



Analysis of γH2AX, 53BP1 and replication factories collocalization in A549 cell nuclei

**Microscopy Image Analysis
using Machine Learning based algorithms**

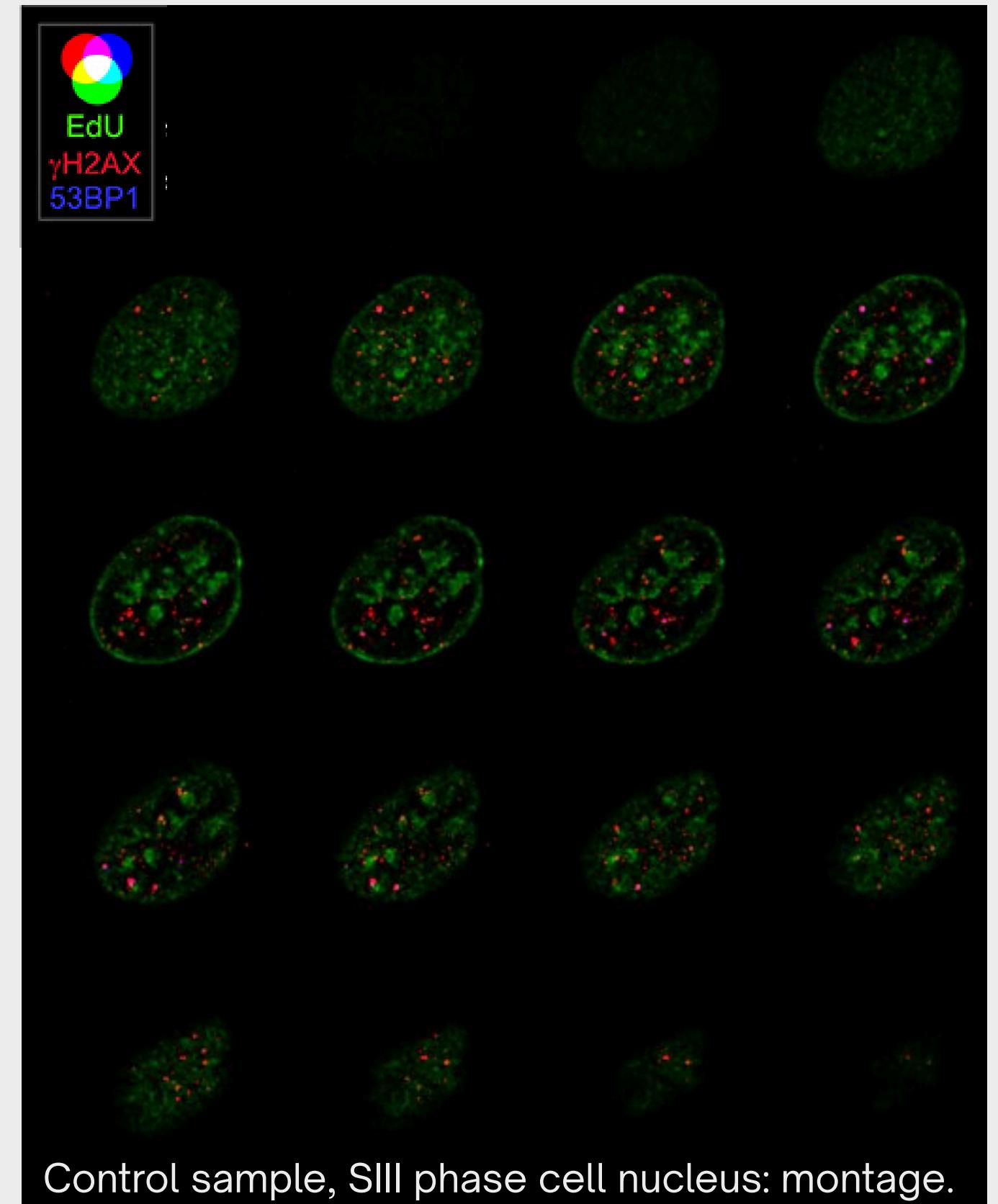
by Daryna Yakymenko

Objectives and Goals

Study of spatial patterns of colocalisation **histone H2AX phosphorylation sites & foci of repair protein 53BP1 recruitment**, caused by the appearance of DNA double-strand breaks (DSBs) at **replication sites** under the action of topoisomerase inhibitors:

- camptothecin (CPT),
- mitoxantrone (MTX),
- etoposide (ETP),

with the aim of extracting contributions on the number of DSBs they provoke at different stages of the G/S phases of the cell cycle, as well as the kinetics of the above-mentioned processes.



Control sample, SIII phase cell nucleus: montage.

Data: Rybak P. et al. Oncotarget (2016)

Data source:

www.impactjournals.com/oncotarget/

Oncotarget, Vol. 7, No. 31

Research Paper

Low level phosphorylation of histone H2AX on serine 139 (γ H2AX) is not associated with DNA double-strand breaks

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Objectives and Goals



Goal # 1

To segment individual foci of γH2AX, 53BP1 and replication factories



Goal # 2

To divide γH2AX foci into classes and isolate the class marking the DSB sites



Goal # 3

To calculate the number of colocated foci of bright-class γH2AX, 53BP1 & replication factories



!!! Data context

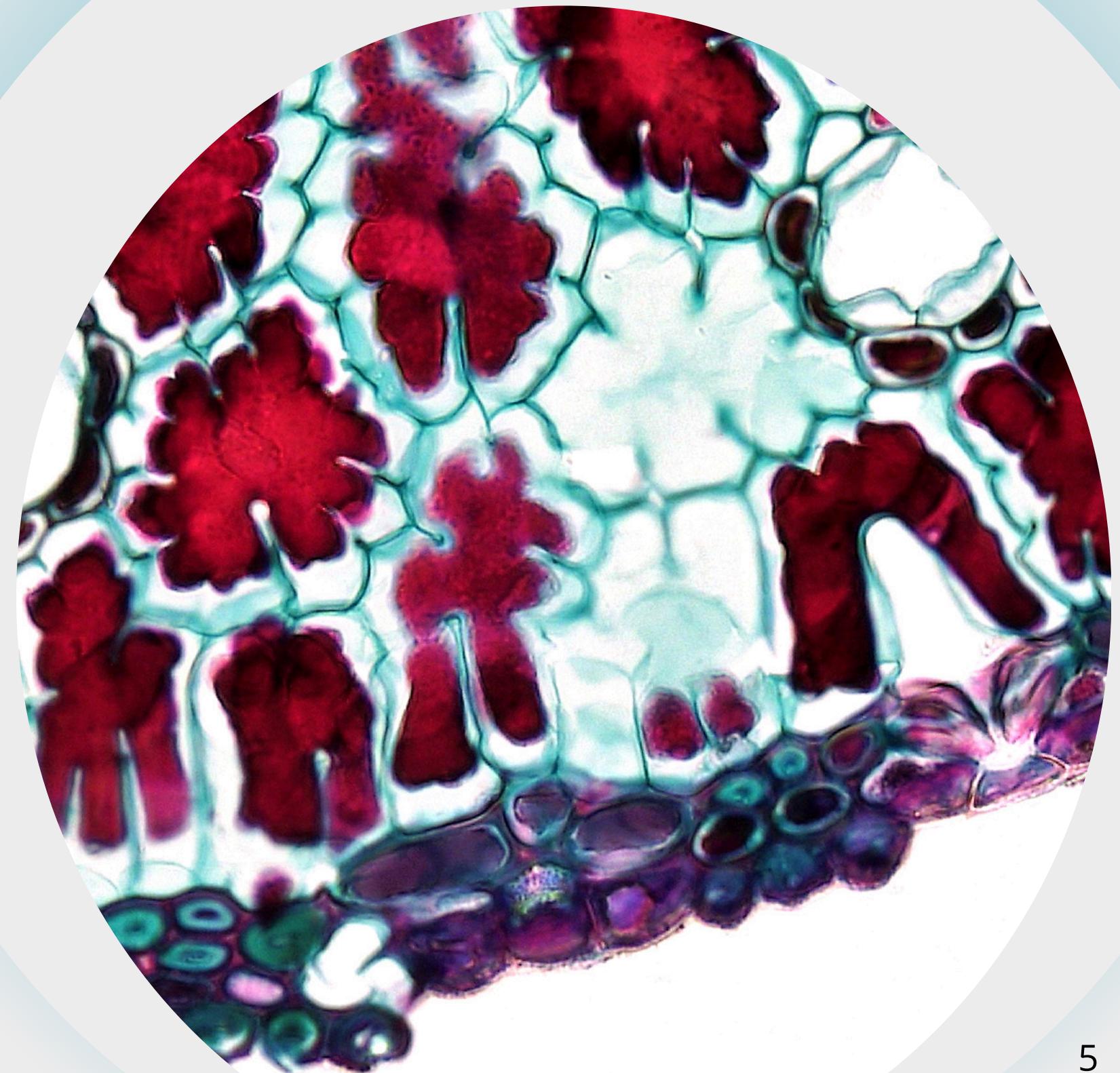
...

Data we are working with are not just symbols/numers etc.

They are containers for information about complicated biological systems and living organisms.

To be able to consciously analyse the data, one must be aware of:

- 1) the object of the experiments analysed
- 2) technique exploited to collect the data (including all its strengths and drawbacks)
- 3) broader context of the study



What will we be analysing?

- **Imaging data**

Collected by means of confocal fluorescence microscopy



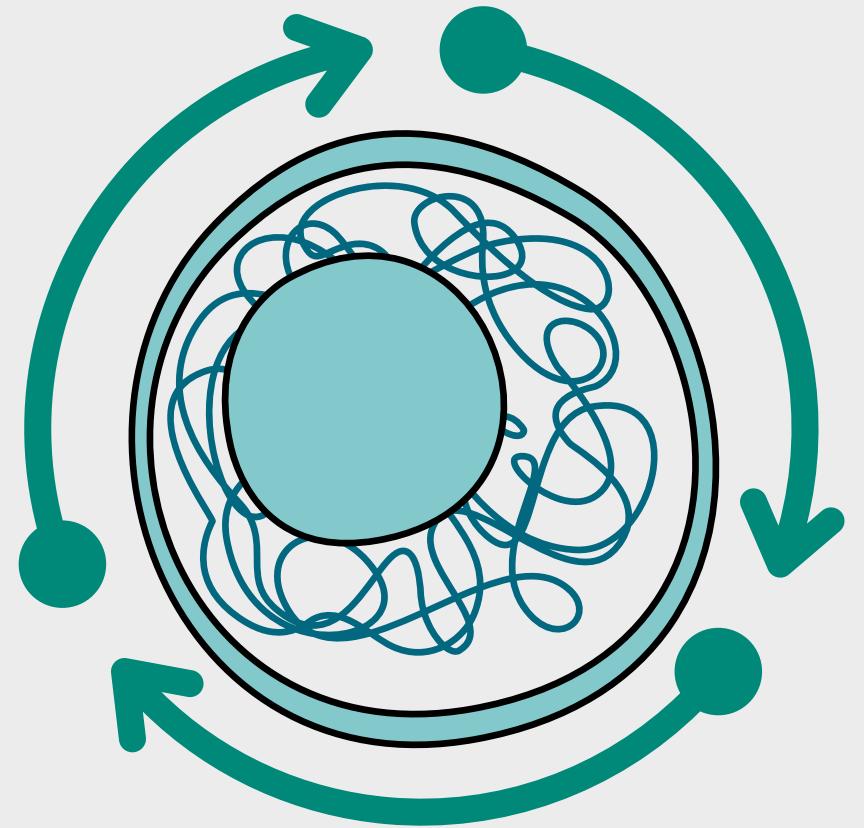
- **Biological process**

Reparation of double strand DNA breaks in nuclear DNA

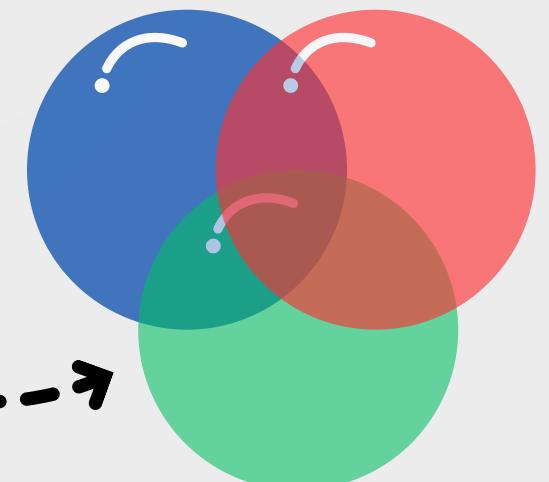


- **Spacial organization of reparation foci of few signals**

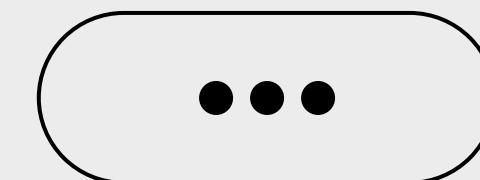
Immunostained 53BP1 protein, phosphorylated histone H2AX (gammaH2AX) and replication factories stained with EdU-Click



