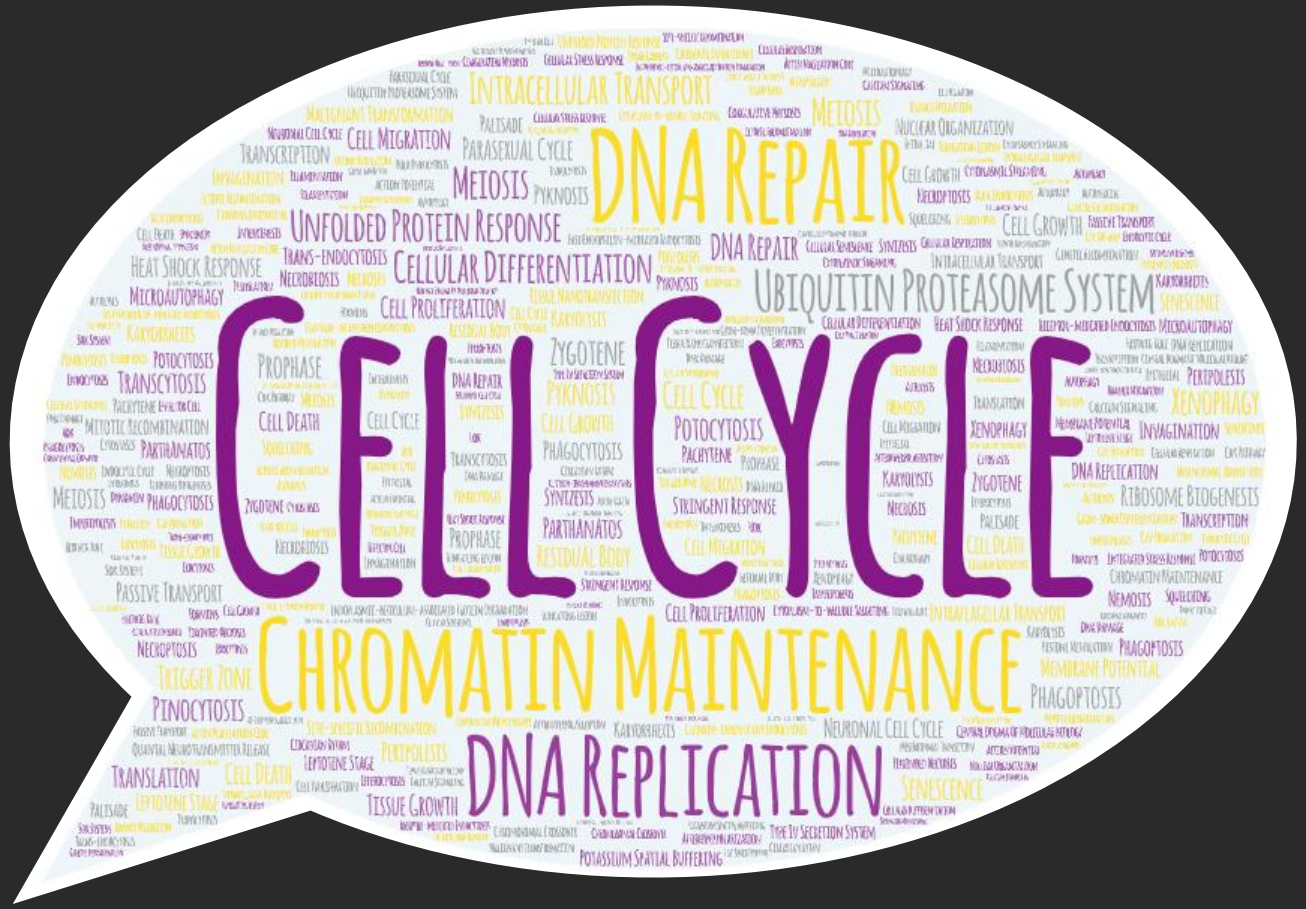
Differential Interference Contrast (DIC) Microscopy

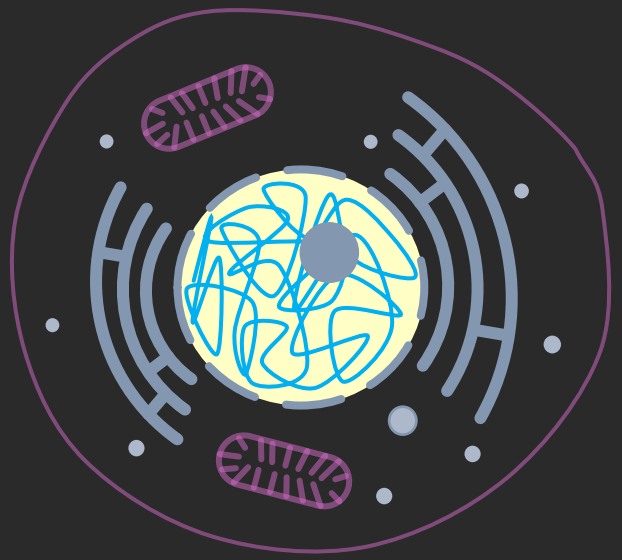






Cells, cells, cells… How to visualize cellular processes inside cells?





eukaryotic cell

Proteins *serve as the primary driving force behind nearly all cellular processes, and their proper functioning is essential for the health and fitness of living organisms.*

Exploring ***protein function*** *represents a fundamental approach to unveiling the molecular nature of vital cellular processes and their related changes underlying disease development.*

Yadav A.K. et al, bioRxiv, 2024

How to visualize proteins inside cells?

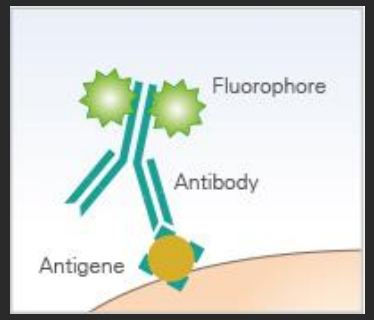
Antibodies

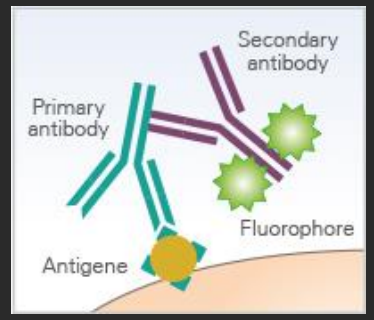
Protein tagging

Immunofluorescence (IF)

An immunofluorescence experiment is based on the following principal steps:

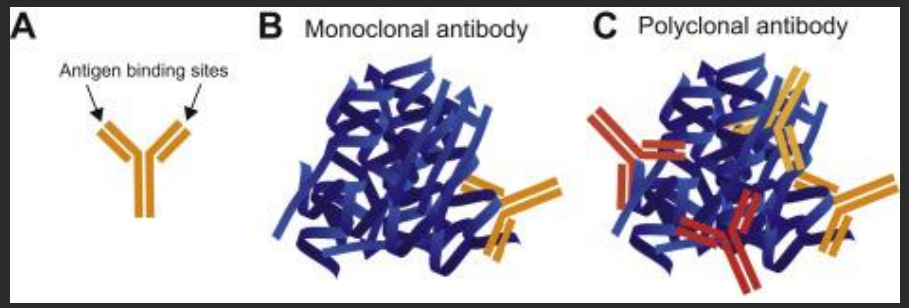
1) Specific antibodies bind to the protein of interest.
2) Fluorescent dyes are coupled to these immune complexes to visualize the protein of interest using microscopy.



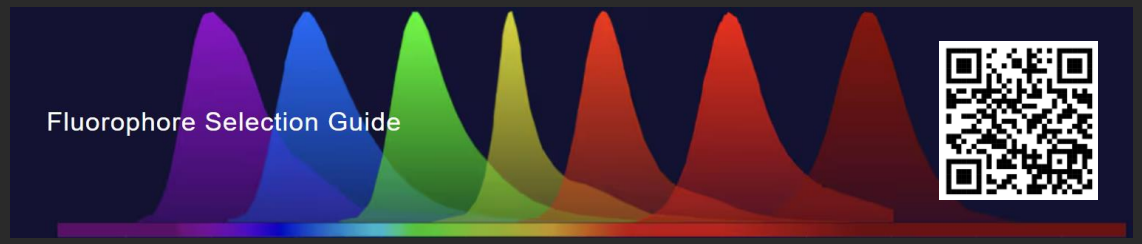


How to select good antibodies?

The Human Protein Atlas



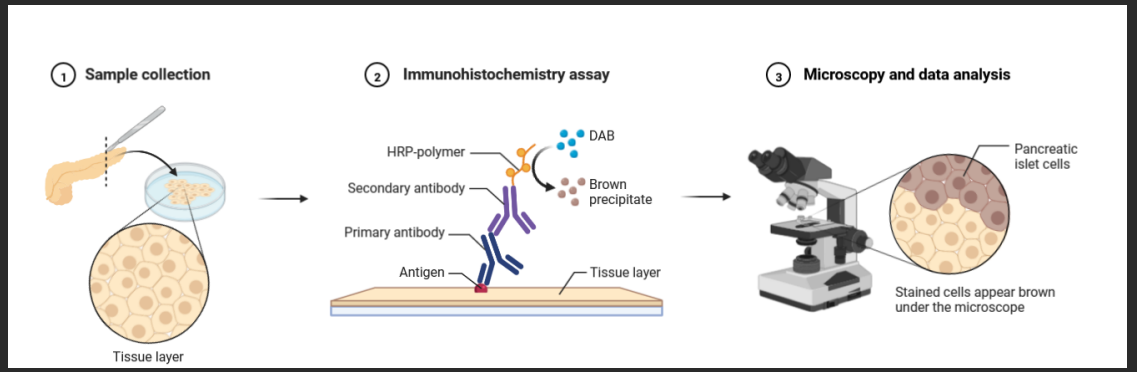
CiteAB Reagent search

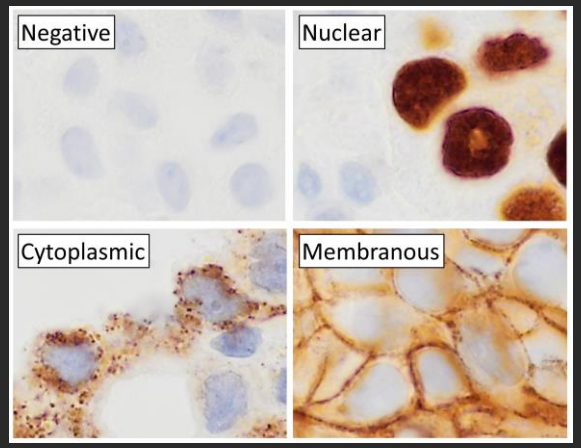


Immunohistochemistry (IHC)

An IIHC experiment is based on the following principal steps:

1) Specific antibodies bind to the protein of interest.
2) Colorimetric detection - Alkaline Phosphatase (AP) and Horseradish Peroxidase (HRP) conjugates are coupled to these immune complexes to visualize the protein of interest using microscopy.





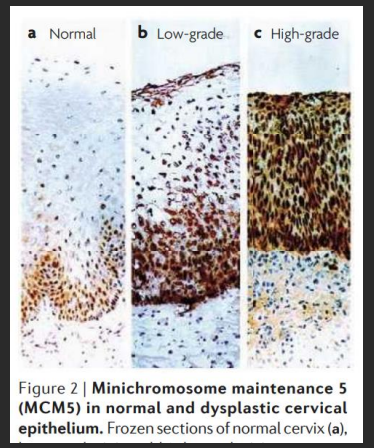
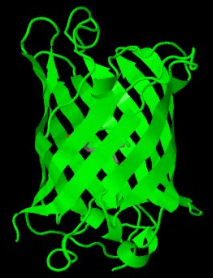


Figure 2 | **Minichromosome maintenance 5 (MCM5) in normal and dysplastic cervical epithelium.** Frozen sections of normal cervix (a),

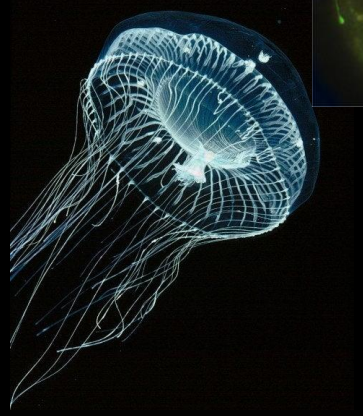
Advantages vs. Disadvantages

The discovery of Green Fluorescent Protein (GFP)



1962

O. Shimomura purifies GFP from *Aequorea victoria*



M. Chalfie expresses GFP in *E. coli*

1992

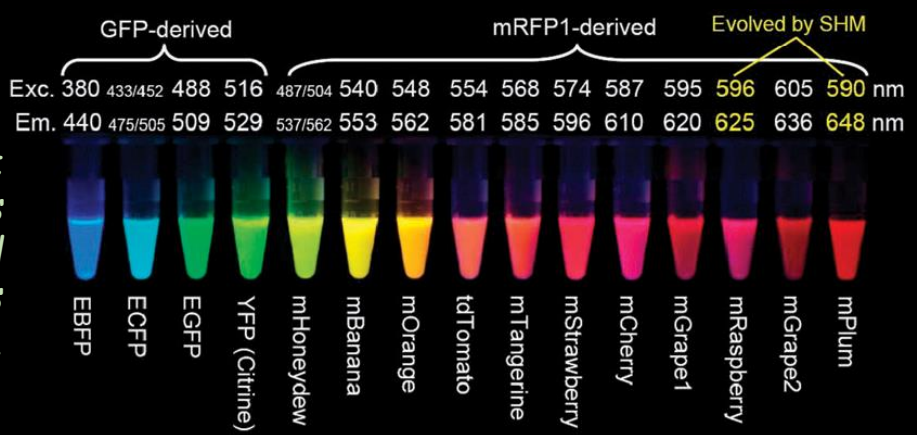
1994

M. Chalfie expresses GFP in *C. elegans*

R. Y. Tsien shows that GFP chromophore is formed in a chemical reaction that requires oxygen.

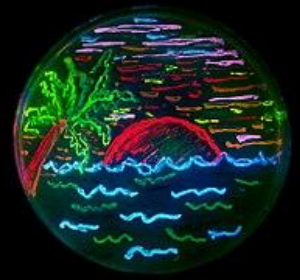
1994-1998

Tsien and collaborators tweak the structure of GFP to produce cyan, blue and yellow variants.



