Module 1: Single-Cell Optical Microscopy Experiments and Image
"Fluorescent Labeling Techniques used in single-cell Research. Part 1.1"

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COLORADO STATE UNIVERSITY

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ASU Arizona State University

Fluorescent Labeling Techniques used in single-cell Research

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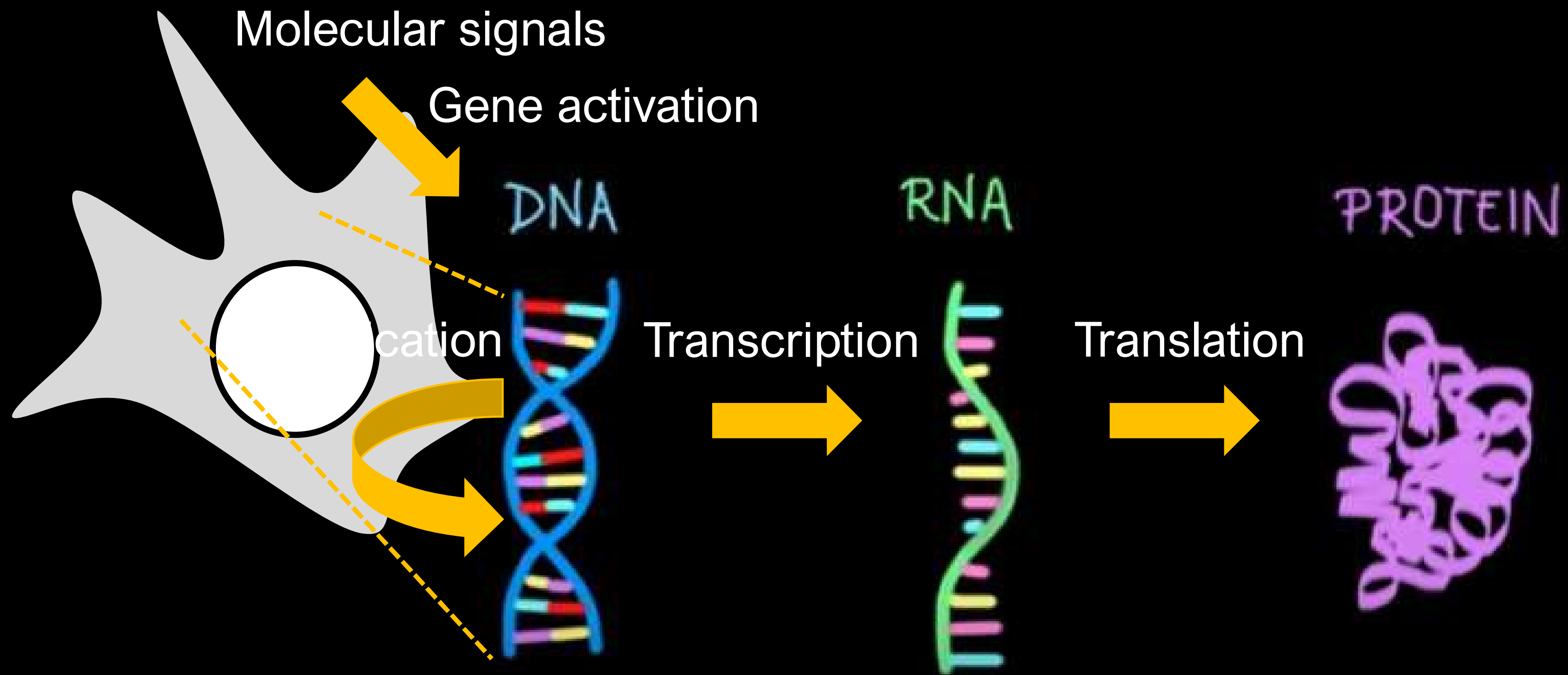
Outline-Part 1.1:

1. The Central Dogma of Molecular Biology
2. Labeling Proteins for Single-Molecule Imaging
a. Methods for labeling proteins
b. Fluorophores

Outline-Part 1.2:

3. Labeling Techniques Employed in Fixed cells
a. Immunolabeling
b. Single-Molecule Fluorescence In situ Hybridization (smFISH)
4. Questions & Practice
5. Supplementary Material



How can we visualize these processes?