Nuclea of cells
in G/SI-V phases



Imaging Data: Confocal Fluorescent Microscopy



(1) Microscopy

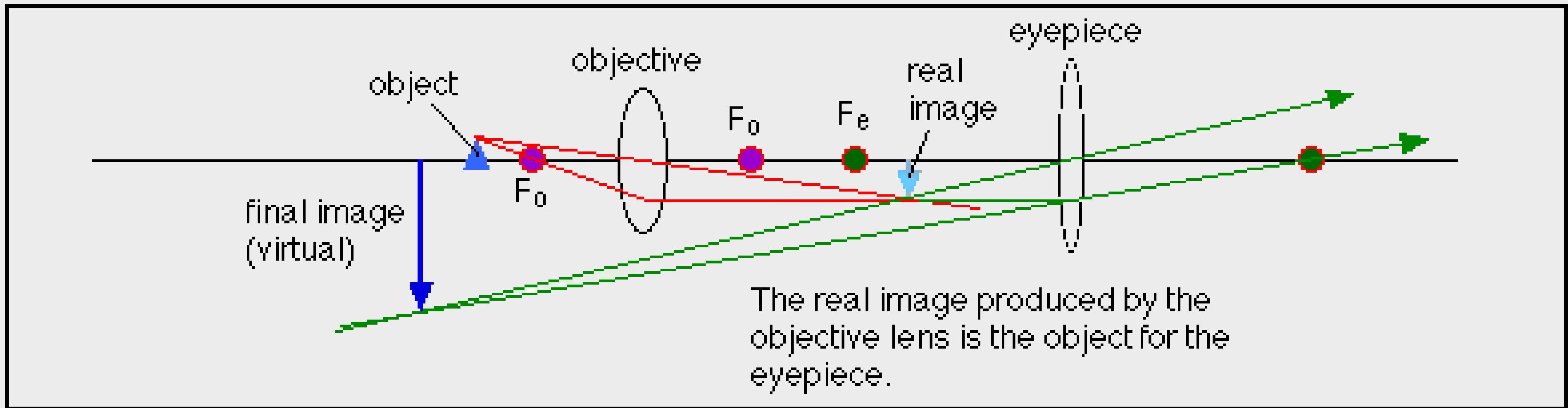


“Microscopy is the technical field of using microscopes to view subjects too small to be seen with the naked eye (objects that are not within the resolution range of the normal eye). There are three well-known branches of microscopy: **optical**, **electron**, and **scanning probe microscopy**, along with the emerging field of X-ray microscopy.

Optical microscopy and electron microscopy involve the **diffraction**, **reflection**, or **refraction** of electromagnetic radiation/electron beams interacting with the specimen, and the **collection of the scattered radiation** or another signal in order to create an image. (...)" © Wikipedia

(1) Microscopy

MICROSCOPE MAKES THINGS LOOK BIGGER



A modern optical microscope (usually fluorescent or confocal) is also used for complex measurements of phenomena occurring in living cells.

(1) Microscopy

The origin of the limited microscopic resolution and image contrast can be understood only by considering the wave nature of light.

Magnification: can be increased by adding magnification stages to a microscope.

Resolution: can't be improved beyond a fundamental limit. Refers to the level of detail that can be recognized in an image, such as small and intricate structures or the distance between closely placed small objects.

The Rayleigh criterion for optical resolution:

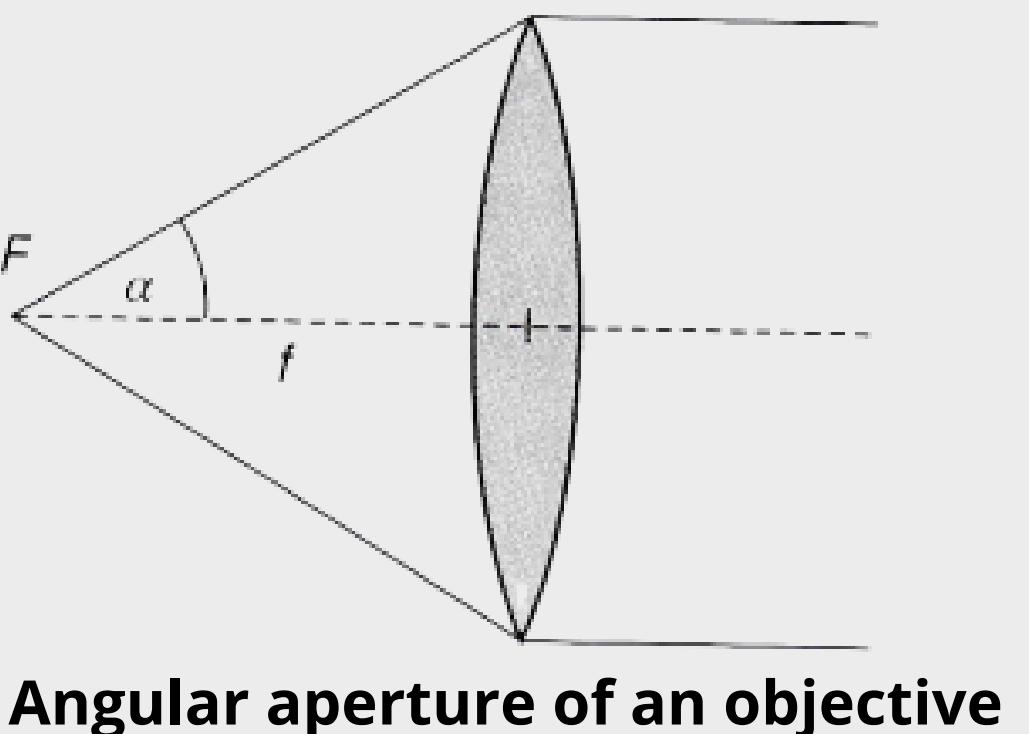
$$d_R = \frac{0.61\lambda}{n \sin \alpha}$$

λ - wavelength of light,

n - refractive index,

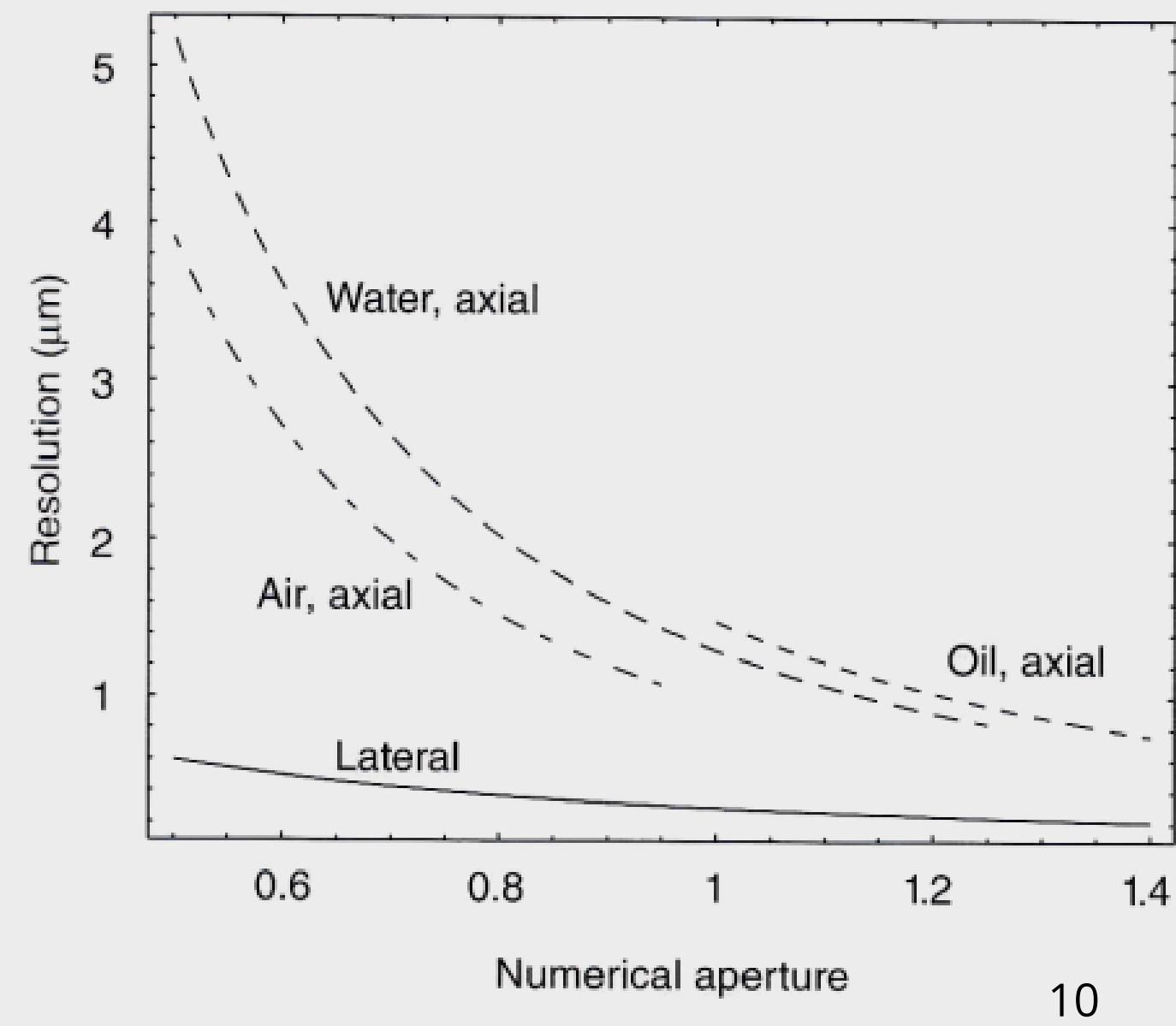
α - half-angle of aperture cone,

$n^* \sin \alpha$ - numerical aperture (NA)

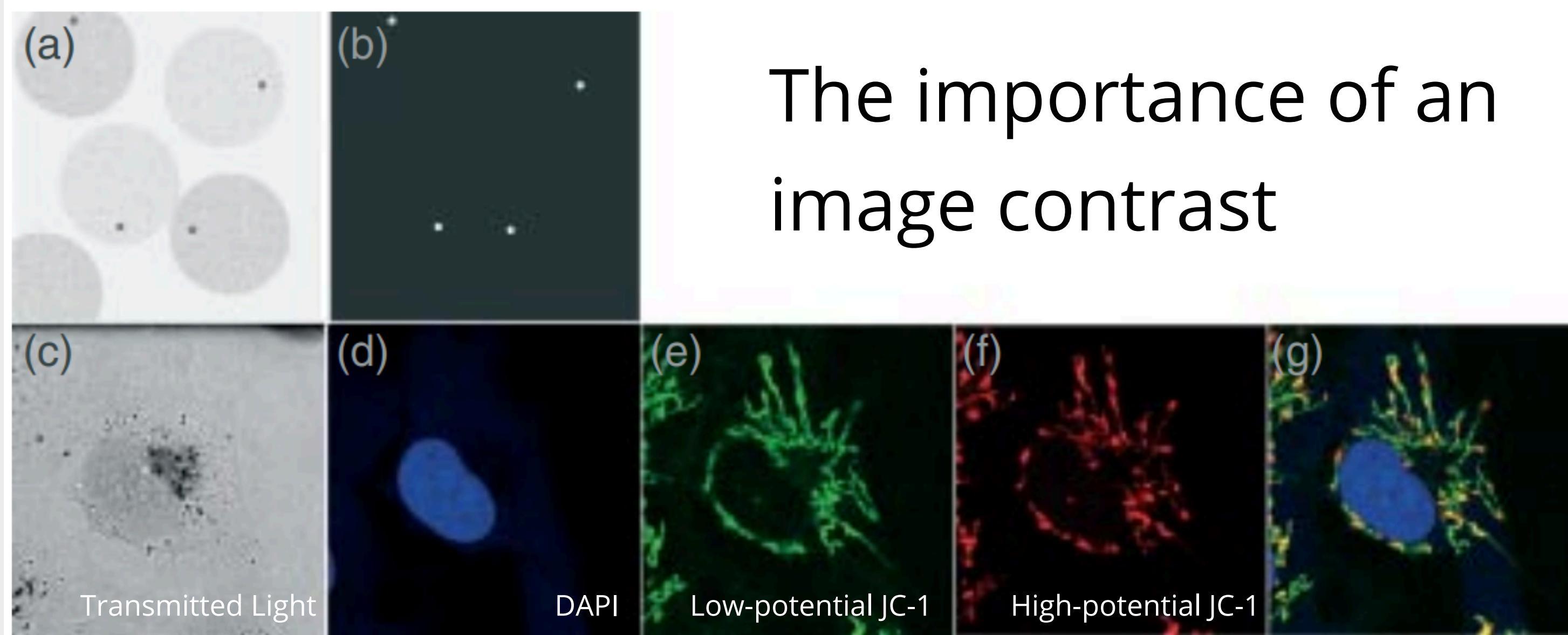


The numerical aperture describes the ability of a lens to gather light.