Discovery of six GFP-like proteins in fluorescent corals, including red protein DsRED (M. Matz & S. Lukyanov)

1999



2000-2002

Tsien produces variants of DsRED including red, orange and pink

Nobel prize: 'for the discovery and development of the green fluorescent protein, GFP'

2008

Osamu Shimomura

Martin Chalfie

Roger Tsien

GFP in bioimaging of cellular processes

196

O. S
puri
fror
vict

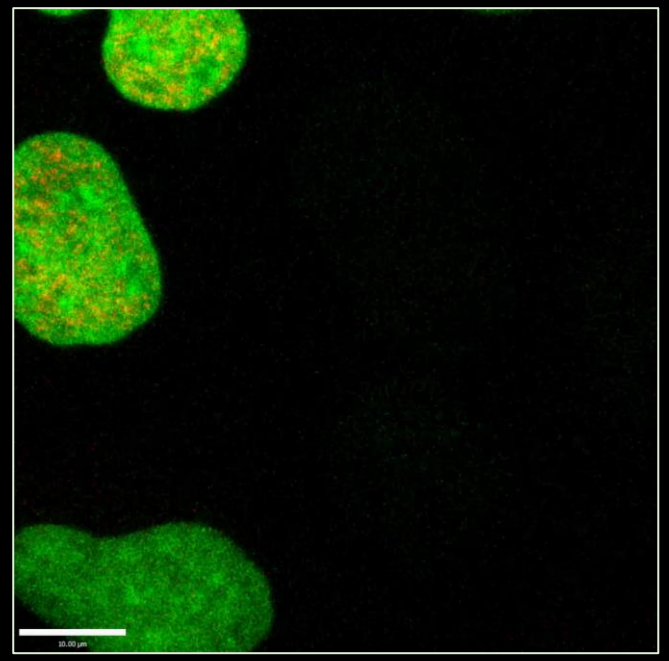
Discovery of six GFP-like proteins in fluorescent corals

2000-2002

Nobel prize: 'for the discovery and development of the green fluorescent protein, GFP'

2008

Imaging of proteins in living cells & development of biophysical techniques (FRAP, FRET, FLIP, FLIM, FCS, FCCS) to study protein dynamics and interaction in living cells.



Production of endogenous MCM4
Bleach-chase: U2OS MCM4-GFP, PCNA-RFP cells, Perkin Elmer Spinning disc, 60x

BUT...

X multiple cell line generation for imaging protein of interest by different fluorescent colours

X fusion GFP with additional tags for protein degradation (AID – auxin inducible degron)

X adding tags for protein purification (FLAG, His etc.)

Tsien produces variants of DsRED including red, orange and pink

Osamu Shimomura

Martin Chalfie

Roger Tsien

oxygen.

FP
FP
FP
P (Citrine)
Honeydew
Banana
Orange
Tomato
Tangerine
Strawberry
Cherry
Grape1
Raspberry
Grape2
mPlum

HaloTag technology: Powerful and Universal tagging

1985

purification & characterization from Xanthobacter, Keuning S et al, J. Bacteriol, 1985

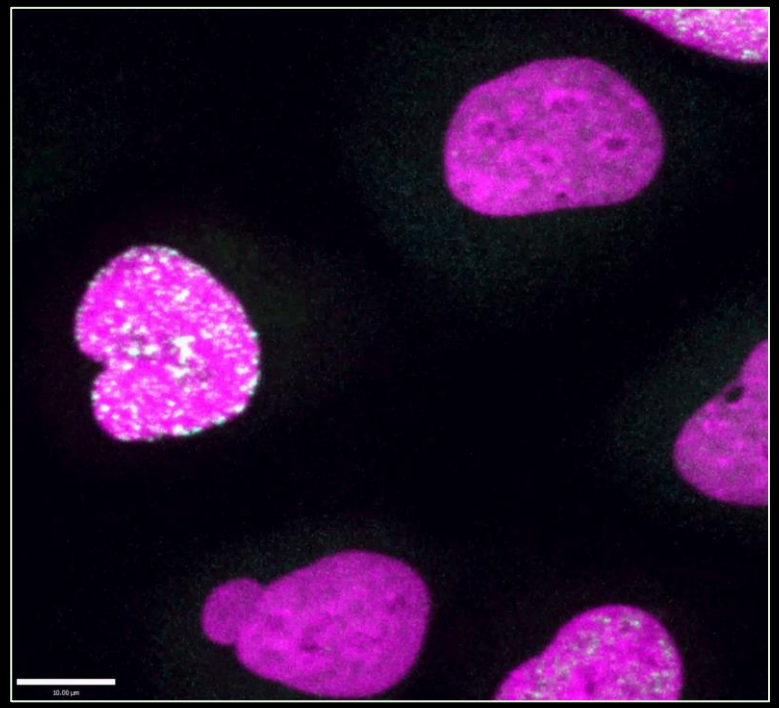
Haloalkane dehalogenas

200

development of JF HaloTag fluorescent ligands by J. Grimm & L. Lavis

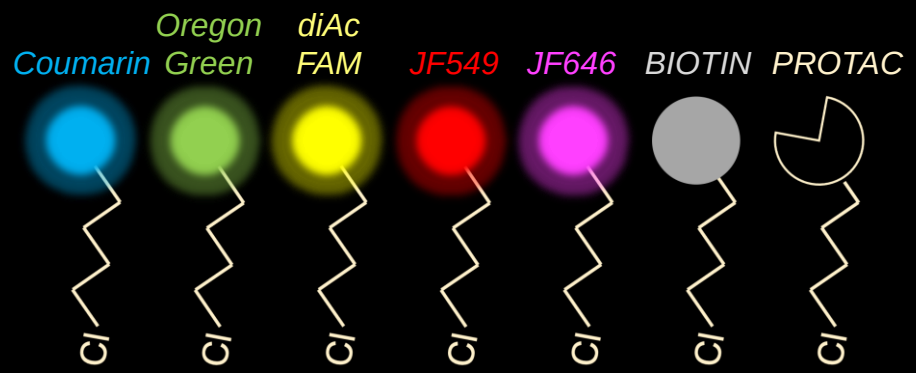
hhmi | janelia Research Campus

Visualization of MCM2-7 helicase turnover in living cells by dual HaloTag labelling protocol



Pulse-Chase: U2OS MCM4-Halo (JF549 pulse, JF646 chase), PCNA-RFP cells, Perkin Elmer Spinning disc, 60x

HaloTag ligands rainbow



HaloTag applications:

- ✓ *in vivo & in vitro cellular imaging by different fluorescent colours*
  - ✓ *subsequential labelling with two Halo ligands*
- ✓ *protein degradation (PROTAC ligands)*
- ✓ *adding tags for protein purification & interaction (BIOTIN ligands, HaloTrap beads)*

Whenever using Imaging approach, make sure that your probes are reliable…

How do you validate your antibody?

Antibody validation techniques

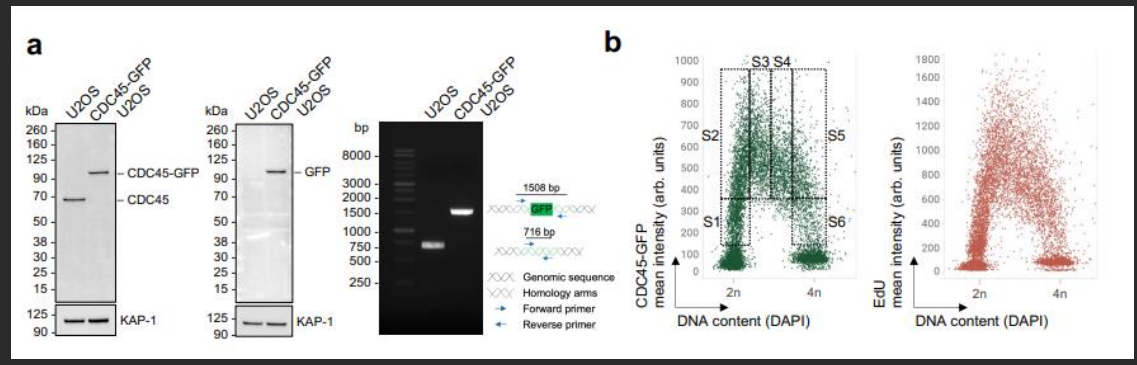
Genetic strategies	Orthogonal strategies	Independent antibody strategies	Tagged proteins/ Recombinant expression	IP followed by MS
Knockdown (down-regulation) or Knockout (removal) methods are used on the target protein to confirm antibody specificity	Antibody independent methods and antibody dependent methods are used in parallel and results compared to confirm the antibody works as expected	Two or more antibodies against different epitopes on the same protein are compared to show if the antibody is specific to the target	Comparison between an over-expressed or tagged protein and a control sample demonstrates antibody specificity	Antibody-target binding is compared to the results when the target is immunoprecipitated and undergoes mass spectrometry

CiteAb https://pubmed.ncbi.nlm.nih.gov/27595404/

How do you validate your cell line?

- ***Genome**: integration of the tag (PCR, sequencing, etc.)*
- ***Protein expression**: level of expression (compared to naïve cells)*
- ***Protein function**: dynamic behavior, interacting partners*
- ***Off-target effects**: cell cycle regulation, genome instability etc.*

An example of cell line validation:



Polasek-Sedlackova et al, Nature Communications, 2022

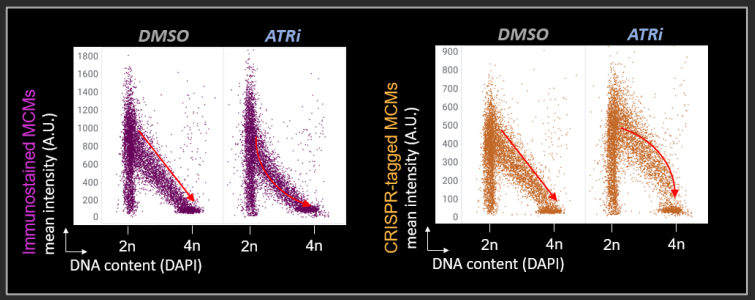
When antibody may appear functional…
Be aware of the detection of different protein variants!

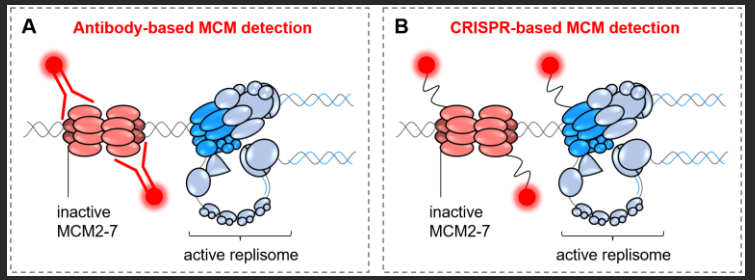
Replication factories (CDC45-GFP)

CRISPR-tagged MCMs

Immunostaned MCMs

Late stage of the S phase, ATRi treatment



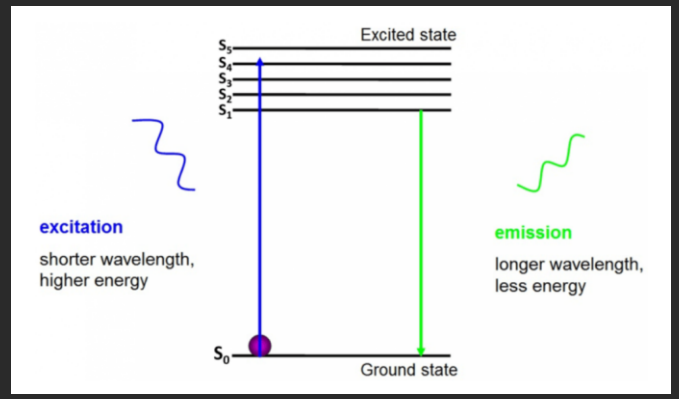


Polasek-Sedlackova et al, Nature Communications, 2022

Be aware of the properties of fluorescent probes...

Reminder: Principle of fluorescence

When an electron is hit with a **photon** of a certain energy range, the electron **absorbs the energy** of the photon and jumps up to a higher energy state (S_1 , S_2 , or S_3). To return to the **ground state** (S_0), the electron **releases the additional energy** as the **emission** of a photon. The energy of this photon is **less** than the excitation energy so it has a **longer wavelength**.



How to select good fluorophores?

Fluorophore Properties:

FluoroFinder

FluoroFinder →

Combination of fluorophores:

Fluorescence SpectraViewer →

Properties of Fluorophores

Excitation maxima (ExMax) is the wavelength of light most efficiently absorbed by the fluorophore.

Emission maxima (EmMax) is the wavelength of light that will be emitted from the fluorophore at the highest intensity.

Quantum yield (ϕ) of a fluorophore is the ratio of the number of **released photons** to the number of **absorbed photons**. The quantum yield is often expressed as a value from 0-1, which 1 being **100%** efficiency of photon conversion.

Extinction Coefficient (ε) of a fluorophore is the amount of light of a specific wavelength that can be absorbed by a given material in solution.

*A fluorophore's **quantum yield** and **extinction coefficient** are often displayed together to describe how bright the fluorophore is shown to be in experimental settings.*

Fluorescence Lifetime is the amount of time that an electron spends in the **excited state** before releasing a photon and returning to the ground state.

Photostability is the ability of a fluorophore to resist damage (photobleaching). Photobleaching is a phenomenon in which a fluorophore will "burn out" and cease to fluoresce after continual, high-intensity excitation.

Widefield Fluorescence Microscopy

In **brightfield microscopy**, the sample is illuminated with **transmitted white light**. This creates an even illumination of the sample under the microscope to observe highly contrasted, stained or naturally pigmented samples. However, brightfield is **not sufficient to distinguish between transparent/translucent, unstained cells or cellular structures to study processes of interest**.

Fluorescence microscopy relies on the use of **fluorophores**, molecules that **emit light of a specific visible wavelength** when exposed to light of a different wavelength. When these fluorophores are bound to a targeted structure of interest, photons emitted from the fluorophore can be used to visualize this structure of interest.

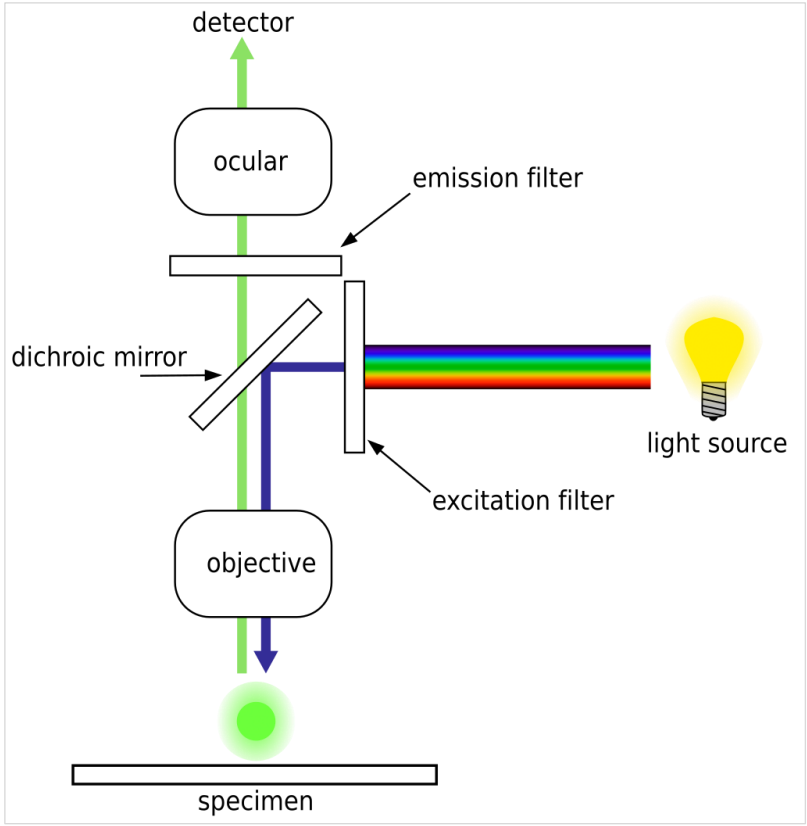
Important components of the fluorescent microscope

Light source: requires intense, near-monochromatic (only one frequency), illumination (e.g. xenon arc, mercury-vapor lamps, and high-power LEDs.).

Excitation Filter: placed within the illumination path of a fluorescence microscope. It filters out all wavelengths of the light source except for the excitation range of the fluorophore or specimen under inspection.

Dichroic Mirror: placed between the excitation filter and emission filter at a 45° angle. The dichroic mirror acts as a **wavelength-specific filter** that transmits fluorescence to the eyepiece or a detector.

Emission Filter: placed within the imaging path of a fluorescence microscope. It filters out the entire excitation range of the fluorophore under inspection and transmits the emission range of the fluorophore.



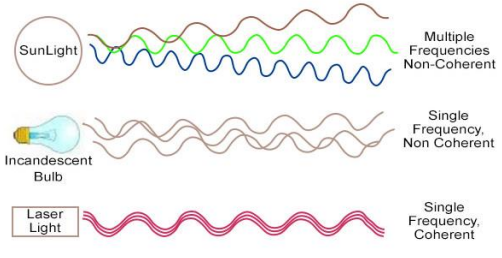
Confocal Fluorescence Microscopy

Confocal Fluorescence Microscopy

Confocal microscopy is a powerful technique that overcomes the limitations of widefield fluorescence microscopy by significantly enhancing **image resolution and contrast.**

Components of Confocal microscope

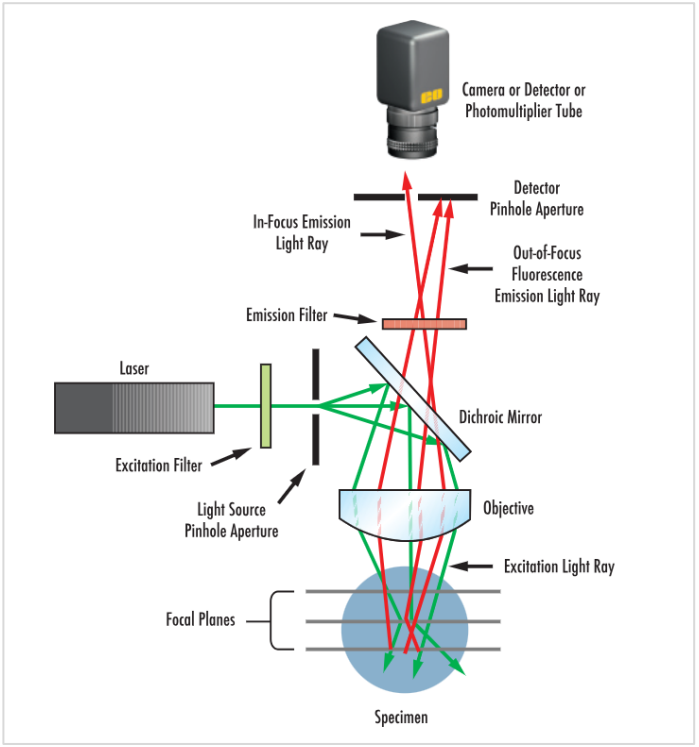
Light source: coherent monochromatic light - Laser (light amplification by stimulated emission of radiation)

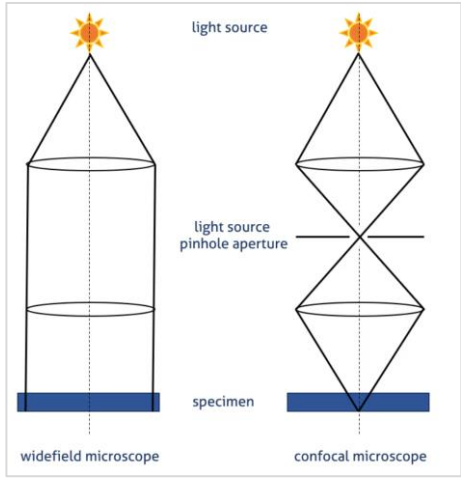


Pinhole: an essential component of confocal setups, it acts as a spatial filter. It prevents any light that is not confocal to the objective focal plane from interfering with the image.

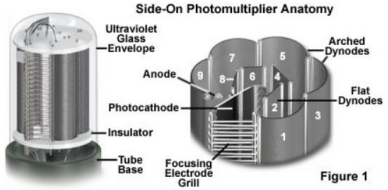
Stepper Motors*:* allows the laser beam to travel incrementally across the sample, obtaining x and y scans collected along the z-axis. This automates three-dimensional data being collected, making it easy to create a **3D image**.

Objective: a **high numerical aperture (NA) objective** is typically used to achieve high resolutions. Water or oil immersion designs are often used to adjust for refractive index mismatch between the aqueous medium many live samples are kept in. Long working distances are required for thick specimens and 3D imaging/optical sectioning.





Photosensitive detectors (photomultipliers, photodiodes, and solid-state charge-coupled devices (CCDs)). In confocal microscopy, **fluorescence emission is directed through a pinhole** aperture positioned near the image plane to exclude light from fluorescent structures located away from the objective focal plane, thus **reducing the amount of light available for image formation**.



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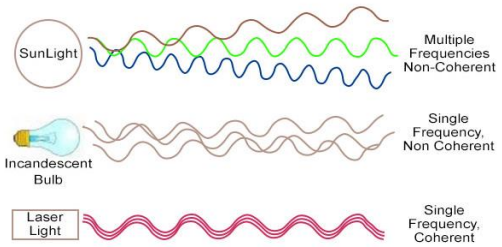

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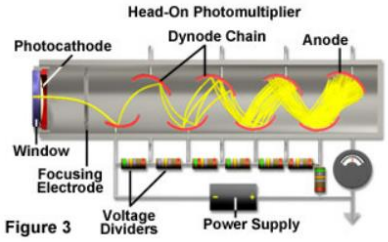


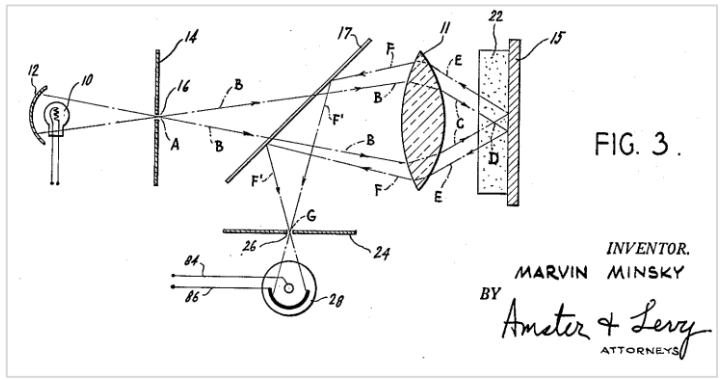
Figure 3

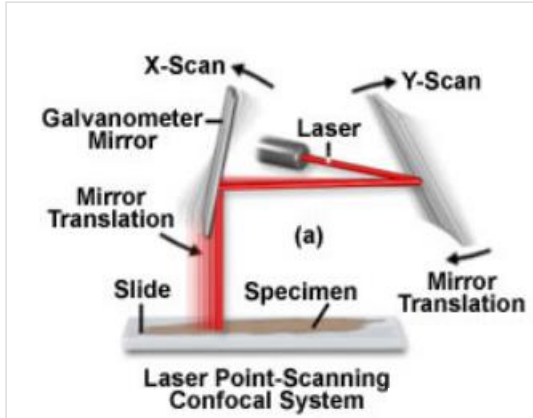
Types of Confocal Fluorescence Microscopy

Laser Scanning Confocal Microscope

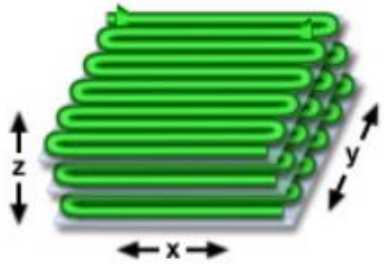
LSCM utilizes a focused laser beam and multiple mirrors (typically 2 or 3 scanning linearly along the x- and the y-axes) to **scan the sample point by point**. While this approach offers exceptional **image quality**, it can be **time-consuming** (large samples or live Imaging).

Marvin Lee Minsky





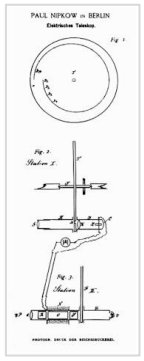
LSCM scanning principle

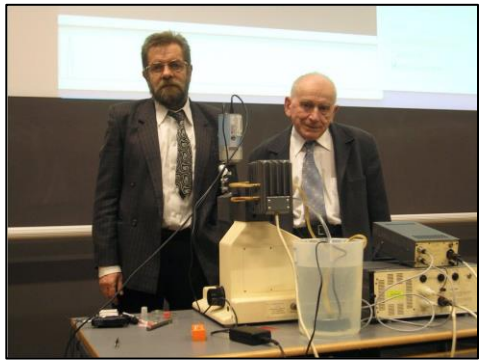


Spinning-Disk Confocal Microscope

Spinning-disk microscope utilises a **spinning disk (Nipkow disk) with multiple pinholes** to simultaneously illuminate different parts of the sample, accelerating image acquisition. This reduces excitation energy and **decreases phototoxicity and photobleaching of a sample**, often making it the preferred system for **imaging live cells or organisms (protein dynamics)**.

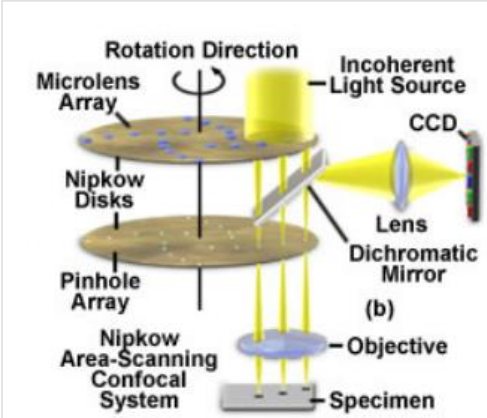
Paul Gottlieb Nipkow



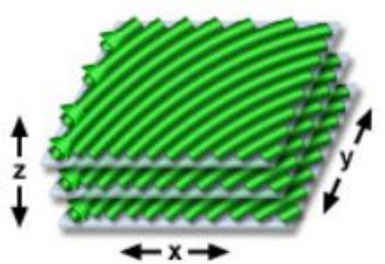


Mojmír Petráň, Milan Hadravský

Yokogava



Spinning-disk scanning principle



Widefield vs Confocal Microscopy: Advantages & Disadvantages

Widefield microscope

✓get images **quickly**
✓ can observe them **directly in the ocular**
✓ Maintenance **cost is low** compared to the confocal microscope.