Axial and lateral resolution as a function of numerical aperture

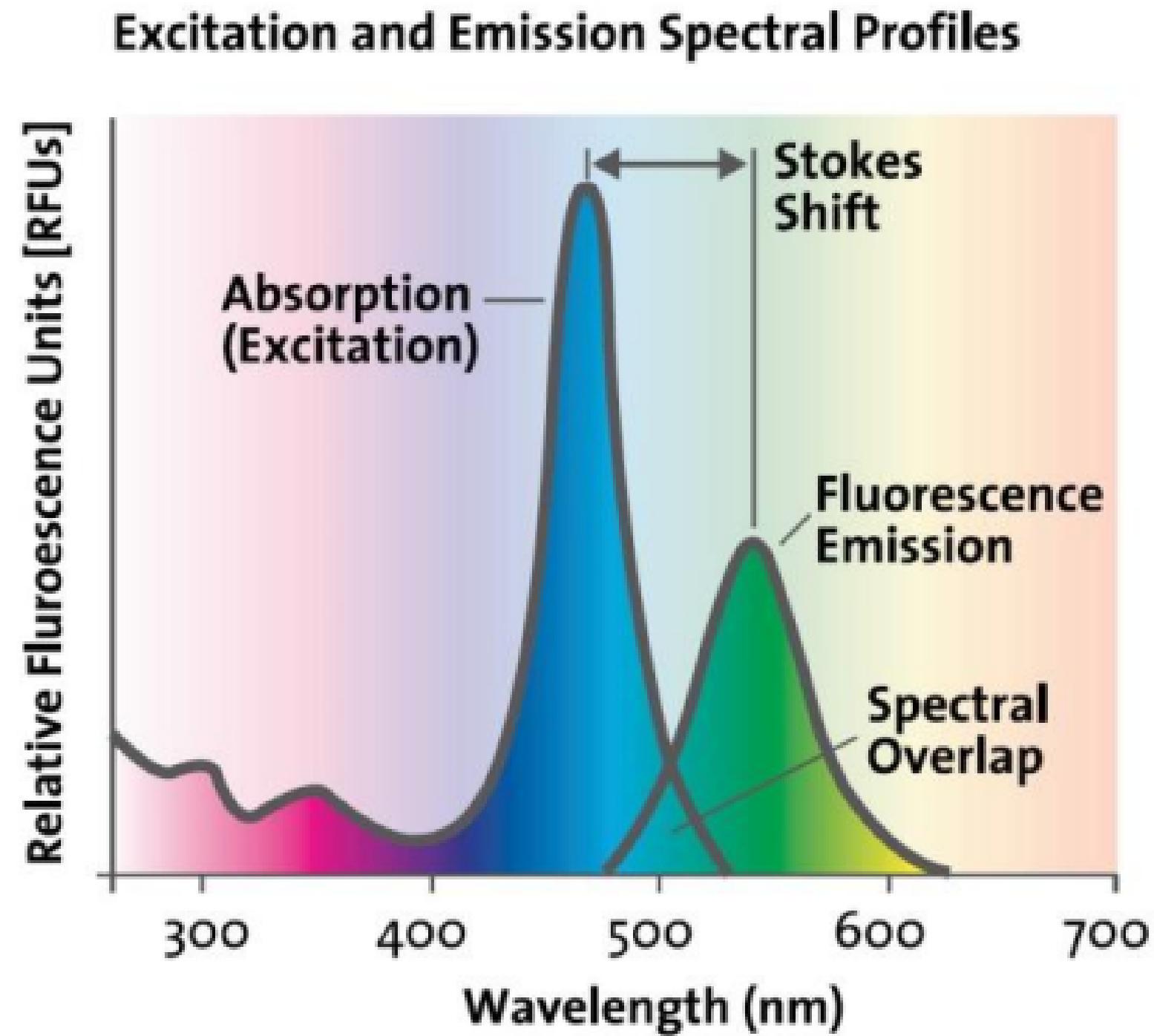
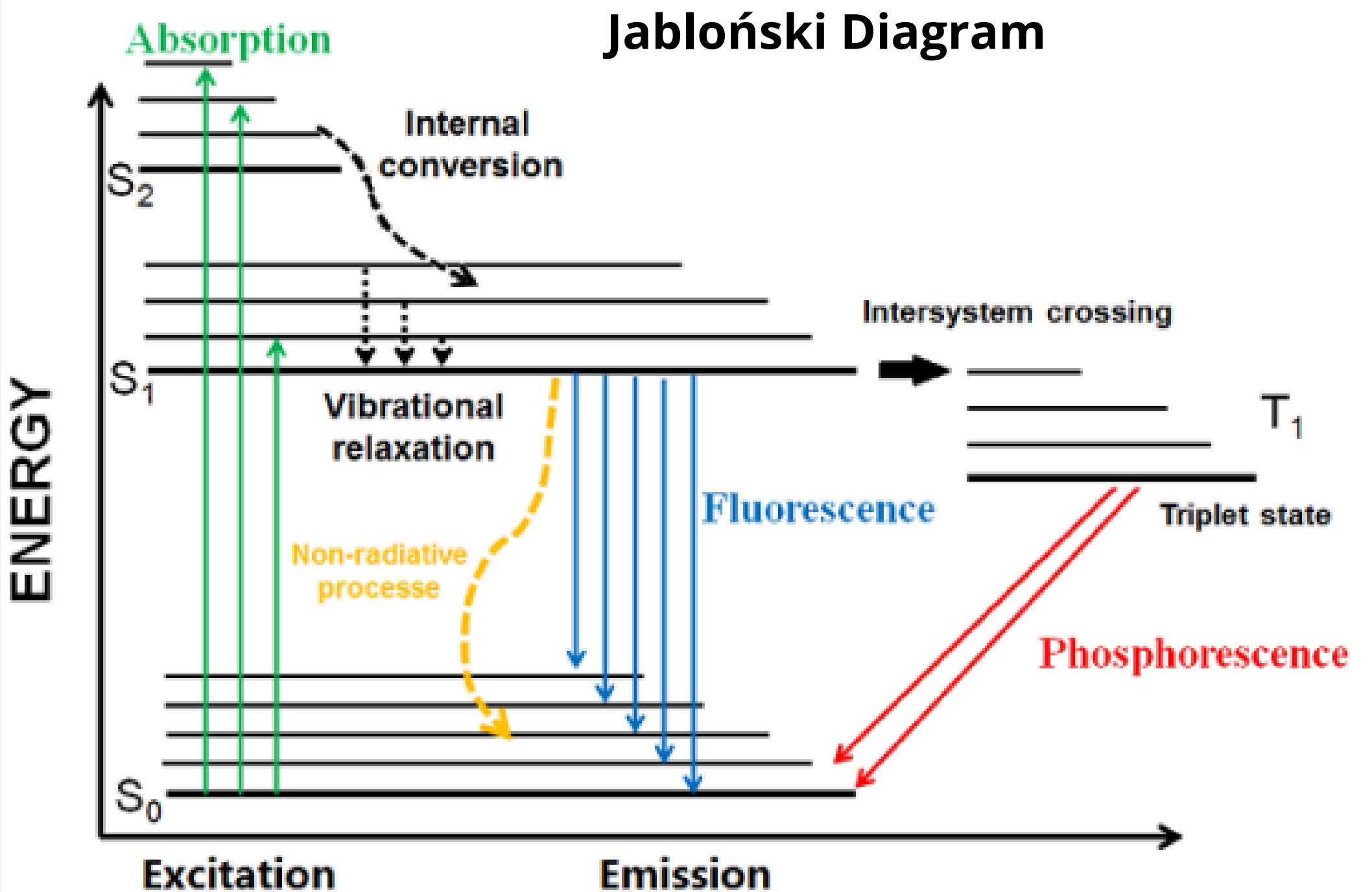


(2) Fluorescent Microscopy



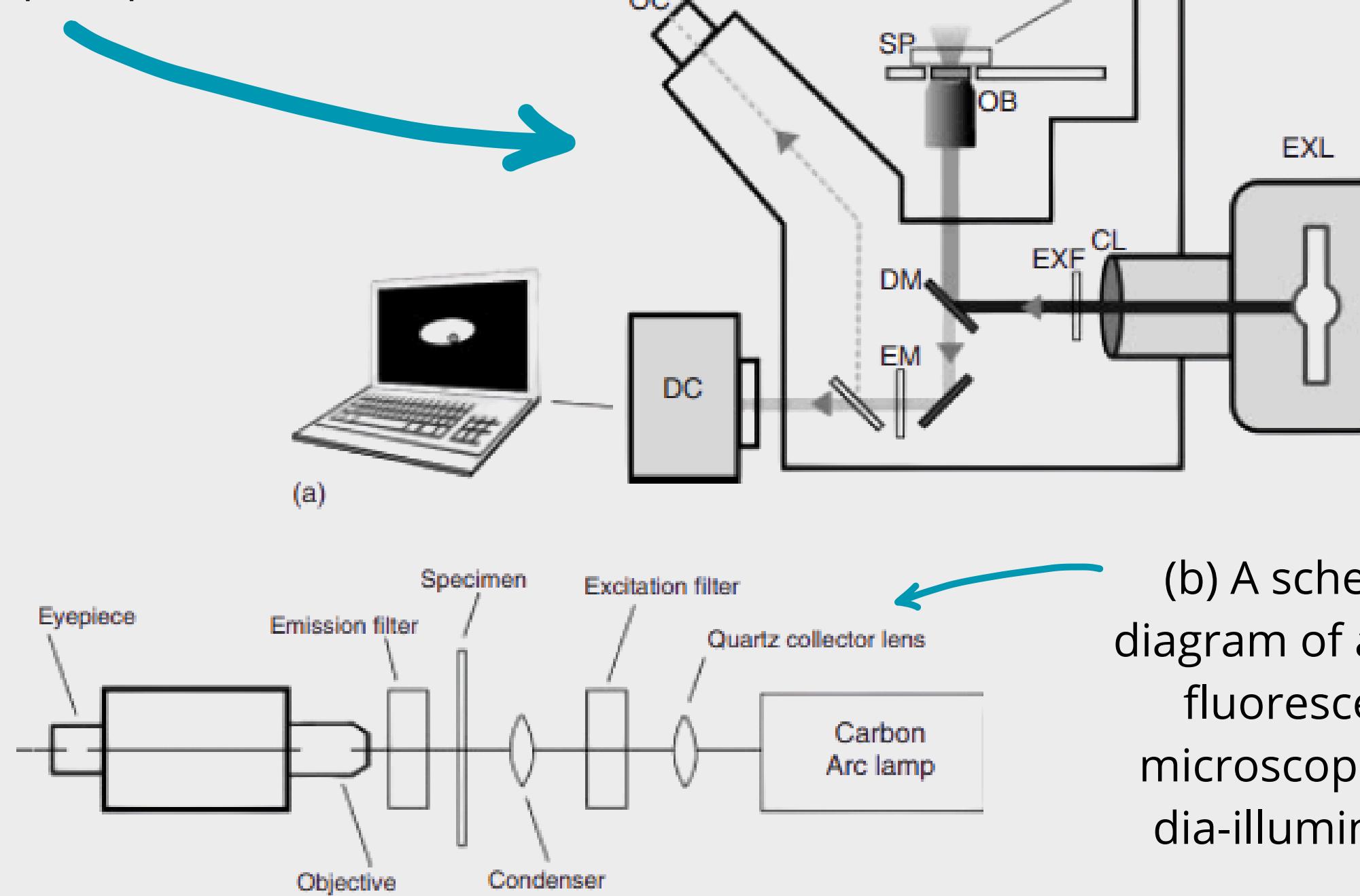
Fluorescence Microscopy: From Principles to Biological Applications, First Edition. Edited by Ulrich Kubitscheck. 2013 Wiley-VCH Verlag GmbH & Co. KGaA.
Published 2013 by Wiley-VCH Verlag GmbH & Co. KGaA. Chapter 3: Fluorescence Microscopy, Fig.3.1.