X a risk of **high background**
X risk of **channel-to-channel bleed-through** *(when fluorescent dyes have overlapping spectral profiles*).
X excitation wavelength bands depend on the **filter sets available**; this can be a limitation.

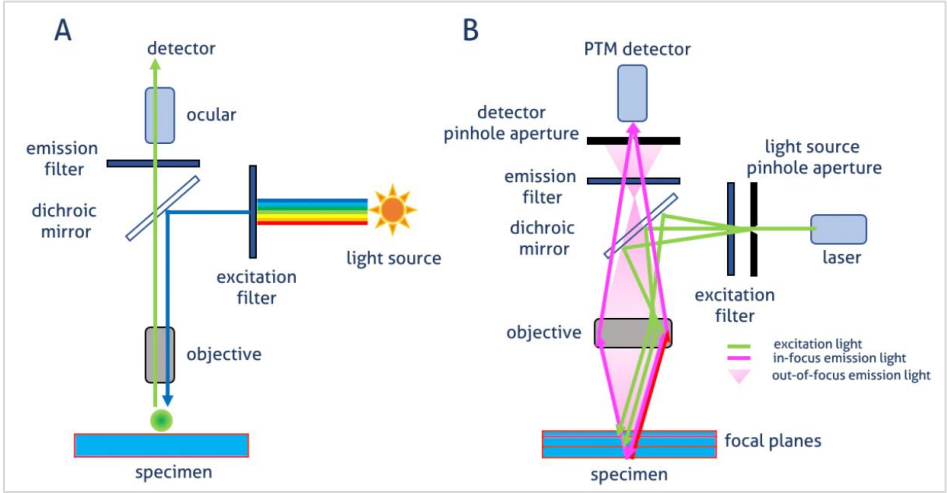
Confocal microscope

✓ **superior image quality** and improve the **signal-to-noise ratio**
✓ flexibility in terms of excitation and emission of wavelength parameters **reduces channel-to-channel bleed-through**
✓ examination and **3D reconstruction** of thick specimens

***X* time-consuming** (depending on the scanning speed)
***X* more complicated image acquisition** procedure compared to widefield
X confocal images are **only obtained digitally** from the PMT detector *(the signal observed through the ocular lens is a widefield image)*

Digital microscopy is a type of optical microscopy that uses optics and a **digital camera** to display images on a monitor of a computer (operation of microscope through **acquisition and analysis software**).

more details about the camera, image acquisition & analysis -> next lecture



Think about your experiment!

For most uses, a widefield fluorescence microscope is sufficient and provides the best trade-off between quality, speed, ease of use, and cost.

A widefield fluorescence microscope is a perfect tool for initial screening, verifying the quality of staining of the sample before approaching the confocal microscope.

Resolution of images

Amount of data (acquired images)

in Microscopy

High-content imaging (screening) technology

High-content imaging technology is mainly based on **automated digital microscopy** and **flow cytometry**, in combination with IT systems for the analysis and storage of the data.

Common workflow:

1) Acquisition of large amounts of data to obtain spatially or temporally resolved information on an event
2) Automated multiparameter analysis of acquired data

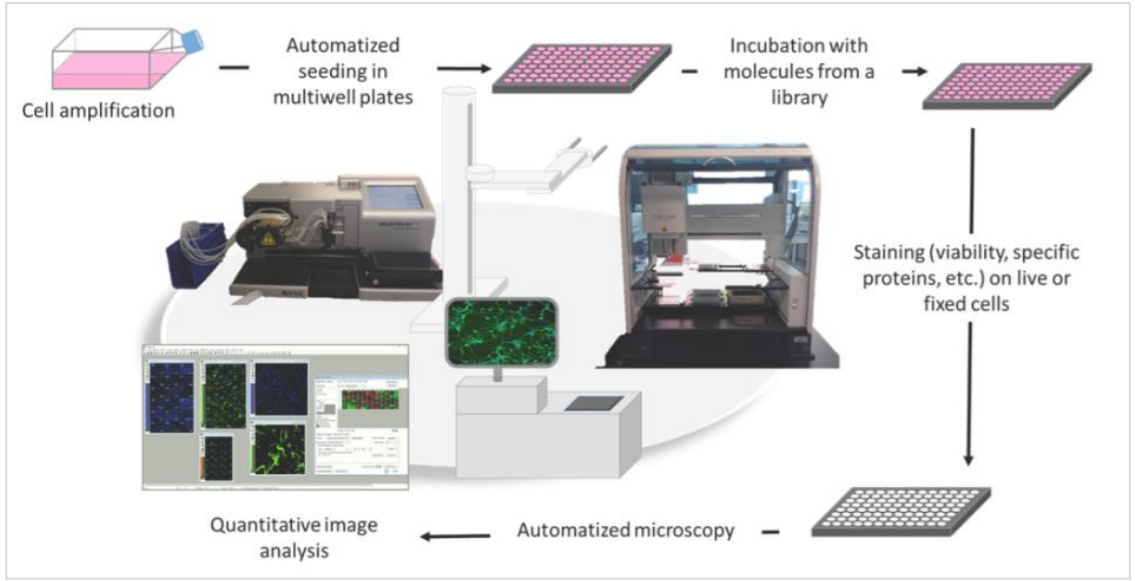
Purpose:

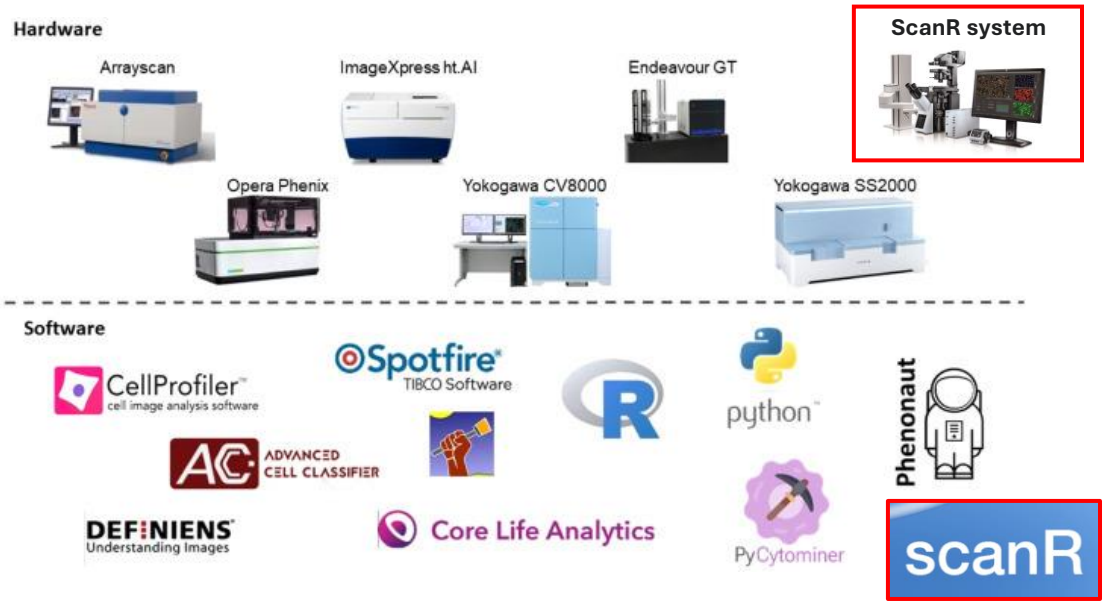
1) fundamental biological research
2) drug discovery to identify substances such as small molecules, peptides, or RNAi that alter the phenotype of a cell in a desired manner.

Options:

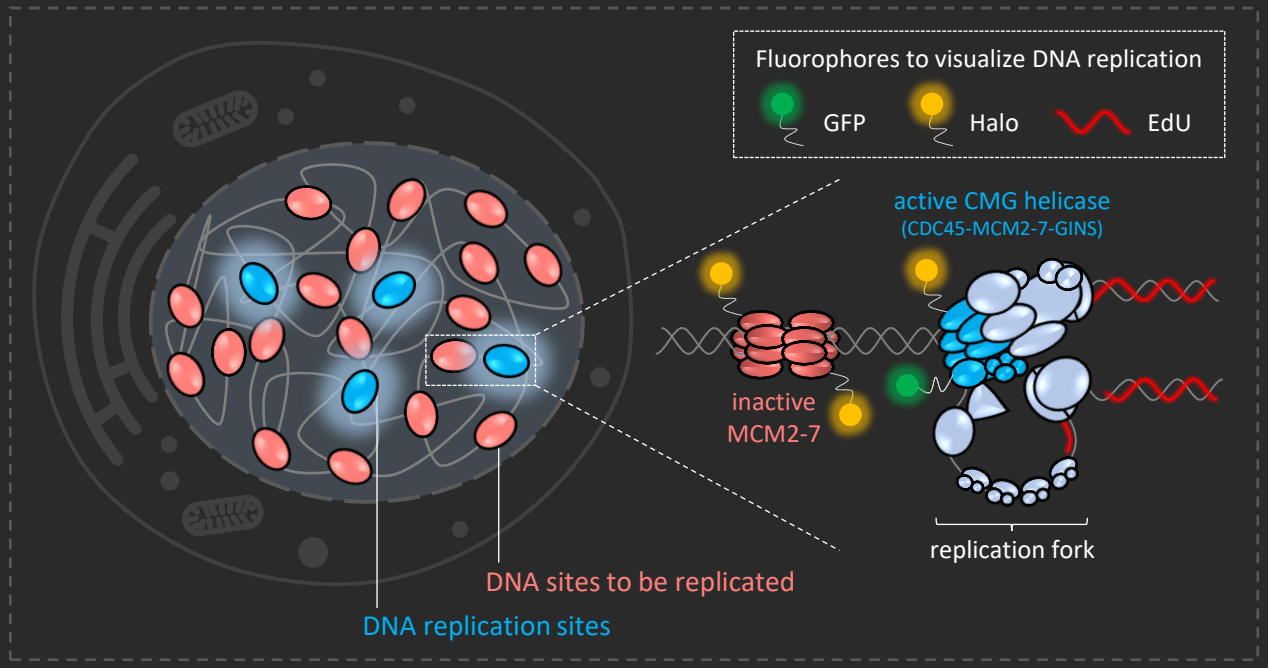
1) Widefield microscopy
2) Confocal microscopy (usually spinning disk)
3) Near-super-resolution (e.g. dual spinning-disk confocal microscopes)
4) Combination of widefield & confocal imaging

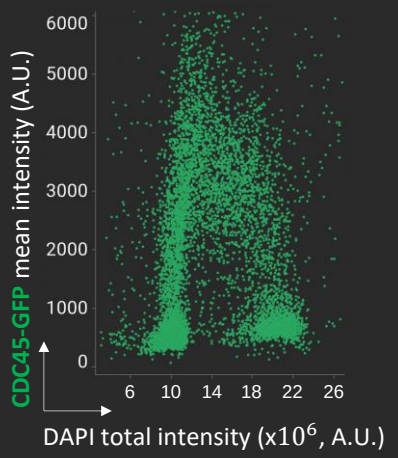
Typical high-content Imaging workflow

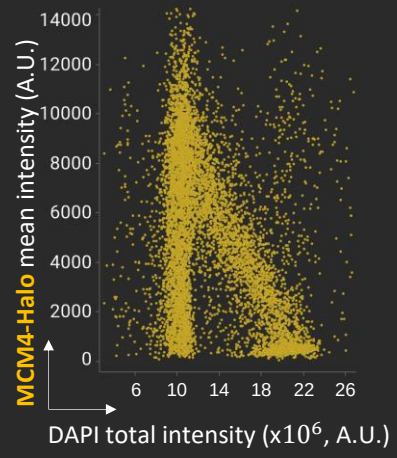


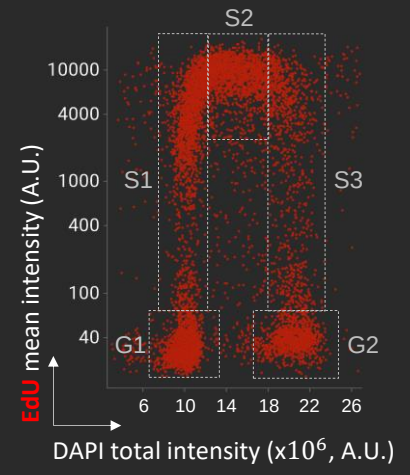


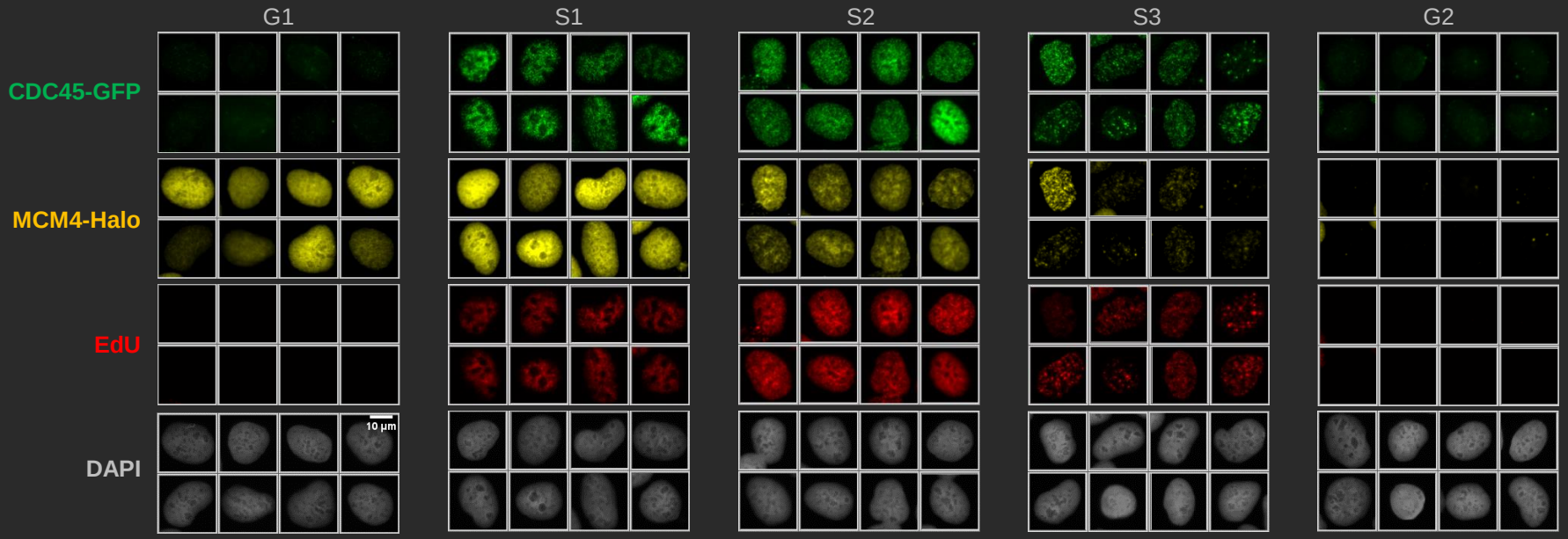
High-content imaging (quantitative image-based cytometry) of DNA replication dynamics