(2) Fluorescent Microscopy

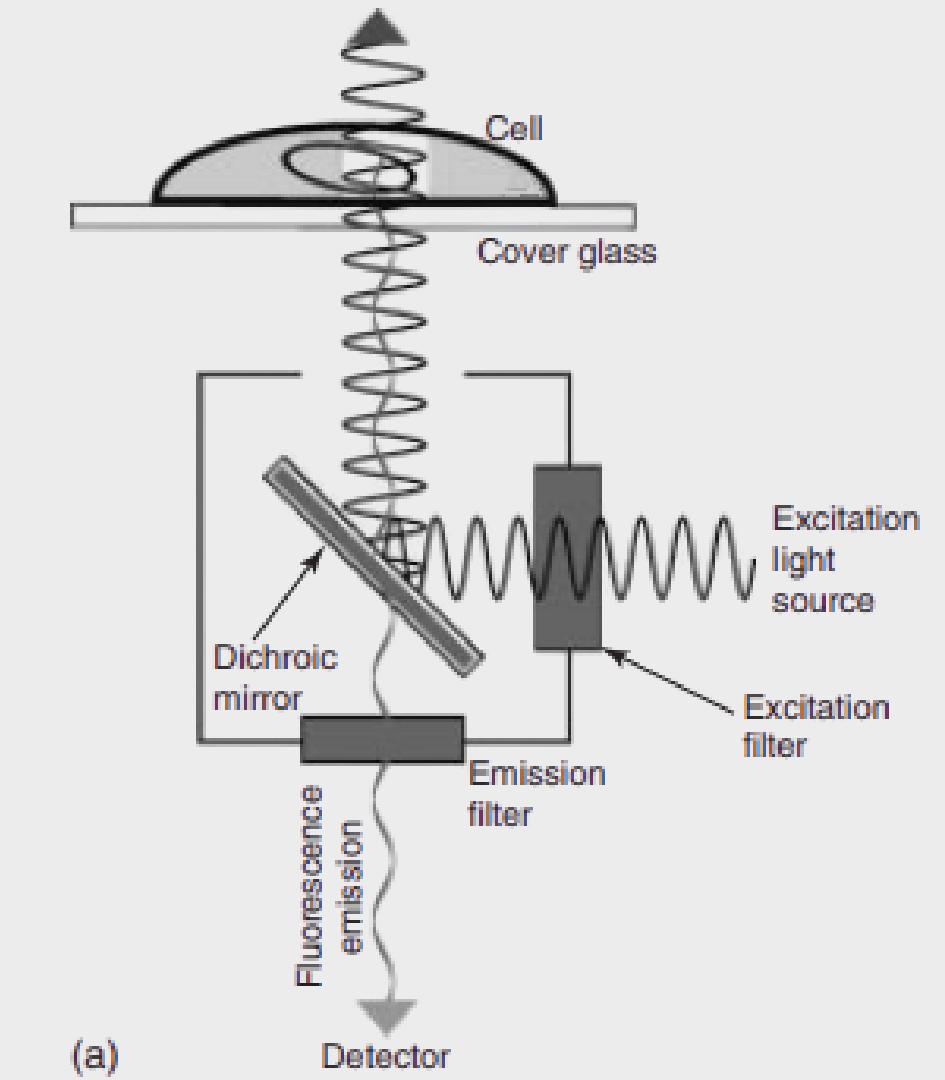


(2) Fluorescent Microscope: principle of operation

(a) A schematic diagram of an inverted fluorescence microscope (epi-fluorescence)



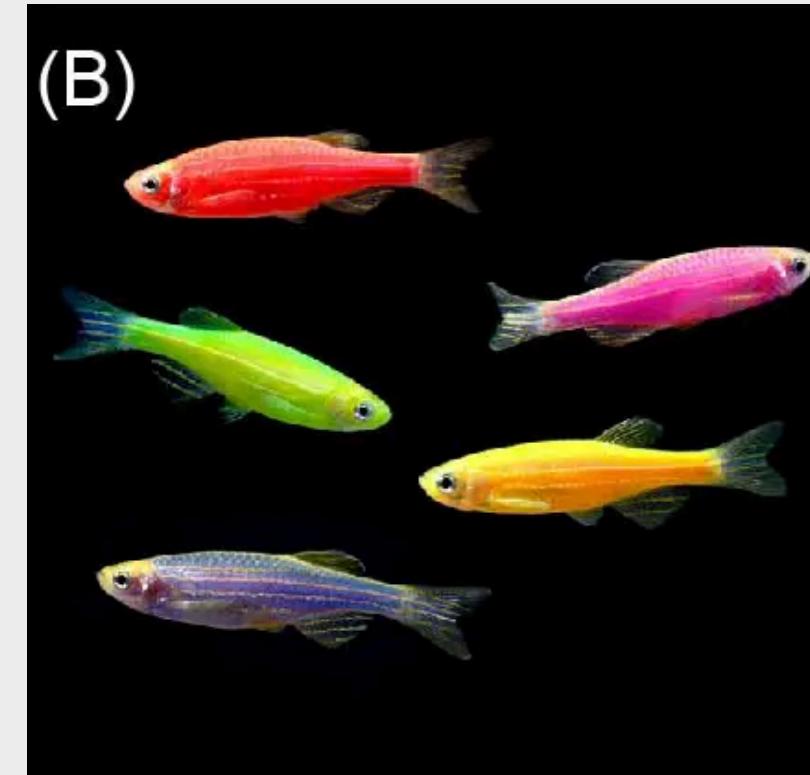
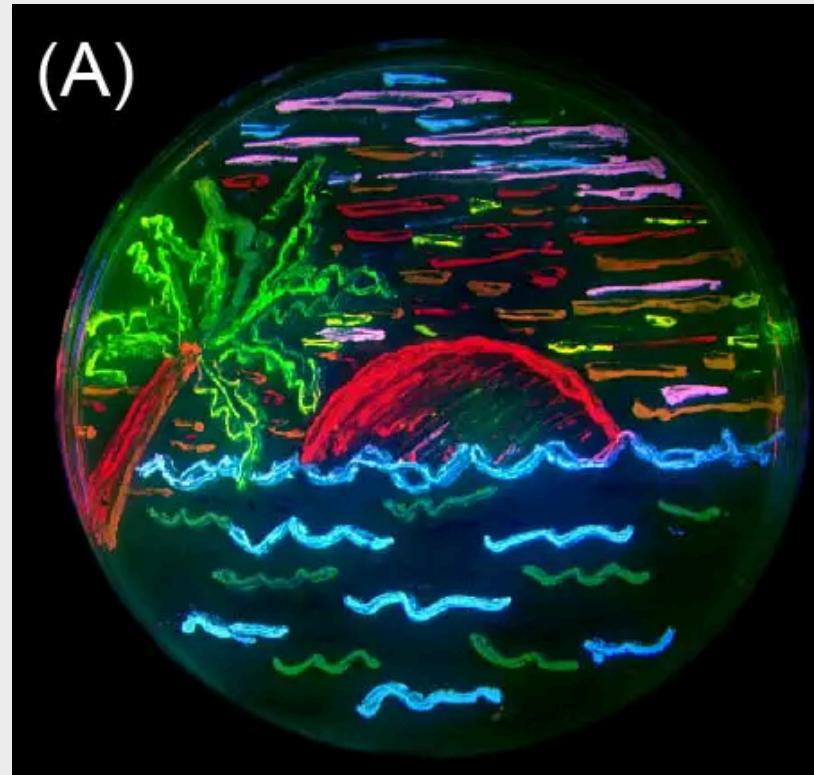
(b) A schematic diagram of an early fluorescence microscope, with dia-illumination



A fluorescence microscope filter block

(2) Fluorescent Microscopy

(A) A painting on a petri dish by using living bacteria expressing 8 different colors of fluorescent proteins (from the lab of Roger Tsien).



(B) GloFish, the first commercially available fluorescent pets created using biotechnology.

(C) Mice with a green fluorescent protein (GFP) inserted into their genomes for neurology studies.



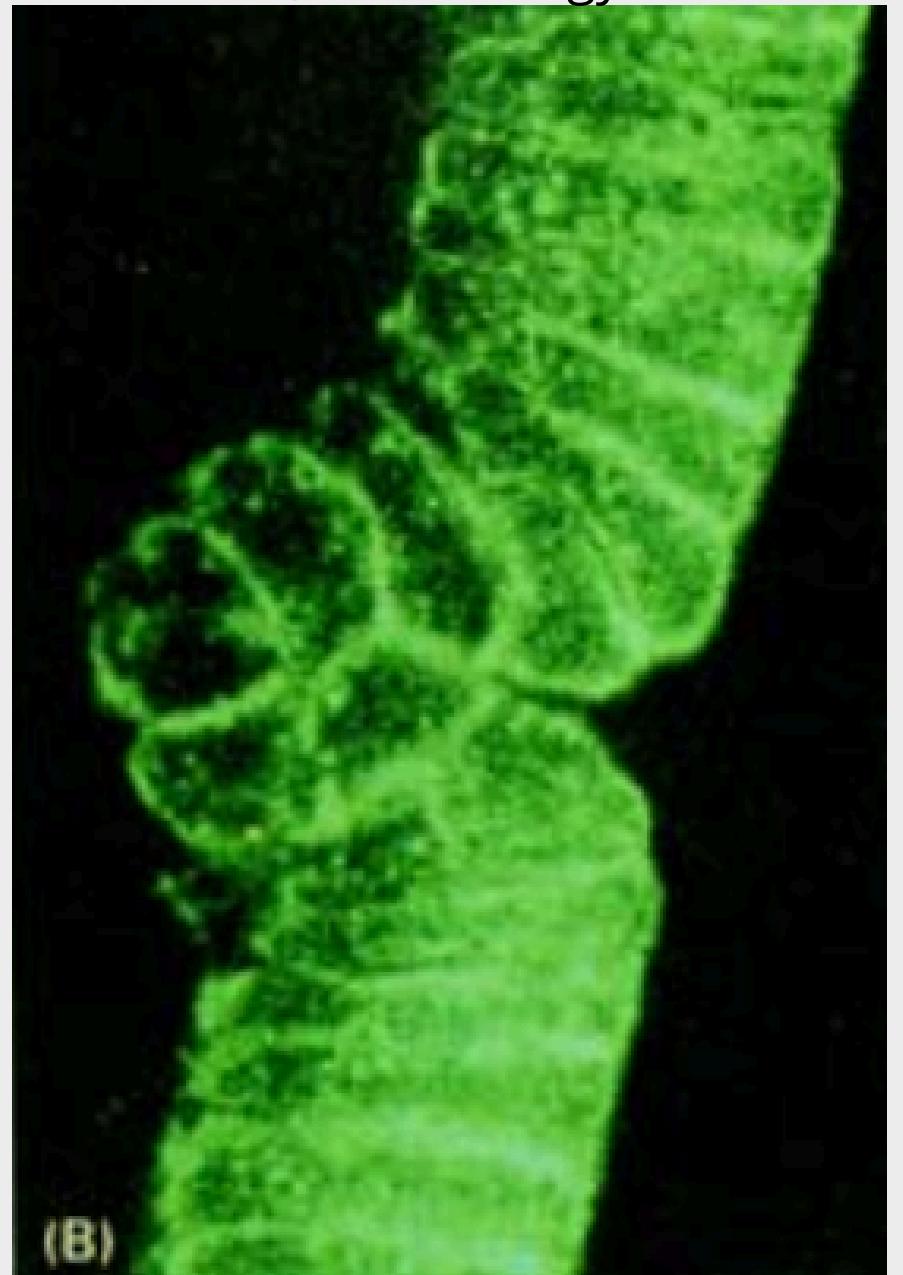
(3) Fluorescence Confocal Microscopy

Fluorescence confocal microscopy

- advantages:

- Specificity – detection of selected molecules
- Very high image contrast
- High image resolution
- Three-dimensional images

Alberts et al., Mol. Biology of the Cell

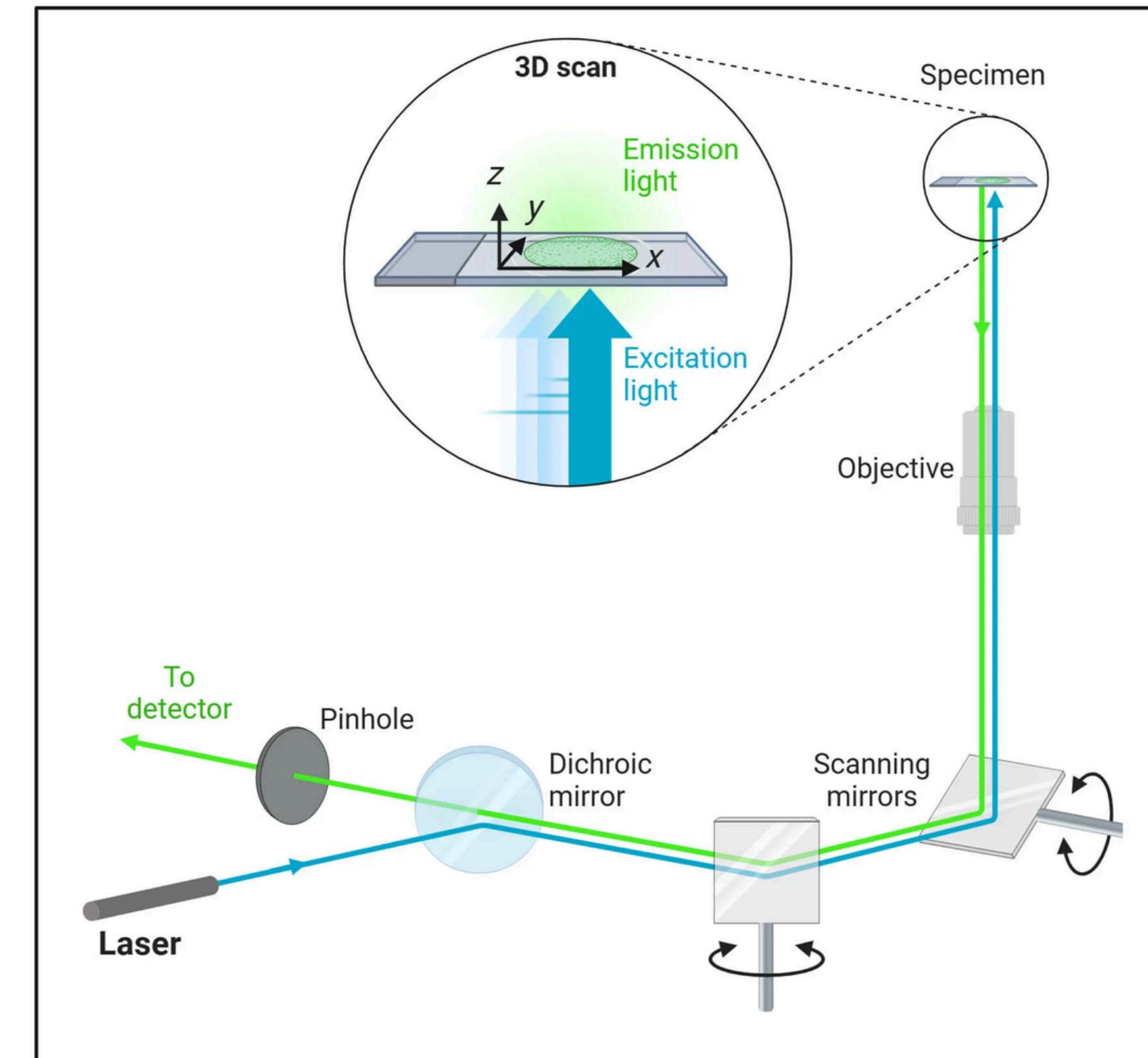


Standard widefield microscope (A) vs. confocal microscope (B)

(3) Fluorescence Confocal Microscopy

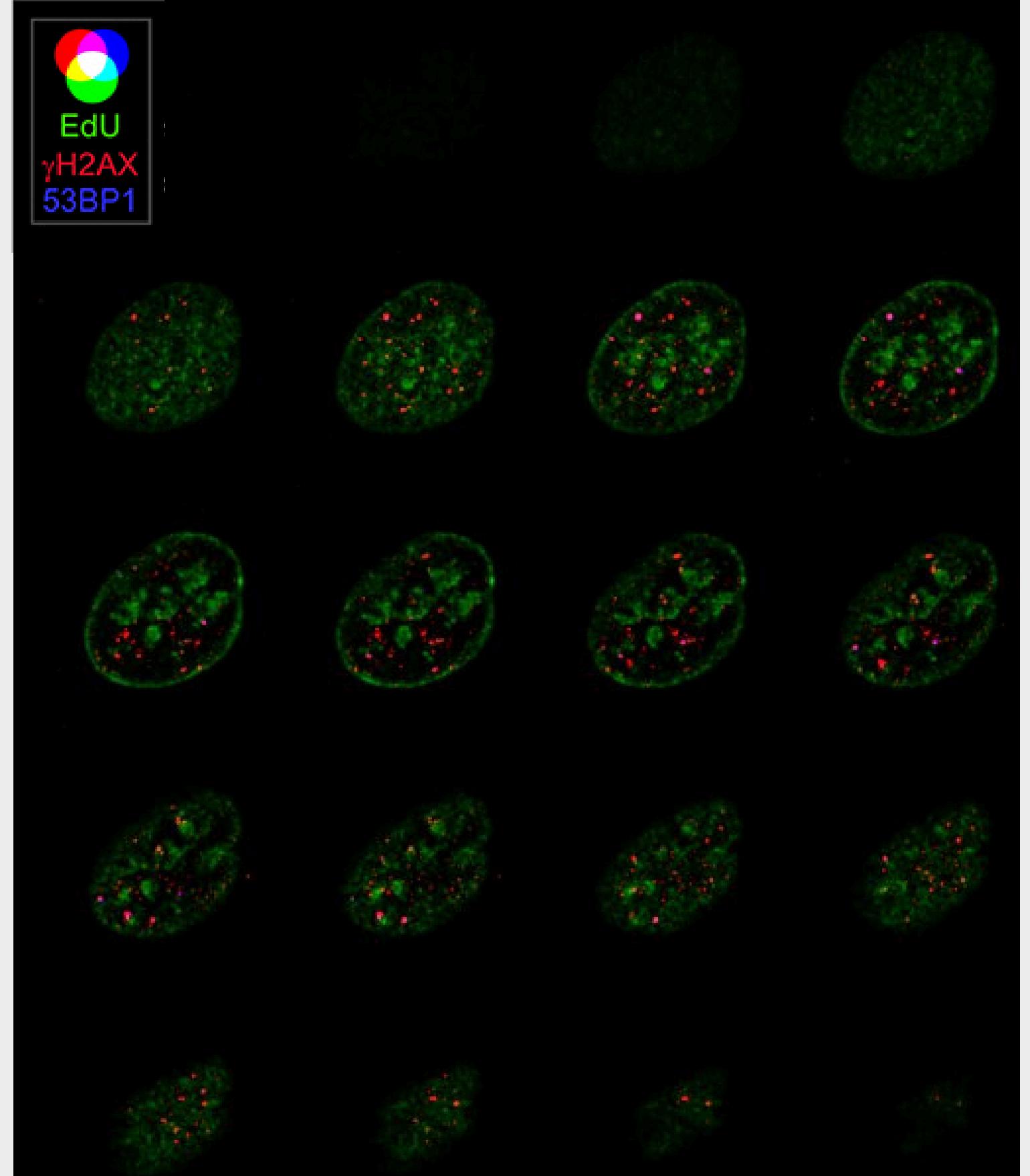
- Confocal imaging focuses both illumination and detection optics on the same diffraction-limited spot, scanned across the sample to build an image.
- Only light from the focal plane is detected, as out-of-focus light is blocked by a pinhole, reducing blur and enabling optical sectioning.
- This setup improves depth resolution and image sharpness, especially in thick or scattering samples.

Confocal Laser Scanning Microscope Principle



(3) Fluorescence Confocal Microscopy

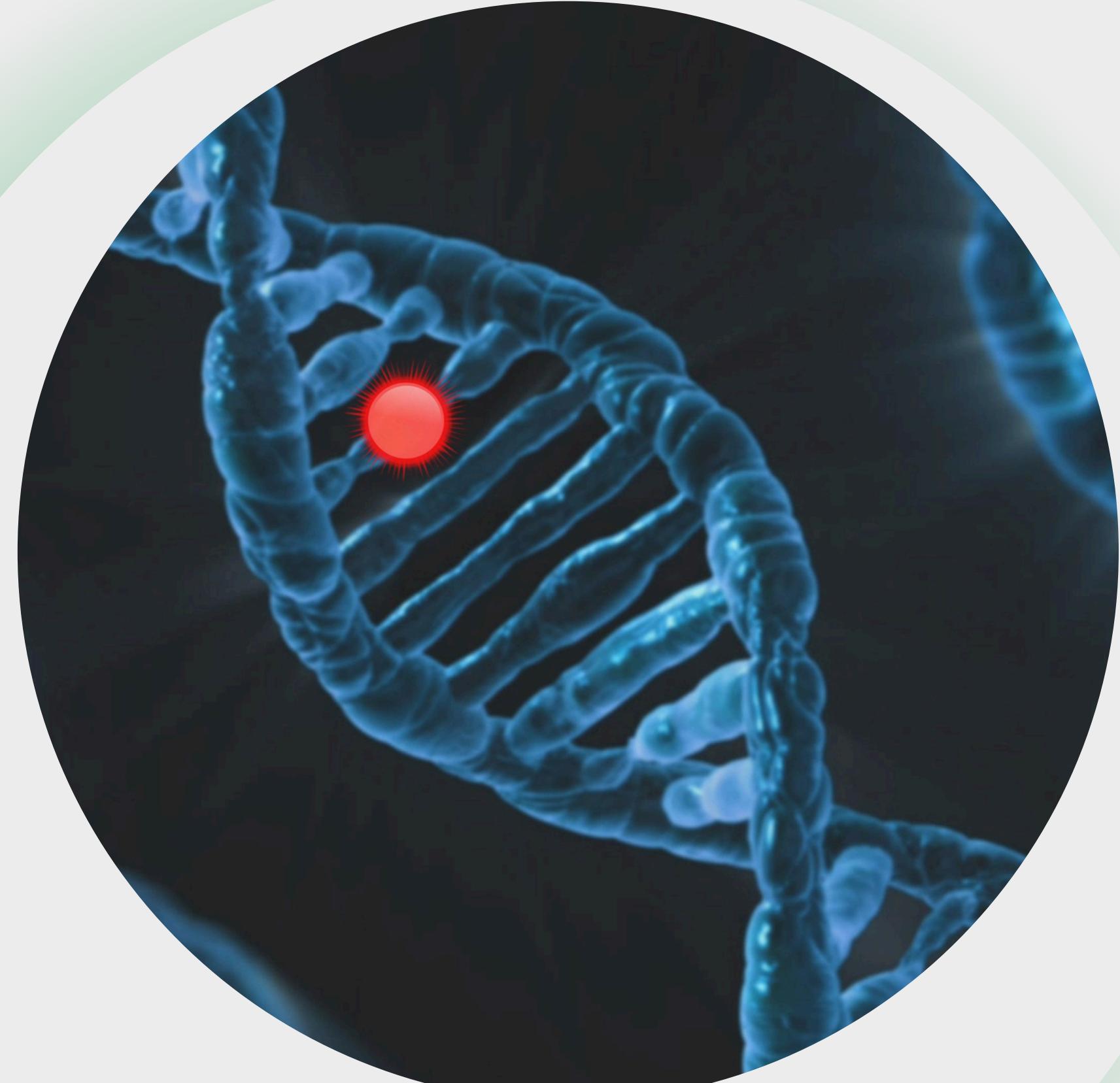
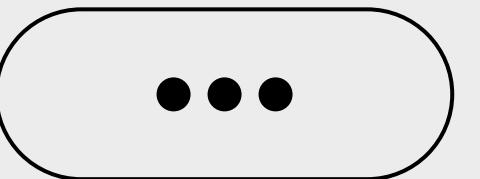
A confocal microscope creates so-called optical cross-sections of the object under examination:



Control sample, SIII phase cell nucleus: montage.

Data: Rybak P. et al. Oncotarget. 2016

Double-strand breaks in DNA



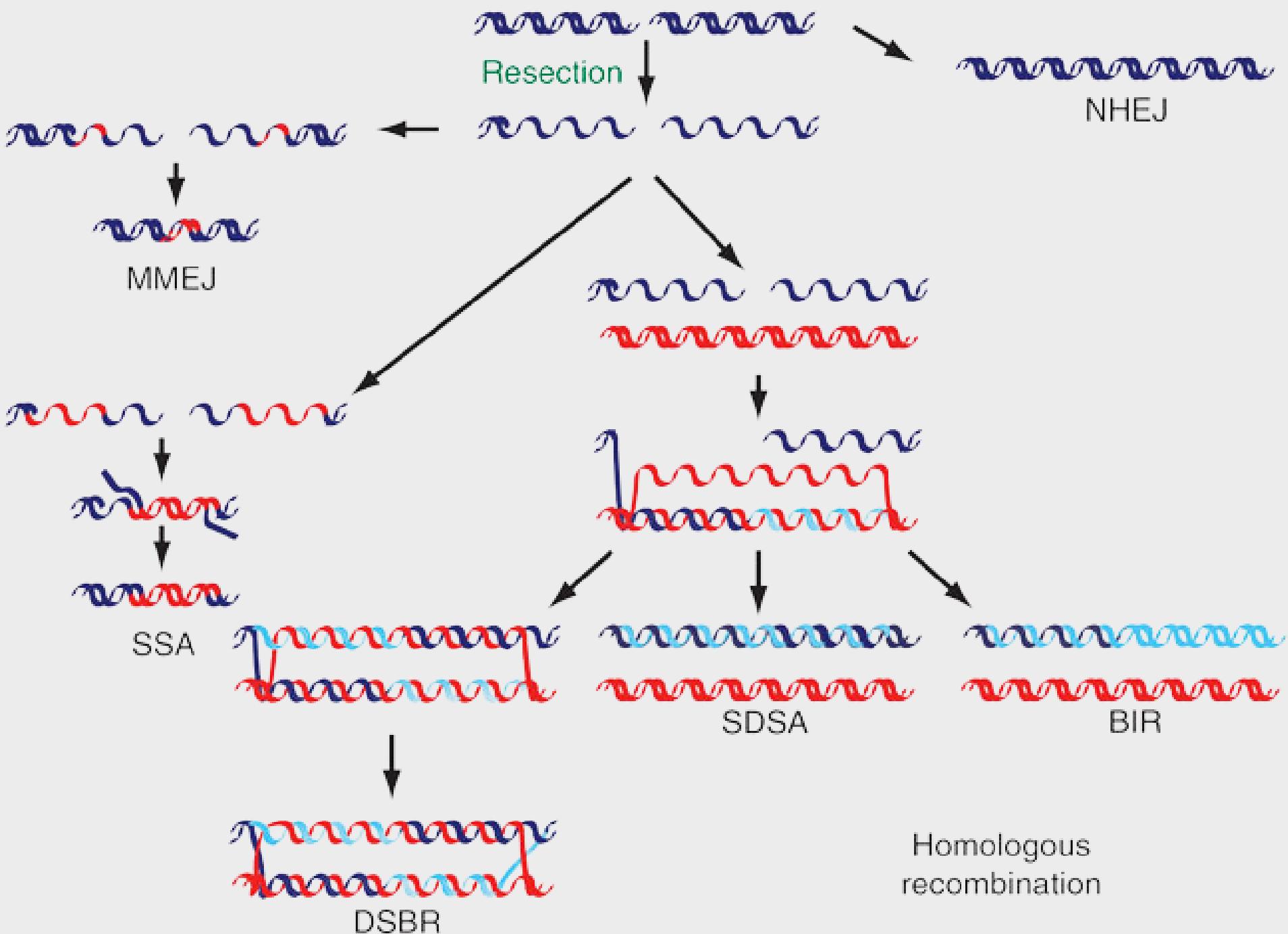
DNA Damage and Repair

DNA damage is constant due to normal metabolism (e.g., ROS) or external factors (UV, radiation, chemicals).