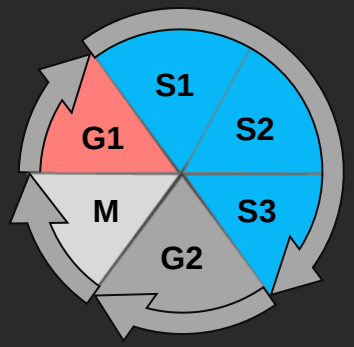












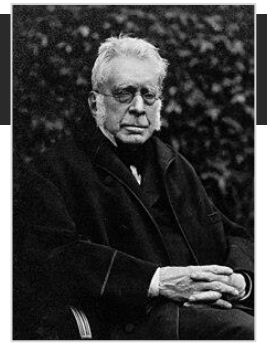
Resolution of images

Amount of data (acquired images)

How far can we go with image resolution?

in Microscopy

Microscope Resolution: Airy Discs



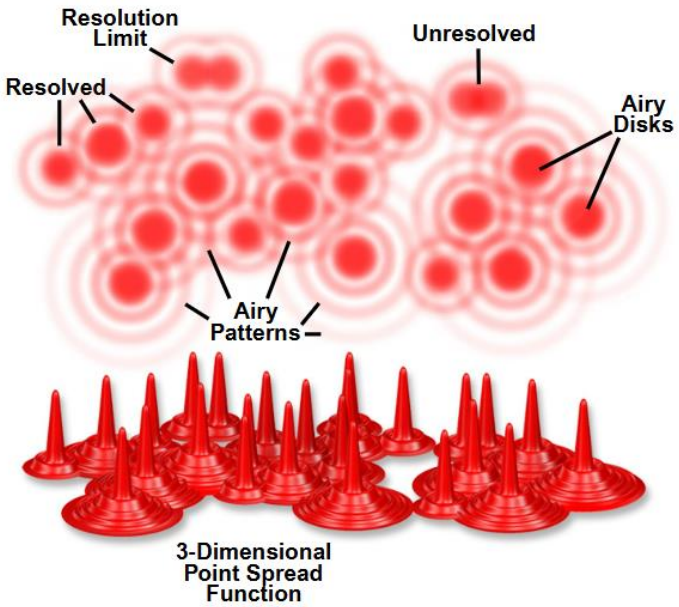
George Biddell Airy

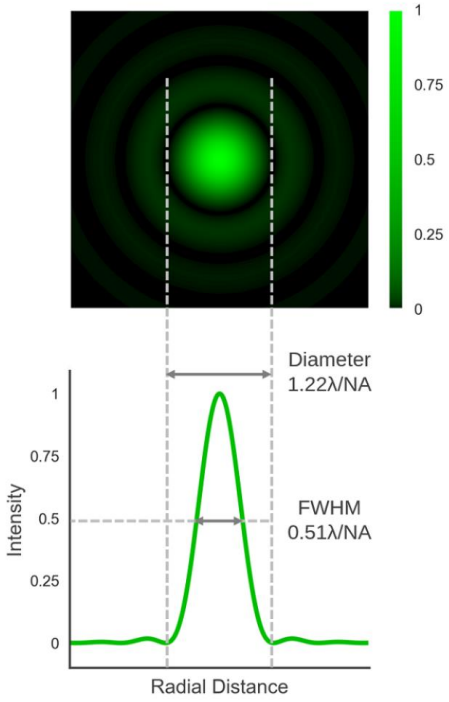
A point object in a microscope, such as a fluorescent protein single molecule, generates an image at the intermediate plane that consists of a **diffraction pattern** created by the action of interference (a central spot (diffraction disk) surrounded by a series of diffraction rings, **Airy disc**).

The size of the Airy disc is given by:
Airy Disc Diameter = 1,22 x λ/NA

λ is the wavelength of the emitted or scattered light
NA is the numerical aperture (objective lens)

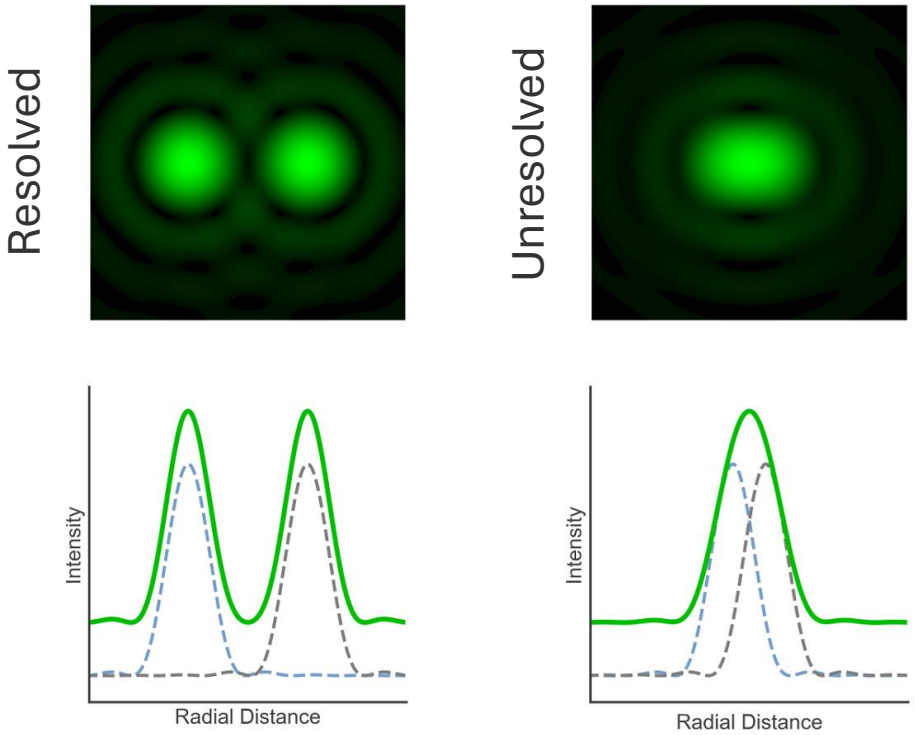
Figure 1 - Airy Patterns and the Limit of Resolution



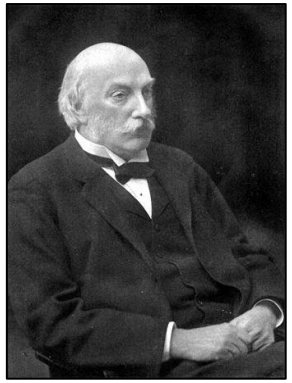


What is Resolution?

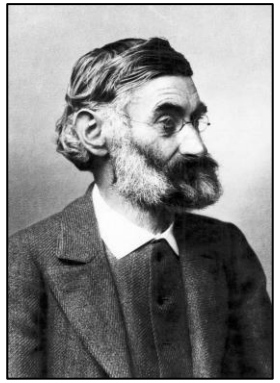
Resolution can be defined as the **minimum separation between two objects** that results in **a certain level of contrast between them**.



Microscope resolution: Rayleigh criterion & Abbe‘s limit



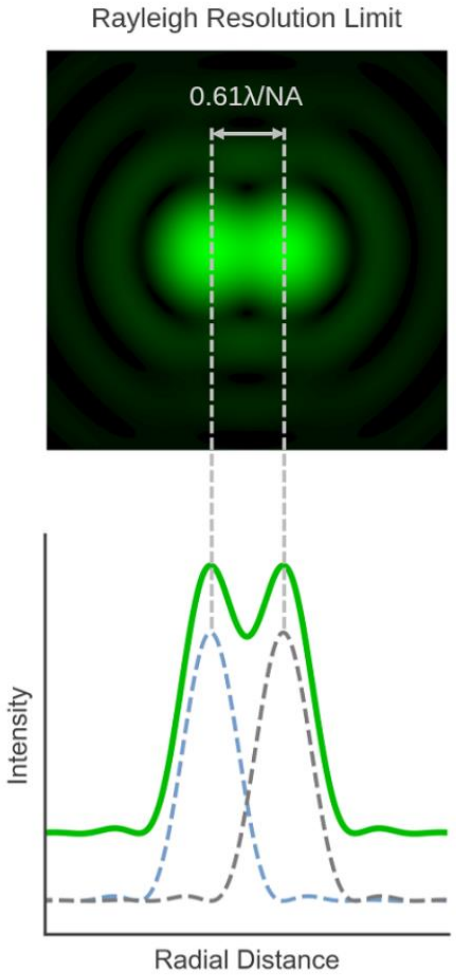
John William Strutt, 3. baron Rayleigh



Enst Abbe

Rayleigh criterion

- is essentially just **different definitions** of what constitutes ***a sufficient level of contrast between the objects*** for them to be resolved
- is defined the resolution limit as the separation where the central maximum of the Airy pattern of one point emitter is directly overlapping with the first minimum of the Airy pattern of the other (**the minimum resolvable separation between the points is the radius of the Airy disc**)



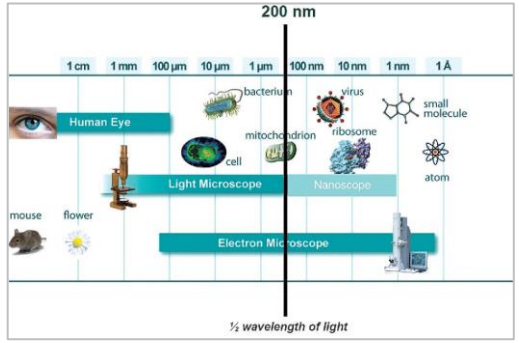
Rayleigh resolution limit = 0,61 x λ/NA

Abbe resolution limit

- **microscope resolution** is determined by the **wavelength of light** and the **numerical aperture of the objective**

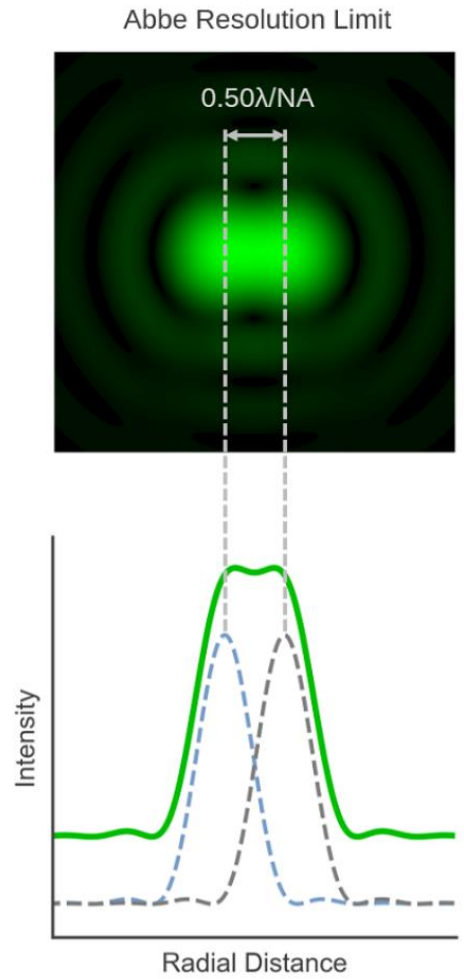
Abbe resolution$_{xy}$ limit = 0,5 x λ/NA

(spot size produced by a 100x objective of numerical aperture 1.4 is approximately 250 nanometers (spatial resolution))



Axial resolution in optical microscopy is even worse than lateral resolution, on the order of 500 nanometers.