Double-strand breaks (DSBs) are especially dangerous — can lead to cell death or cancer-causing genomic rearrangements.

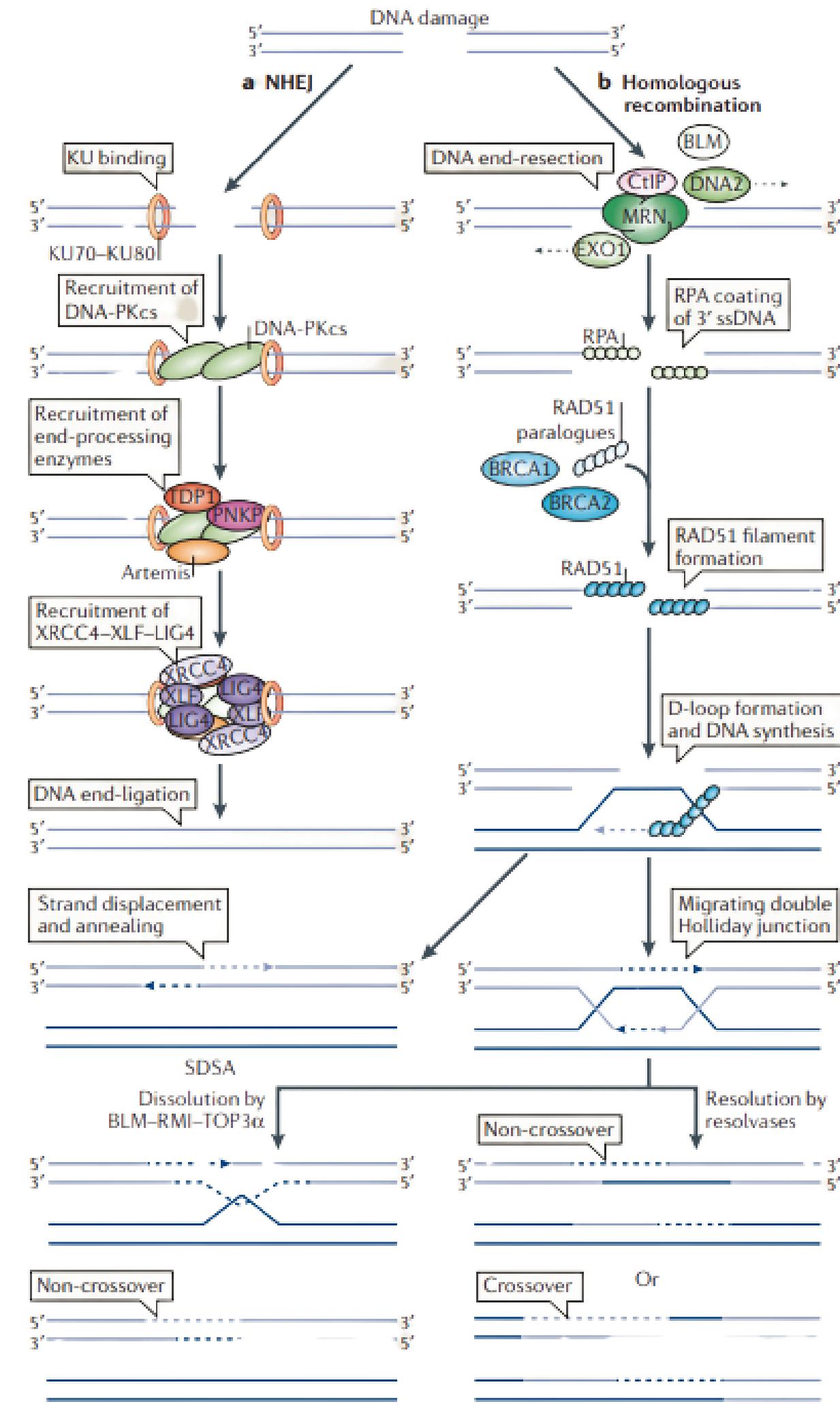
DSBs can be repaired using several different mechanisms:

- NHEJ (nonhomologous end-joining);
- MMEJ (microhomology-mediated end-joining);
- SSA (single-strand annealing);
- SDSA (synthesis-dependent strand annealing);
- BIR (break-induced replication).



Understanding these repair systems is key in genetics and cancer research.

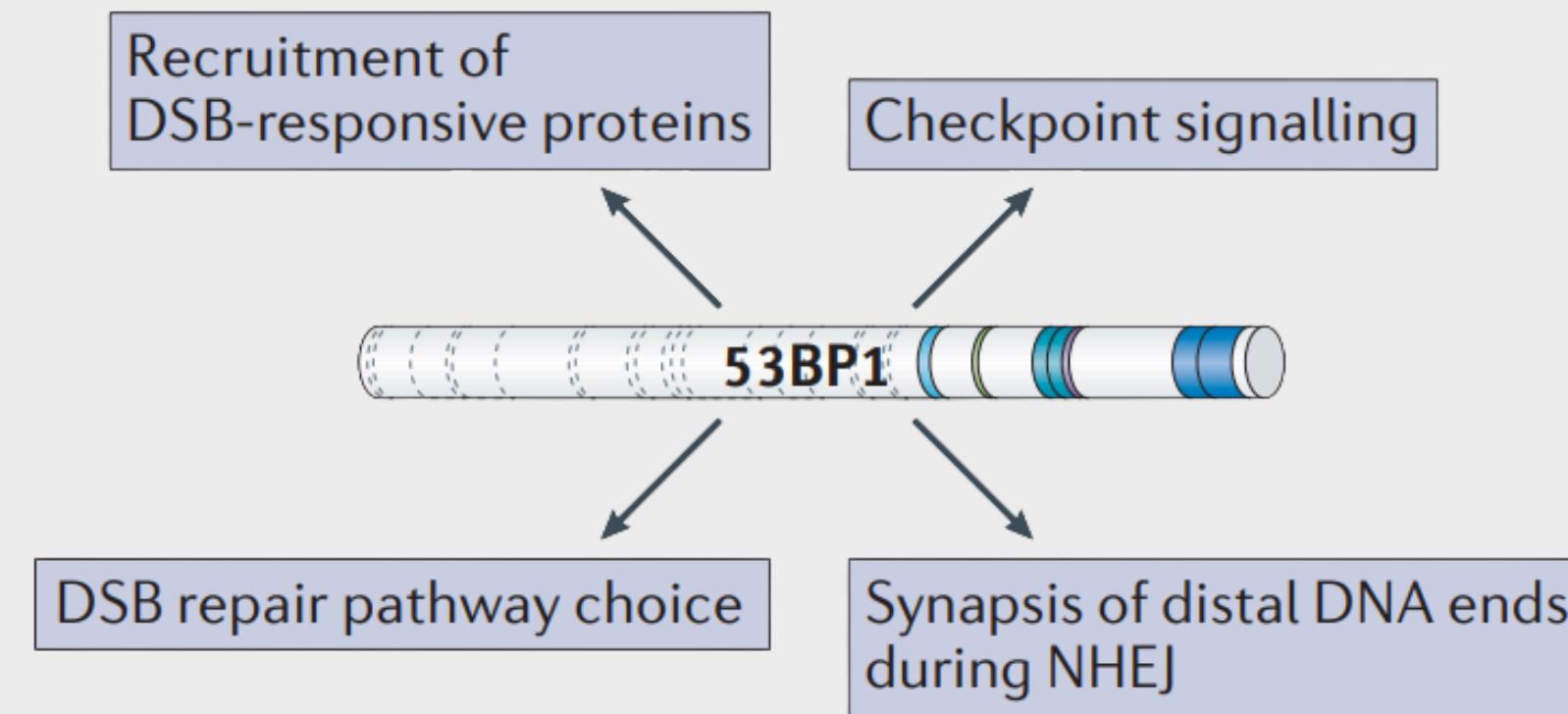
Huertas, P. Nat Struct Mol Biol (2010)



The two main DNA double-strand break (DSB) repair pathways in eukaryotic cells are non-homologous end-joining (NHEJ; a) and homologous recombination (b).

53BP1

- p53-binding protein 1 (53BP1) is a crucial component of DSB signalling and repair in mammalian cells.
- 53BP1 is a key regulator of DSB repair pathway choice.



- During G1, it promotes non-homologous end-joining (NHEJ)-mediated DSB repair by antagonizing long-range DNA end-resection, which is essential for DSB repair via homologous recombination.
- During S-G2, breast cancer 1 (BRCA1) and its interacting partner CtBP-interacting protein (CtIP) counteract 53BP1-RIF1 and 53BP1-PTIP complexes to promote DNA end-resection and thus homologous recombination-mediated DSB repair.

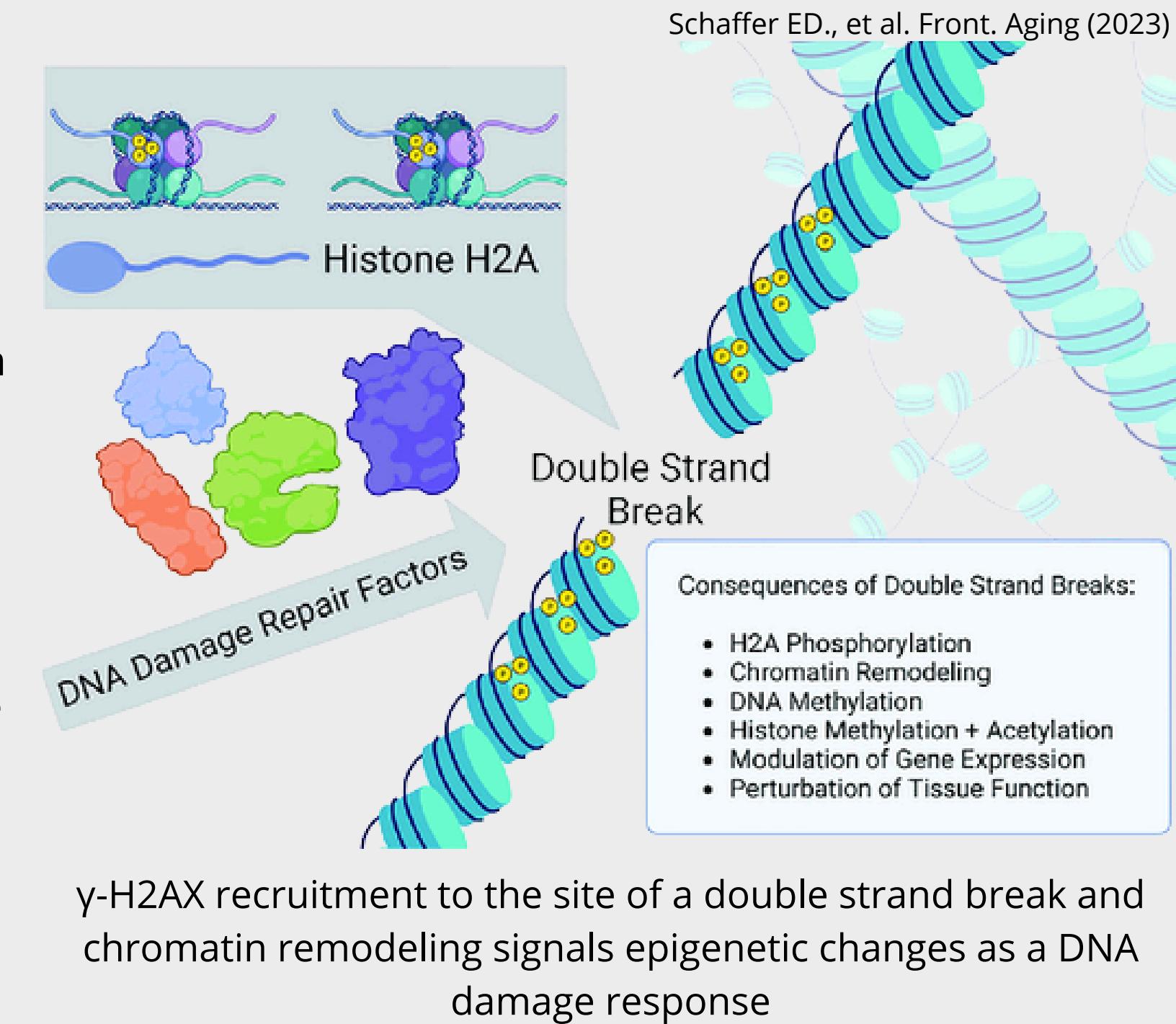
γ H2AX

- “Phosphorylation of the Ser-139 residue of the histone variant H2AX, forming γ H2AX, is an early cellular response to the induction of DNA double-strand breaks.
- This phosphorylation event is now one of the most well-established chromatin modifications linked to DNA damage and repair.
- As γ H2AX is formed de novo, it is a more reliable DSB marker than other repair proteins that are present in cells even when DNA is not damaged.”