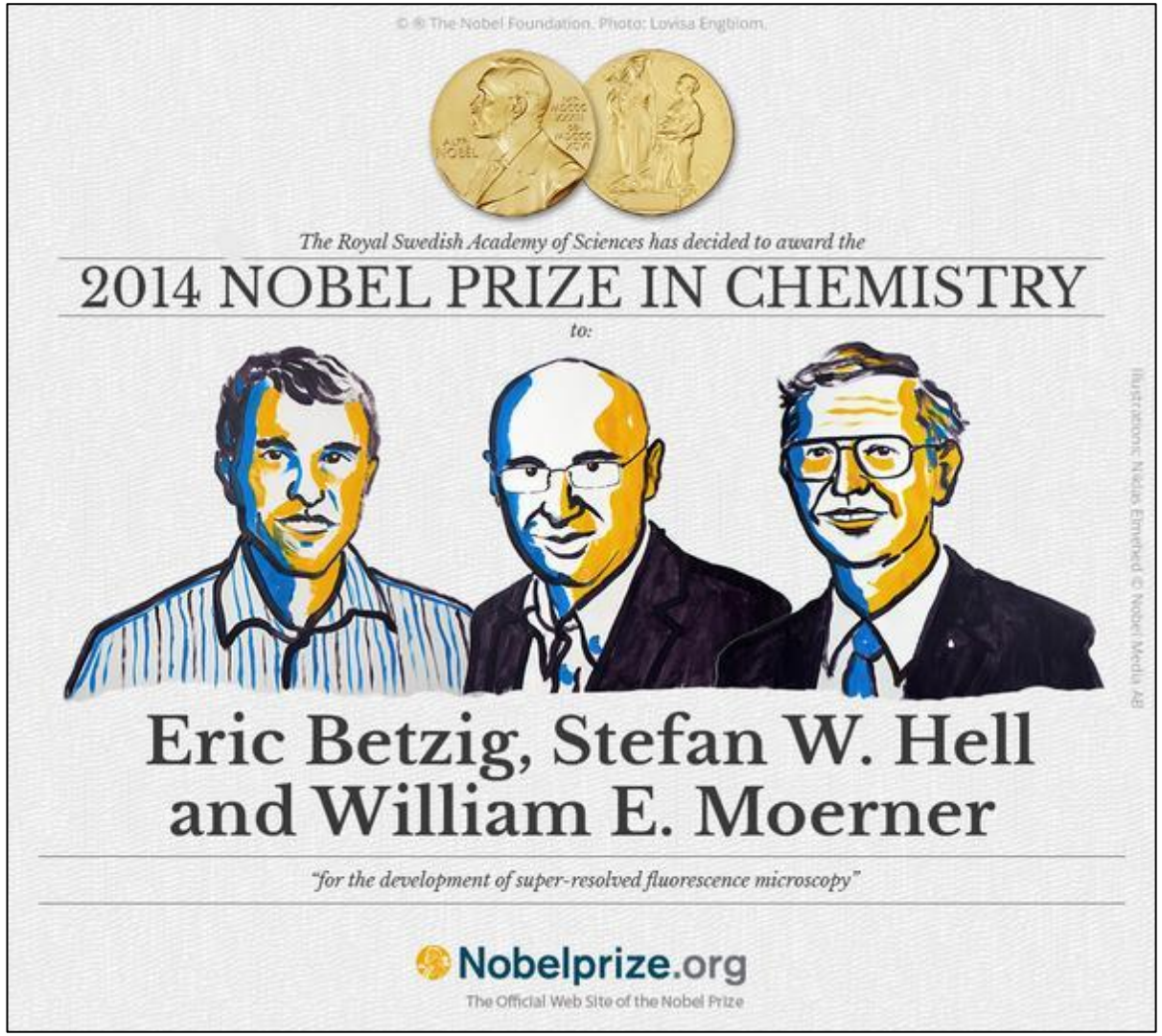
Abbe Resolution$_z$ = 2λ/NA^2



How to increase resolution?

decreasing the imaging wavelength, increasing the numerical aperture, or using an imaging medium having a larger refractive index

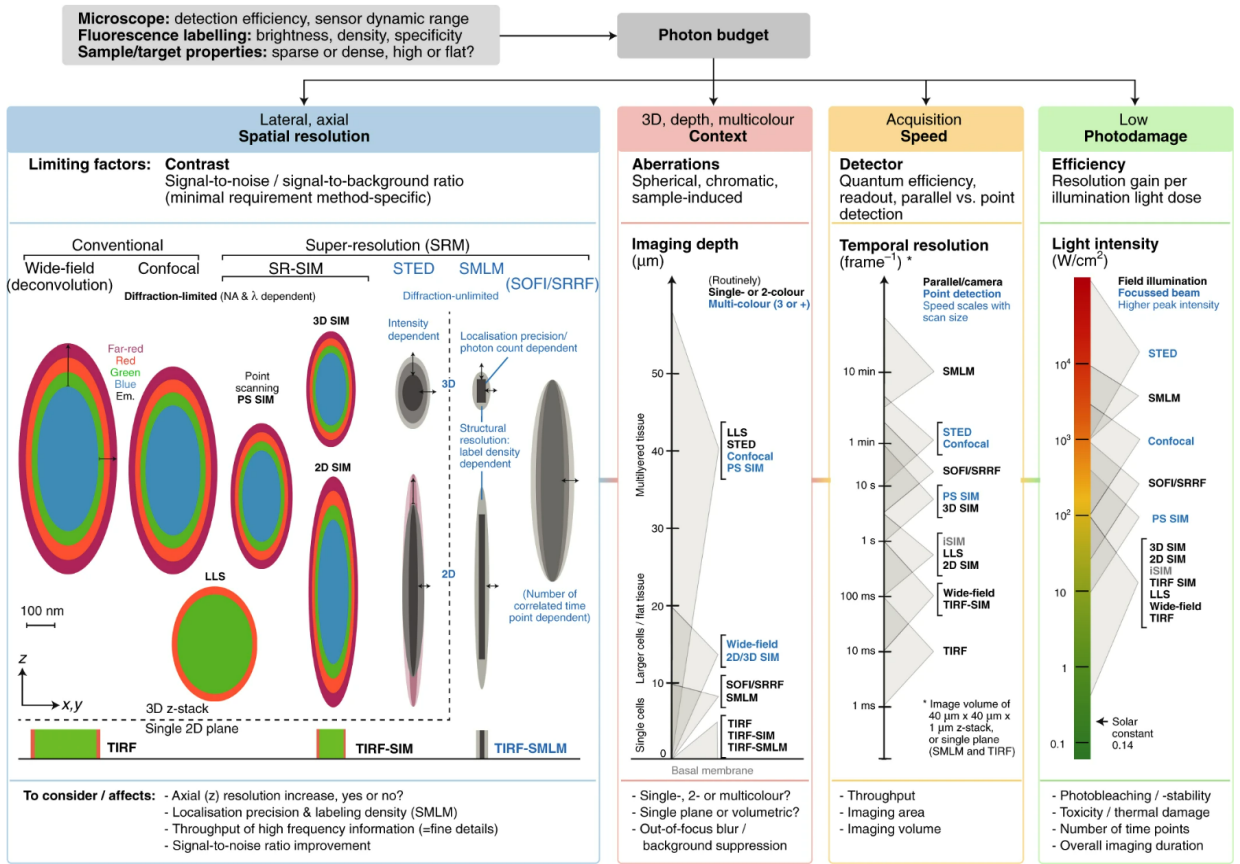
Super-Resolution Microscopy



REVIEW ARTICLE | FOCUS
https://doi.org/10.1038/s41556-018-0251-8
nature cell biology

Super-resolution microscopy demystified

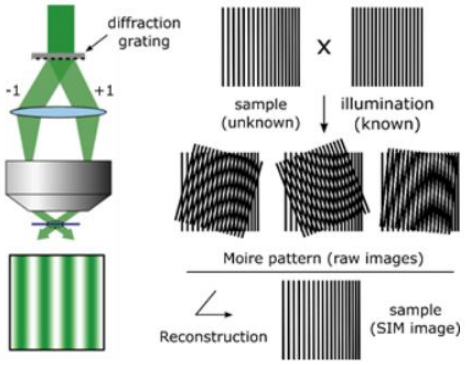
Lothar Schermelleh[1*], Alexia Ferrand[2], Thomas Huser[3], Christian Eggeling[4,5], Markus Sauer[6], Oliver Biehlmaier[2] and Gregor P. C. Drummen[7,8*]



Super-Resolution Microscopy

Structural Illuminated Microscopy (SIM)

- relies on **movable diffraction grating** has been inserted into the excitation path
- *Under **homogeneous illumination**, the PSF of the objective means that objects separated by a small distance, or in other words, being organized at high frequency, are not visible. But, under **structured illumination**, the overlap between the high-frequency organization of the objects within the sample and the high frequency of the illumination stripes creates a pattern of lower frequency, which is well collected by the objective.*
- To **reconstruct the final super-resolved image** (SR-image), several raw images must be collected, each acquired at a different orientation of the structured illumination.
- *2D-SIM SR-image, 9 raw images are required (3 translations x 3 rotations)*

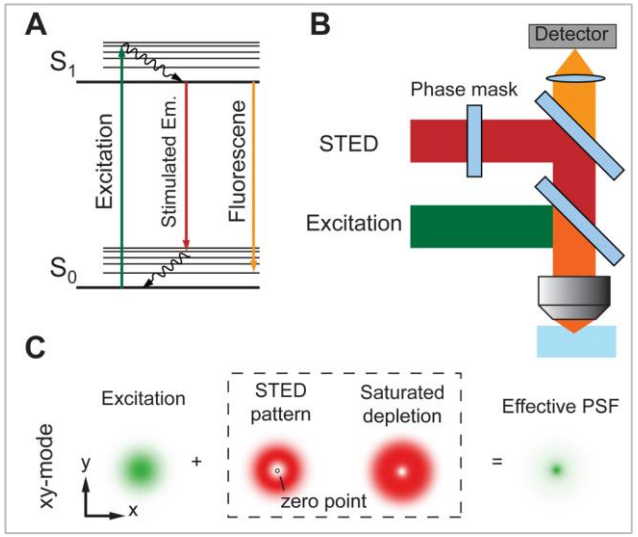


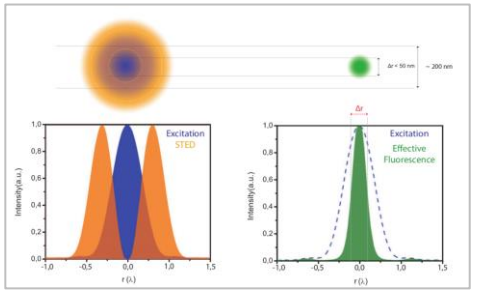
✓ **2x increase in spatial resolution** (~100 nm in xy)
✓ **fast acquisition** in comparison to STED, PALM/STORM
✓ labeling using **conventional fluorophores**
✓ up to **3 simultaneous color imaging** (other only 2)

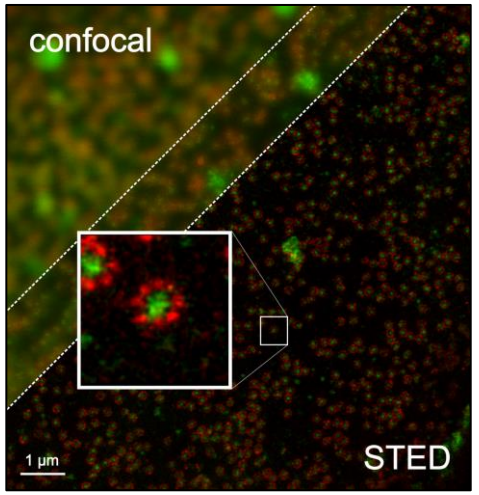
***X* lateral spatial resolution** is not as good as other super-resolution techniques
***X* risk of artifacts** during image reconstruction
***X* sensitive to out-of-focus light** and so difficult on thick or too densely labeled samples

Stimulated emission depletion (STED) microscopy

- creates super-resolution images by the **selective deactivation of fluorophores**, minimizing the area of illumination at the focal point, and thus enhancing the achievable resolution for a given system
- double laser design allows for **excitation** and **stimulated emission** (depletion laser) to be used together for STED







✓ lateral resolution is typically **between 30 and 80 nm**

***X* 2 simultaneous color imaging**
***X* specific fluorophores**

Super-Resolution Microscopy

Photo-activated localization microscopy (PALM)

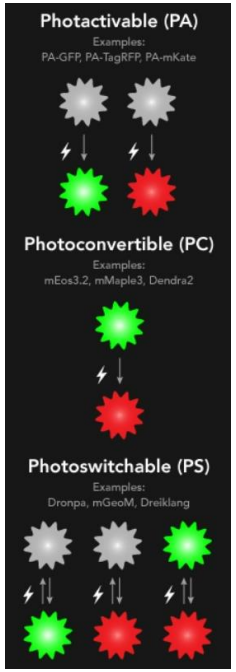
- uses **photoactivatable fluorophores** to resolve spatial details of tightly packed molecules. Once activated by lasers, fluorophores emit for a short period but eventually bleach. The laser stochastically activates fluorophores until all have emitted.

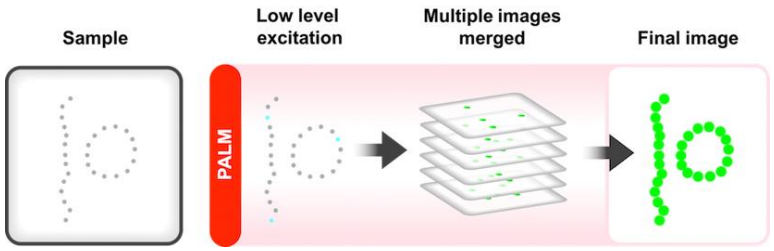
Three commonly used types of fluorophores are:

Photoactivatable Fluorophores: PAmCherry, PA-GFP, which emit light upon activation with UV.