© Mah, LJ., et al. Leukemia (2010).

- “A growing body of evidence demonstrates, however, that while induction of DSBs always brings about phosphorylation of histone H2AX, the reverse is not true - the presence of γ H2AX foci should not be considered an unequivocal marker of DNA double-strand breaks
- The lack of a link between low level phosphorylation γ H2AX sites and double-strand DNA breaks in cells exposed to topoisomerase I or II inhibitors.”

© Rybak, P. et al. Oncotarget. (2016)

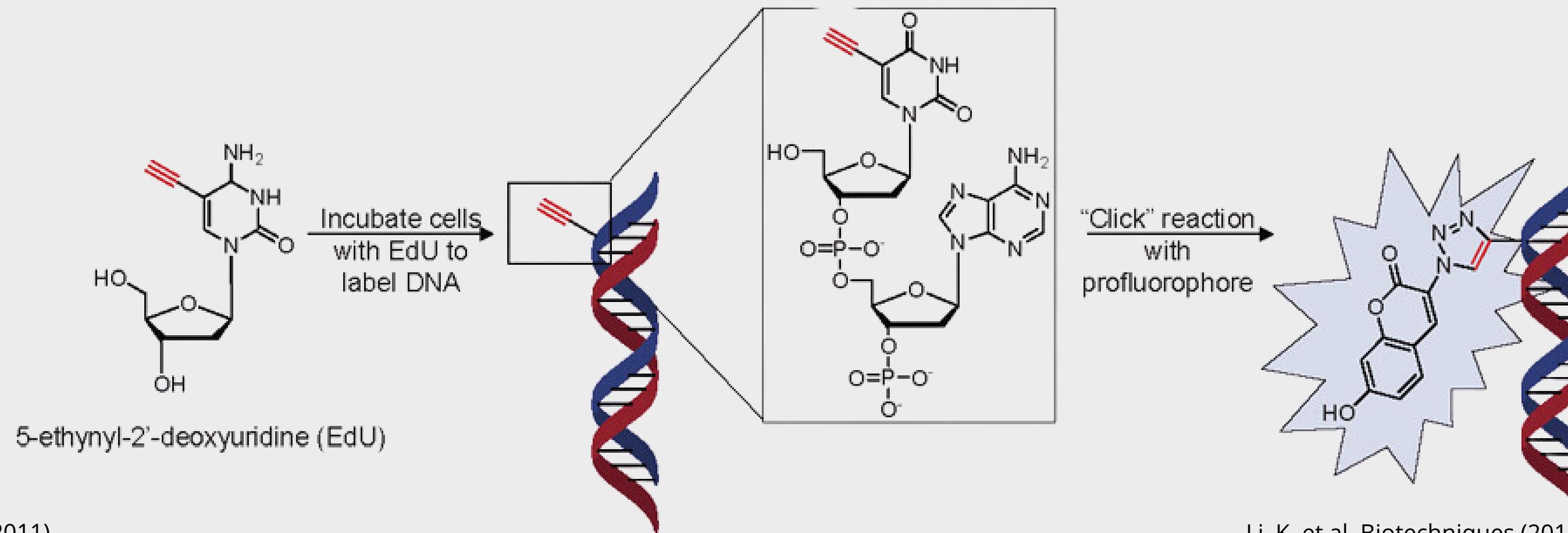


γ -H2AX recruitment to the site of a double strand break and chromatin remodeling signals epigenetic changes as a DNA damage response

Staining techniques: Click reaction for cell proliferation

DNA replication occurs in microscopically visible complexes at discrete sites in the nucleus. They form replication foci, which consist of DNA associated with replication machineries, and are the places of accumulation of newly synthesized DNA.

Replication foci can be revealed by pulse-labeling cells with thymidine analogs such as 5-bromo-2'-deoxyuridine (BrdU) or 5-ethynyl-2'-deoxyuridine (EdU), which are incorporated into DNA during active DNA synthesis and subsequently detected by fluorescent probes.

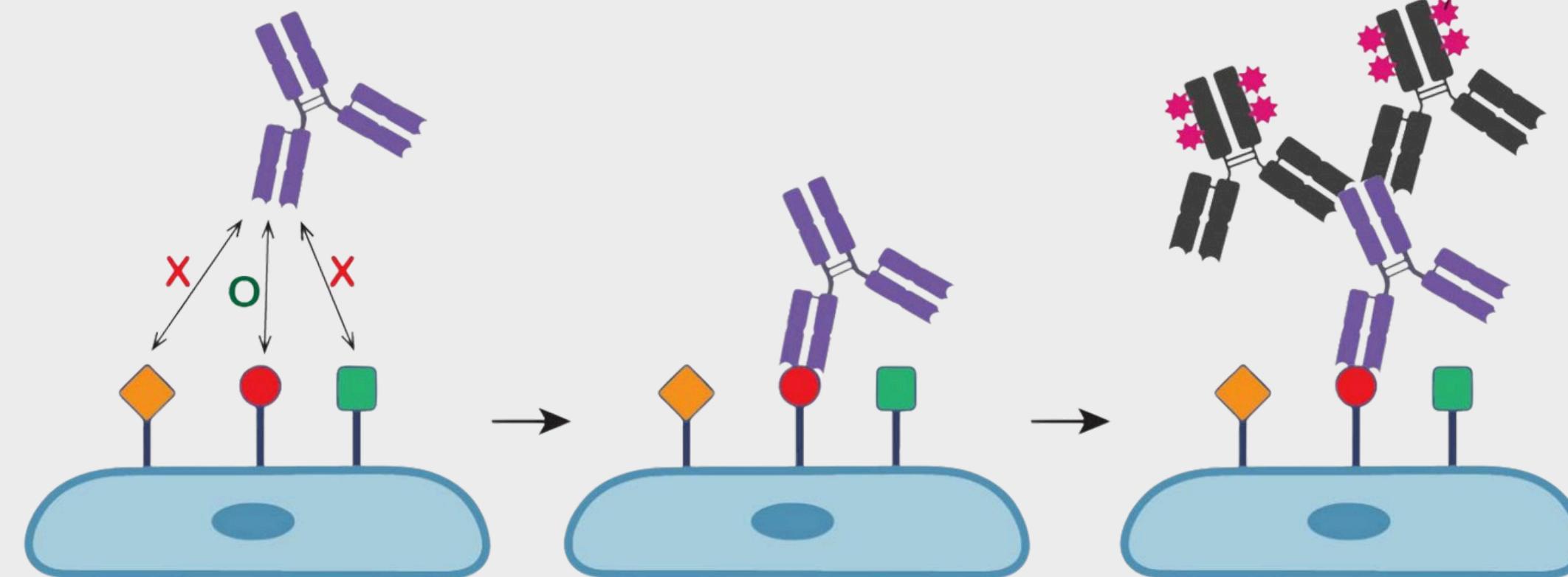
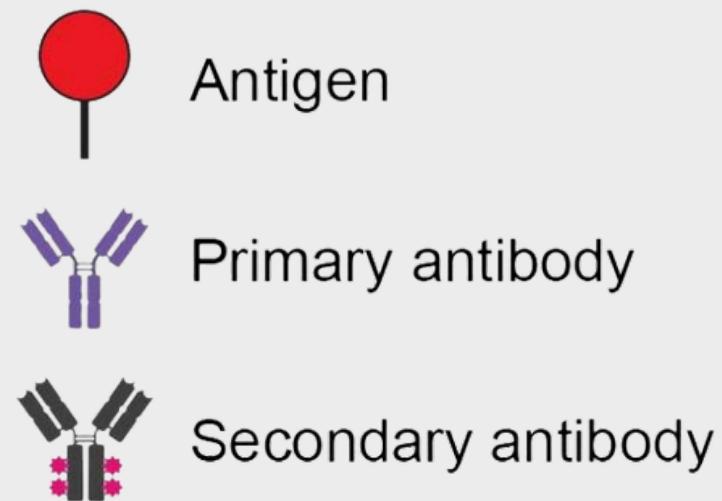


Montecucco, A. Springer (2011).

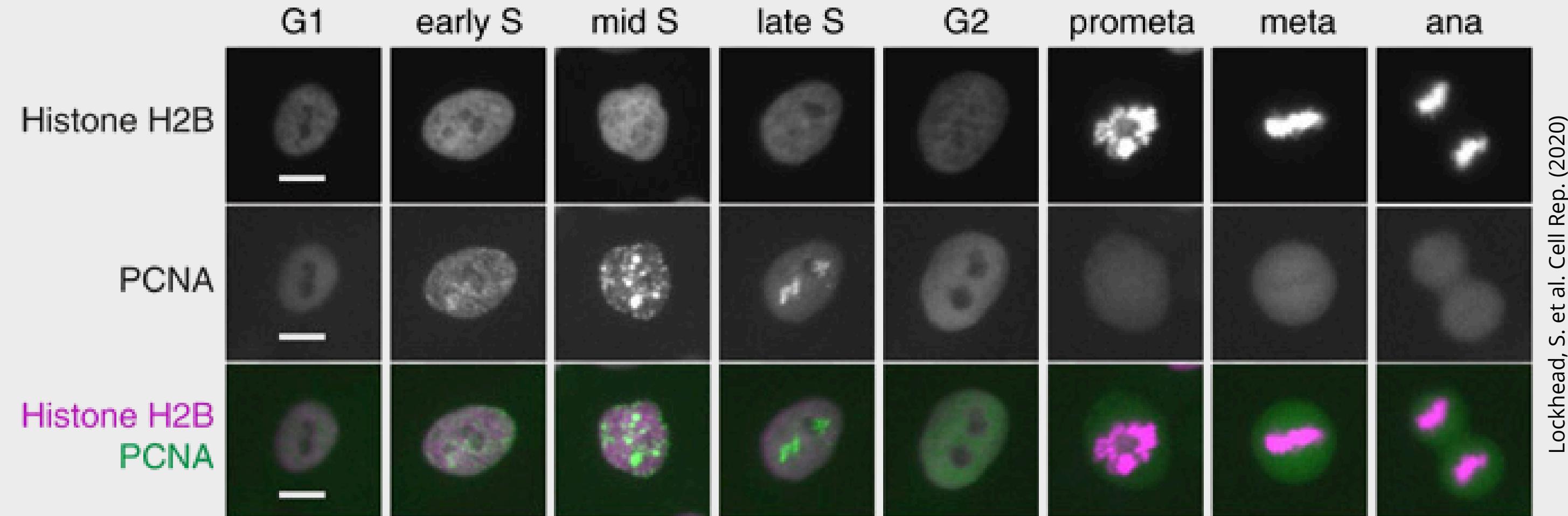
Leonhardt, H. et al. J Cell Biol. (2000)

Li, K. et al. Biotechniques (2010)