Photoconvertible Fluorophores: Fluorescent proteins that change their emission spectrum upon activation with UV light (e.g., mEOS proteins).

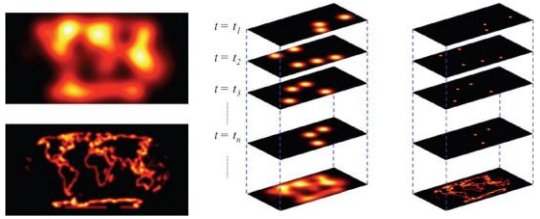
Photoswitchable Fluorophores: Typically chemical dyes (e.g. Alexa Fluor 647, DyLight555) which can switch between dark, non-fluorescent and bright, fluorescent states repeatedly.





✓ **resolution reaching almost 20 nm**
✓ using photoactivatable or photoconvertible proteins enables **the counting of molecules**, for example, in complexes and clusters

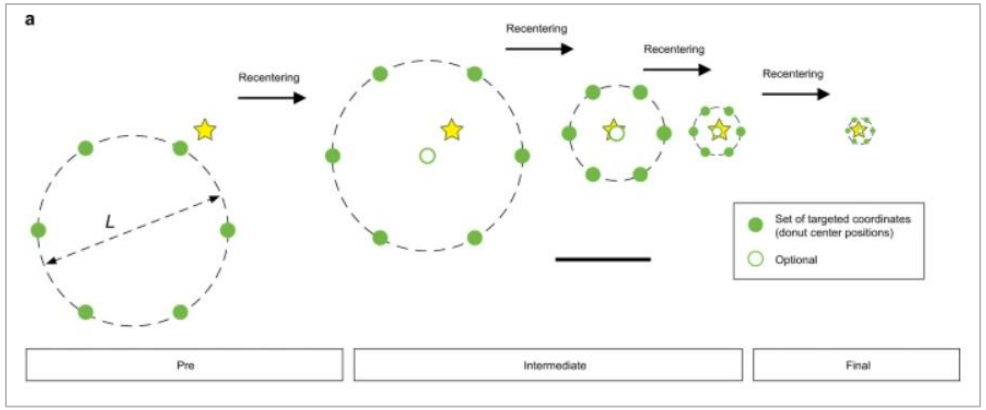
X slow (PALM requires 1000 (or more) raw images)

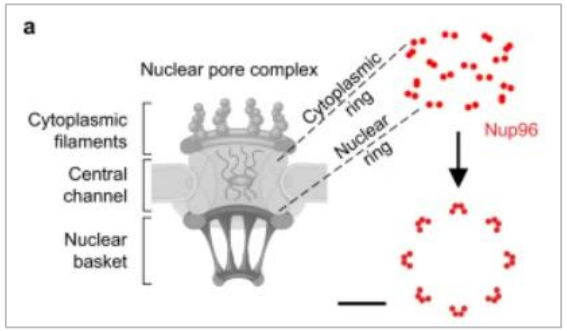


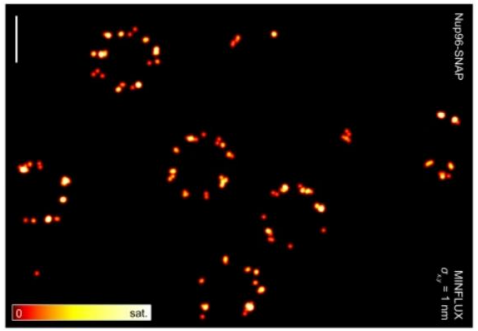
MINFLUX

- combines aspects of **single-molecule localization microscopy** (SMLM, PALM) and **STED microscopy** that can achieve far higher spatial and temporal resolution, while requiring far fewer fluorescence photons
- can attain **1–3 nm resolution** in three dimensions

A donut-shaped scanning beam triangulates the emitters one at a time



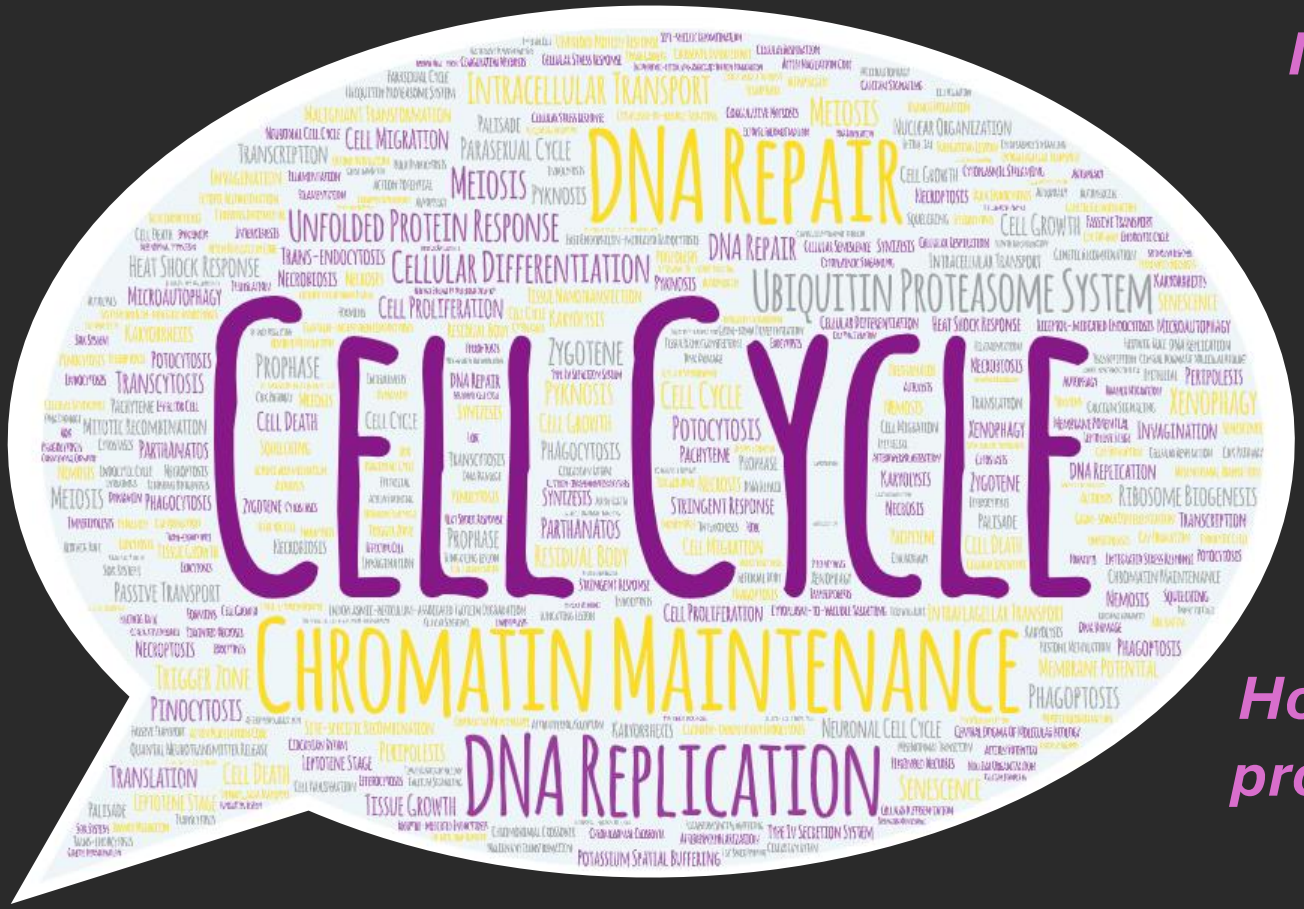




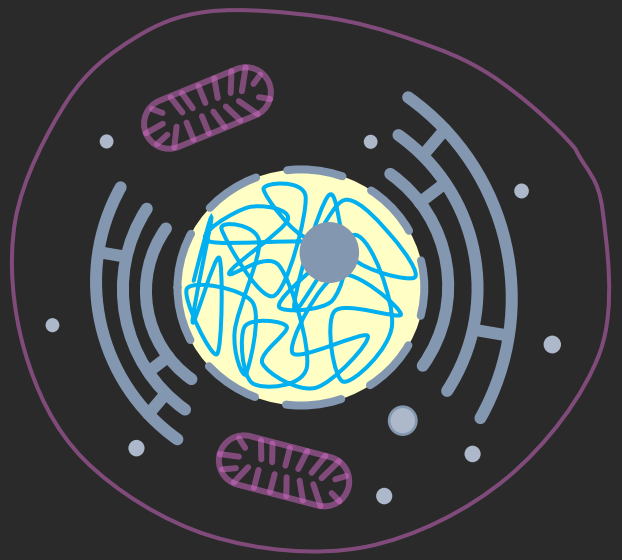
Schmidt et al, Nature Communications, 2021

Fixed Cells, cells, cells… How to *capture* cellular processes inside cells?

In real time?



How can we study protein dynamics?



eukaryotic cell

Proteins *serve as the primary driving force behind nearly all cellular processes, and their proper functioning is essential for the health and fitness of living organisms.*

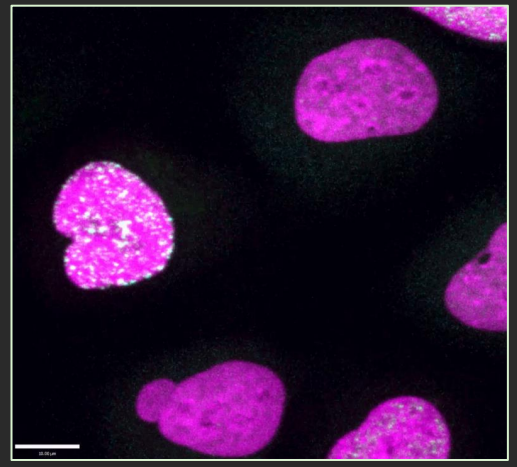
Exploring ***protein function*** *represents a fundamental approach to unveiling the molecular nature of vital cellular processes and their related changes underlying disease development.*

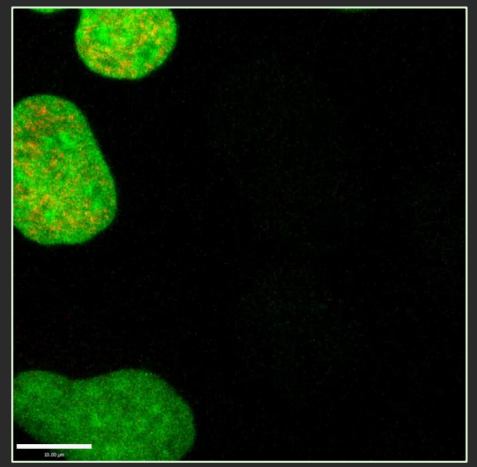
Yadav A.K. et al, bioRxiv, 2024

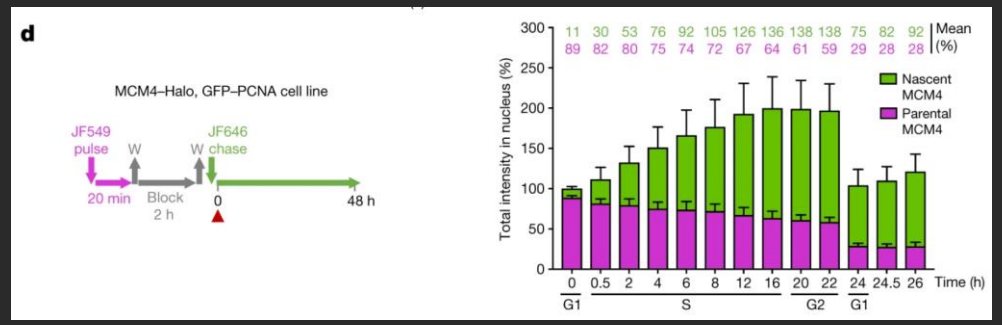
Studying protein dynamics

Live cell imaging

- capturing living cells by **time-lapse microscopy**
- **Types of live cell Imaging:**
  - Phase contrast microscopy
  - DIC microscopy
  - Fluorescence microscopy (widefield, confocal – spinning disk)