Staining techniques: immunofluorescence staining

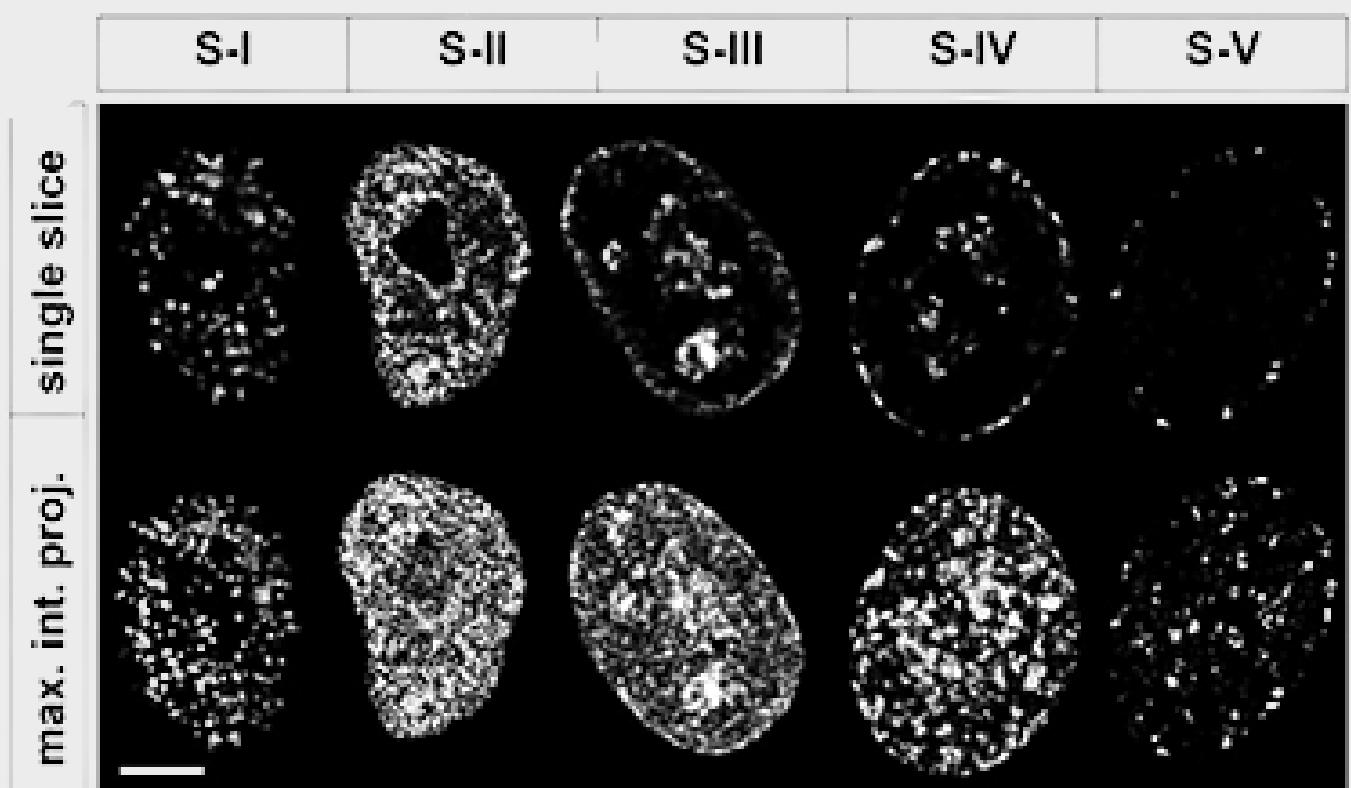


In Rybak *et al.* (2016), γ H2AX and of 53BP1 foci were detected with fluorescence immunostaining



Lockhead, S. et al. Cell Rep. (2020)

The distribution pattern of replication foci within the nucleus changes according to a precise program during S phase



Rybäk P. et al. Oncotarget (2016)

A549 synthesis timing

S-subphase

8h:

S-I

S-II

S-III

S-IV/V

1 h

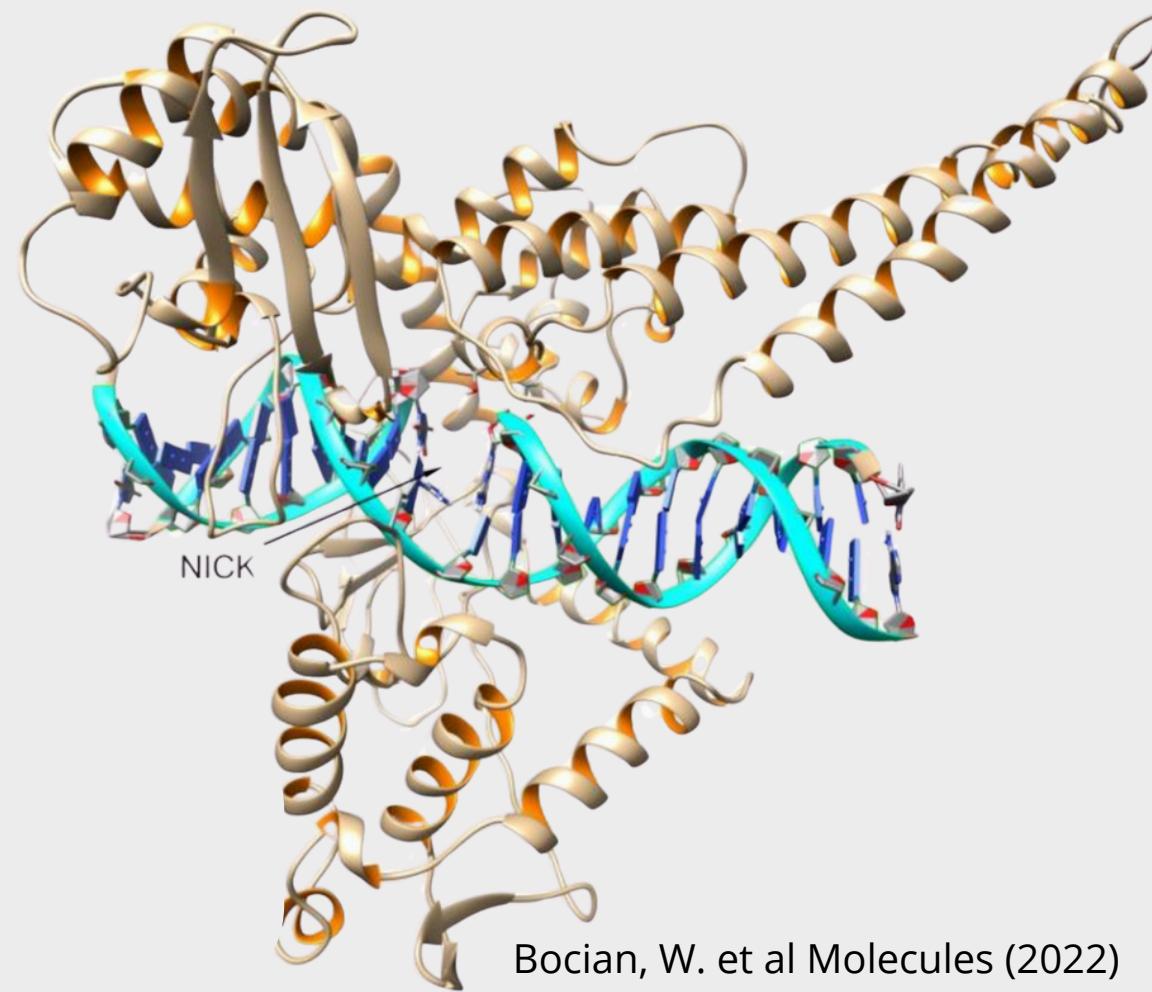
3,5 h

< 3 h

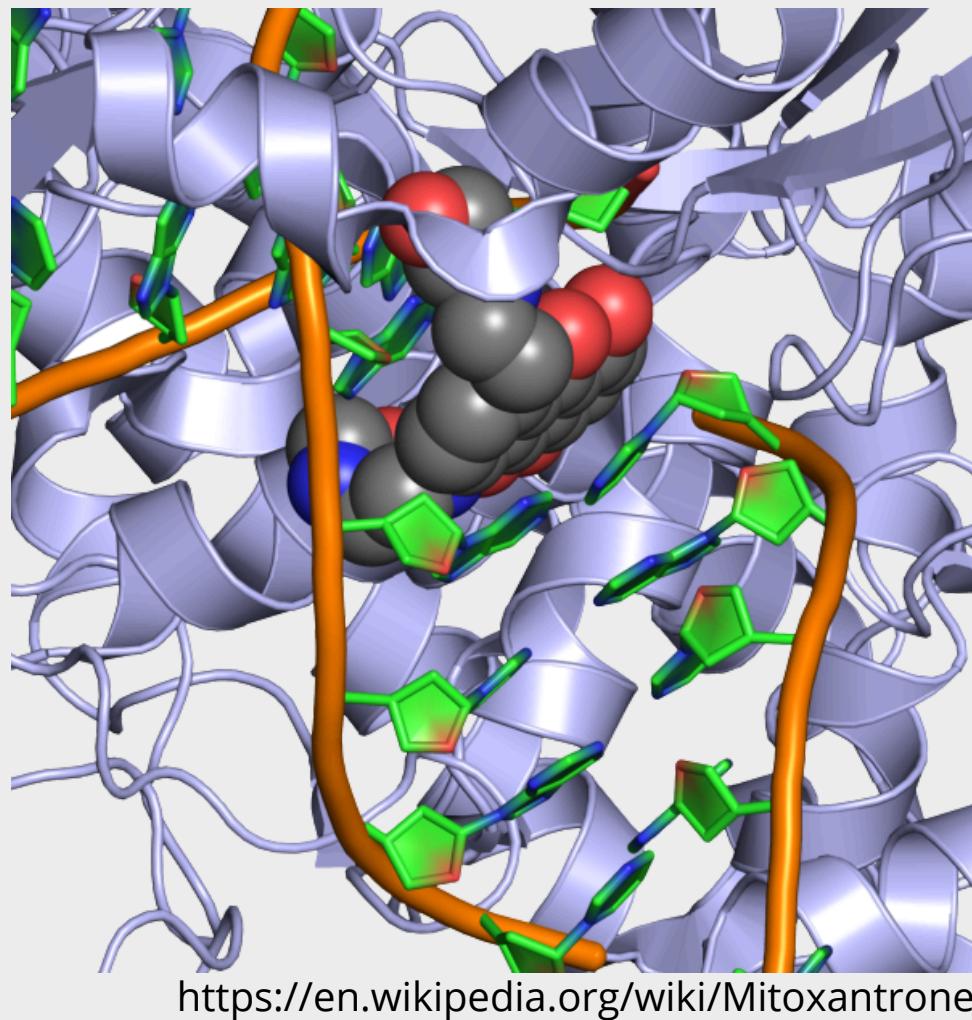
< 1 h

Treatment

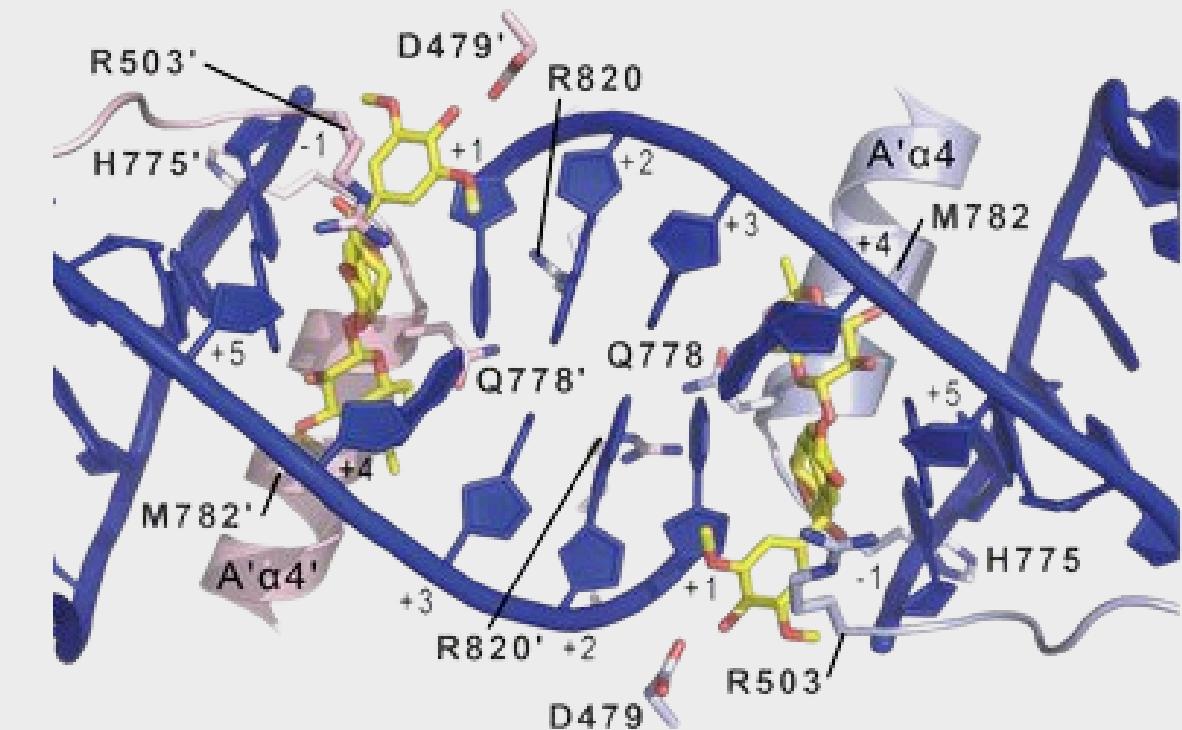
Camptothecin (CPT)



Mitoxantrone (MTX)



Etoposide (ETP)



Camptothecin binds to the topoisomerase I and DNA complex (the covalent complex) resulting in a ternary complex, and thereby stabilizing it. This prevents DNA re-ligation and therefore causes DNA damage which results in apoptosis.

CPT induces DNA double-strand breaks (DSBs) and γH2AX exclusively in S-phase, at the sites of DNA replication.

Mitoxantrone is a type II topoisomerase inhibitor; it disrupts DNA synthesis and DNA repair in both healthy cells and cancer cells by intercalation between DNA bases.
MTX induces γH2AX preferentially (although with lesser exclusivity than CPT) in DNA replicating cells.

Etoposide is in the topoisomerase II inhibitor family of medication. It is believed to work by damaging DNA.

ETP induces γH2AX foci in all phases of the cell cycle, with only weak preference to induce damage in DNA of replicating cells or at DNA replication sites.

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