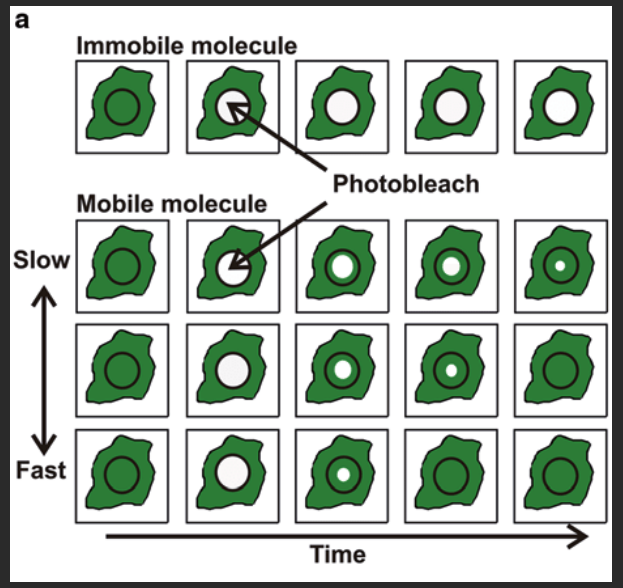




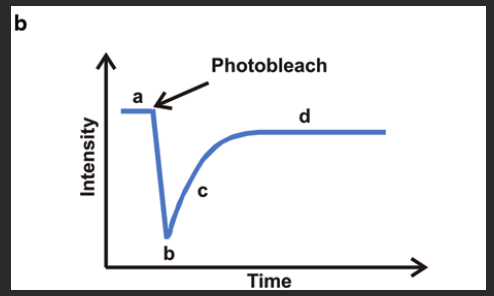


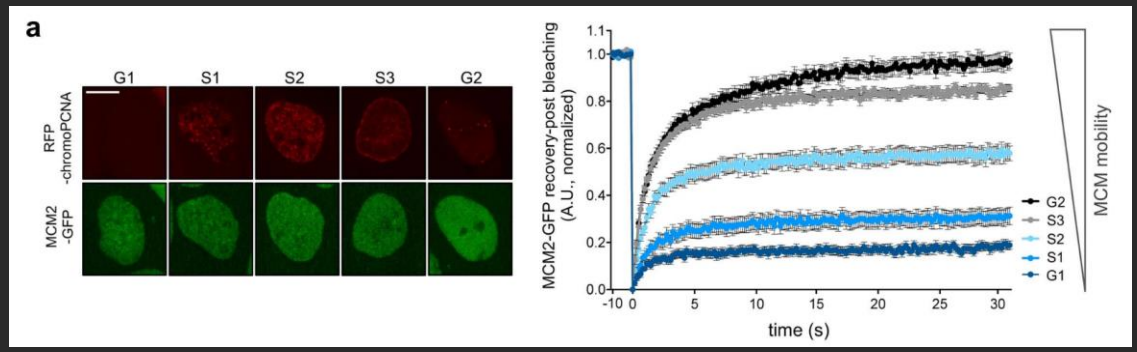
Sedlackova et al, Nature, 2020

Fluorescent Recovery After Photobleaching (FRAP)



- is a method for determining the **kinetics of diffusion** through tissue or cells
- to investigate the 3-dimensional diffusion and binding of molecules inside the cell



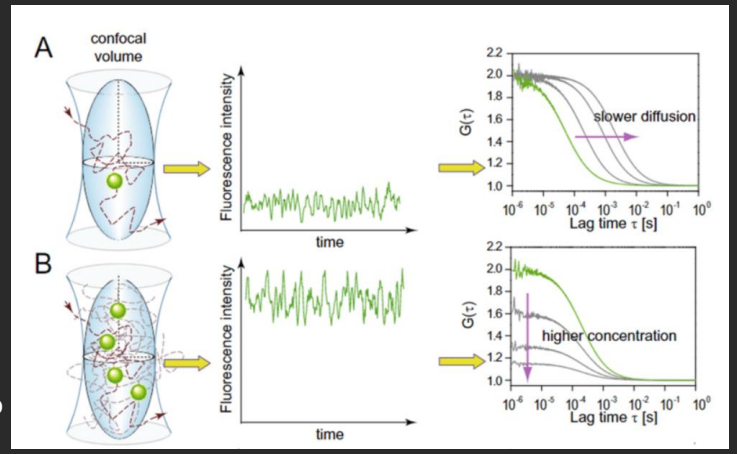


Sedlackova et al, Nature, 2020

Studying protein dynamics

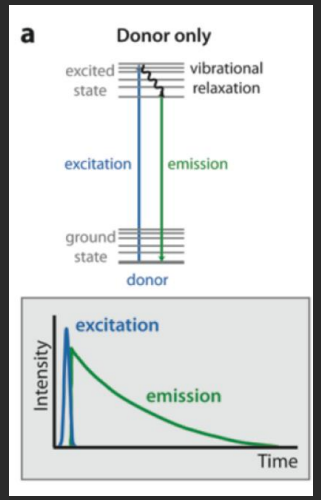
Fluorescence correlation spectroscopy

- FCS measures **fluctuations in fluorescence intensity** coming from any physical, chemical, or biological effects on the fluorophore of interest.
- **Fluorescence cross-correlation spectroscopy (FCCS)** correlates signals from two different fluorophores, detected in two separate channels.



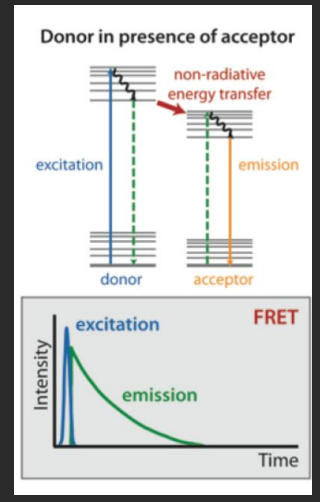
Fluorescence lifetime imaging microscopy (FLIM)

- is an imaging technique where the **fluorescence lifetime variation** across the sample creates the contrast in the image.
- used in biomedical imaging, where tissue and cells are stained with one or more fluorescent dyes.
- **fluorescence lifetime of the dye** depends on the local micro-environment, and FLIM provides an additional dimension of environmental information over other imaging techniques, such as widefield fluorescence.



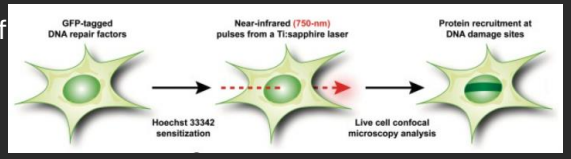
Förster resonance energy transfer (FRET)

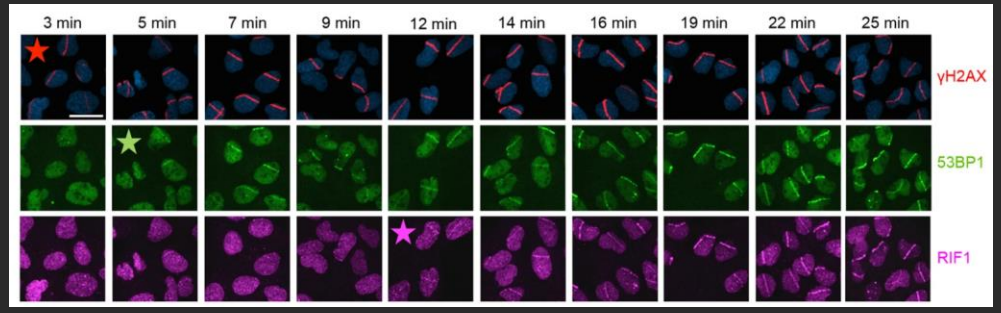
- is a mechanism describing **energy transfer between two light-sensitive molecules**
- One common pair of fluorophores for biological use is a **cyan fluorescent protein (CFP) – yellow fluorescent protein (YFP)** pair.
- **Applications:**
  - **Proteins:** protein-protein interactions, protein conformation, including secondary structures and protein folding
  - **Chemosensor:** changes in the cellular environment due to such factors as pH, hypoxia, or mitochondrial membrane potential
  - **Signaling pathways, polymer folding dynamics**, etc.



Laser Microirradiation and the DNA Damage Response

- analyzes protein recruitment dynamics of fluorescently tagged proteins to laser-induced DNA damage sites using confocal fluorescence microscope



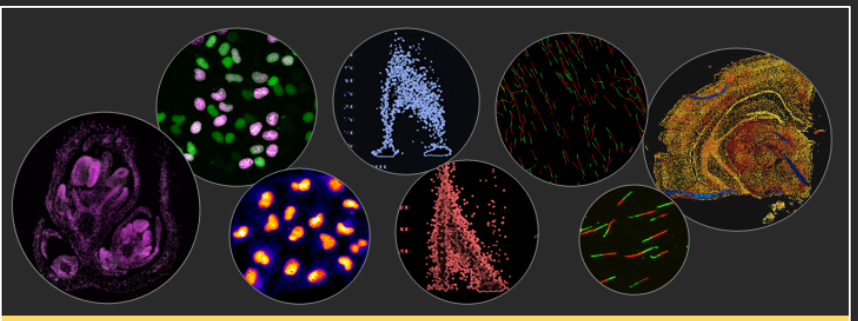


Ochs et al, Nature, 2019


That's all Folks!

Want to know more?

Want to know more?



ScanR workshop:

Pushing the boundaries of high-content imaging

22.-23. August 2024

Institute of Biophysics, Brno, Czechia

CONFIRMED SPEAKERS

Vaclav Bacovsky
Institute of Biophysics, CZ
Kamila Burdova
Institute of Molecular Genetics, CZ
Jana Dobrovolna
Institute of Molecular Genetics, CZ
Panos Galanos
Danish Cancer Institute, DK
Pavel Moudry
Palacky University, CZ

Hana Polasek-Sedlackova
Institute of Biophysics, CZ
Kumar Somyajit
University of Southern Denmark, DK
Luis Toledo
Biorigin & Nodus Oncology, DK
Manoel Veiga
Olympus/Evident, DE

HANDS-ON SESSIONS

SpinSR system: from high-content imaging to super-resolution
led by Tomas Pop and Hana Polasek-Sedlackova
When CellSens meets ScanR
led by Tomas Jendrulek and Vaclav Bacovsky
Beyond image analysis limits with TruAI deep learning module
led by Manoel Veiga
Tips & Tricks on how to present high content imaging data
led by Kumar Somyajit

Registration & more info:

IBP EMBO EVIDENT SVEN BIOLABS S.R.O.

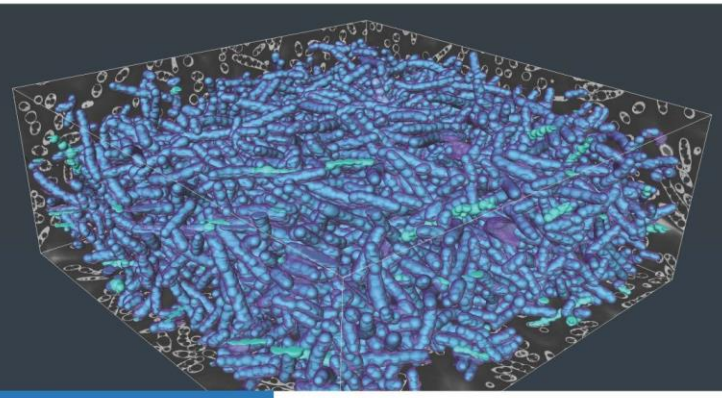
CZECH BIOIMAGING · ISI CAS Institute of Scientific Instruments, The Czech Academy of Sciences · FACULTY OF SCIENCE Charles University · EURO-BIOIMAGING INTERIM WEB ACCESS PORTAL

ADVANCED METHODS OF SCANNING ELECTRON MICROSCOPY AND RAMAN SPECTROSCOPY

2-4 DECEMBER
START FROM 9:00

INSTITUTE OF SCIENTIFIC INSTRUMENTS OF THE CAS
KRÁLOVOPOLSKÁ 147, BRNO, CZECH REPUBLIC

Learn principles of Electron microscopy and Raman spectroscopy in three days and try operating microscopes and preparing biological specimens. The course is aimed at university students and young scientists in the life sciences and soft materials who are planning to use advanced imaging and analytical methods in their research.



REGISTRATION

2.12. 9:00 - 17:00 **FREE lectures**
Hands-on in labs 1000 CZK
3.12. 9:00 - 17:00
4.12. 9:00 - 16:00

GUEST EXPERTS

- JANA NEBESÁŘOVÁ
- MARIE VANČOVÁ
- MARTIN BAČÍK

Attend the entire course and receive:

- **Certificate**
- **Voucher** for the vernisage on 4.12. 17:00 (Mahenova Knihovna , Kobližná 4, Brno) exhibition „**mikrosvět obrazEM**"

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120 seconds: Meet the Brnoregion Microscopy

Brno a elektronová mikroskopie? Jedno bez druhého si nejde představit. V #brnoregion existuje celá řada firem, výzkumných institucí a univerzit, které se zabývají přímo elektronovými mikroskopy, další pracují s jejich výsledky. Jste mezi nimi? Přijďte na akci 120 vteřin, získejte nové kontakty, inspiraci a představte svou práci partnerům z mikroskopické platformy.

Co můžete očekávat?

- Co je nového v brněnské mikroskopii a jaké akce nás čekají?
- **20 krátkých prezentací** zajímavých projektů souvisejících s mikroskopií, po kterých bude následovat networking.
- **Příležitost představit** svou práci těm, kteří pracují na podobných projektech.
- **Možnost nabídnout** svá jedinečná řešení, jako jsou přístroje, software a výjimečné vybavení, a navrhnout spolupráci.
- Příležitost **požádat o spolupráci, pomoc** nebo potřebné vybavení či řešení **dodavatele**.
- **Seznámíte se** s rozvojem mikroskopie a vědy v **#brnoregion** a zapojíte se do něj.

Program

- 15.00–15.15 | registrace
- 15.15–15.30 | přivítání a novinky z mikroskopie v #brnoregion
- 15.30–16.00 | **10 krátkých prezentací** inspirativních mikroskopických projektů
- 16.00–16.15 | *přestávka na kávu*
- 16.15–16.45 | **10 krátkých prezentací** inspirativních mikroskopických projektů
- 16.45–16.50 | závěr
- 16.50–18.30 | navazování kontaktů

120 sec

References

Useful resources:

- ZEISS: Microscopy from the very beginning: https://www.its.caltech.edu/~bi177/private/Microscopy%20from%20the%20very%20beginning.pdf
- Carl Zeiss Online Campus: https://zeiss-campus.magnet.fsu.edu/
- Olympus/Evident Microscopy Resource Center: https://www.olympus-lifescience.com/en/microscope-resource/

Other used resources:

https://andor.oxinst.com/learning/view/article/most-popular-microscopy-techniques
https://en.wikipedia.org/wiki/Microscopy
https://www.atlantisbioscience.com/others/9-types-of-microscopy-and-how-to-select-the-right-scope/
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