Image modified from the Kahn Academy site (see supplementary material)

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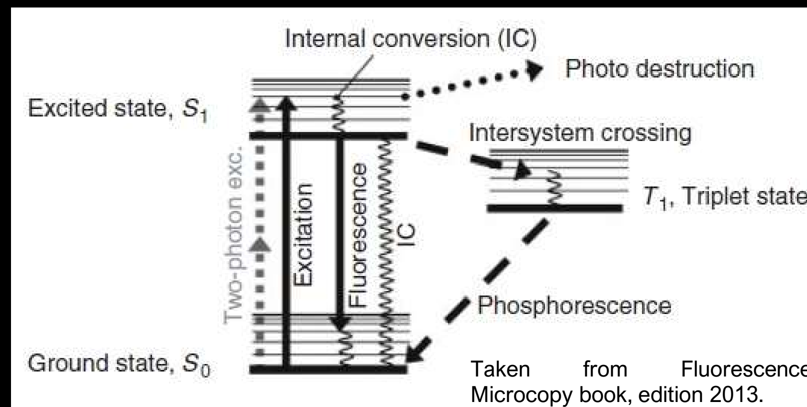
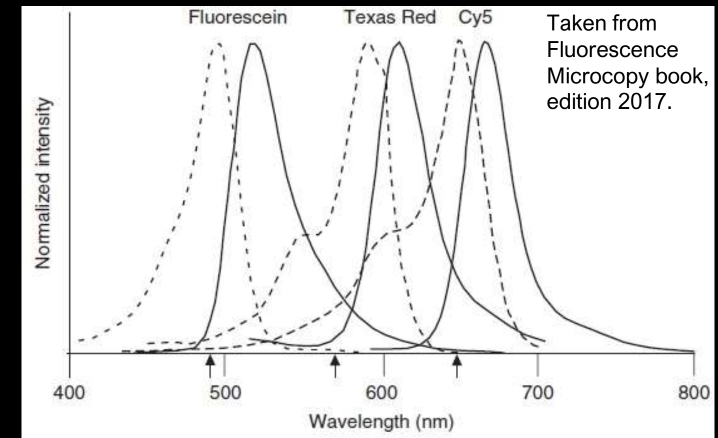
Fluorescence microscopy allows biological processes to be studied as they occur in space and time, at the cellular and molecular levels.



Taken from Fluorescence Microcopy book, edition 2013.

Jablonski diagram, displaying the energy levels and possible routes of excitation & de-excitation of a fluorophore.



Taken from Fluorescence Microcopy book, edition 2017.

Fluorescence excitation (dashed lines) and emission (full lines) spectra of three different fluorophores. The arrows indicate the laser used to excite these dyes.

Common approaches for protein labeling

1. Dye molecules linked to the protein of interest
2. Genetically encoded fluorescent proteins

Critical properties of fluorescent labels

1. Location of the fluorophore
2. Brightness, blinking & photostability
3. Location of the protein of interest

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- **Antibodies** are proteins and part of the immune system capable of recognizing intruders like virus and bacteria. Each antibody recognizes a specific antigen.

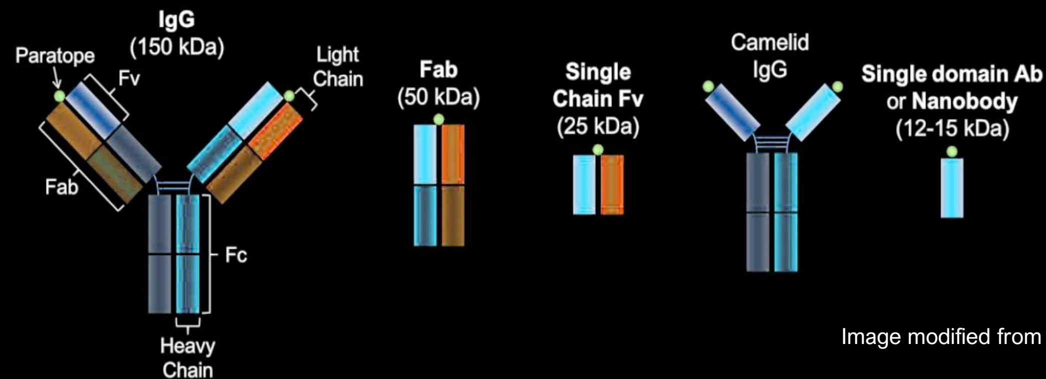
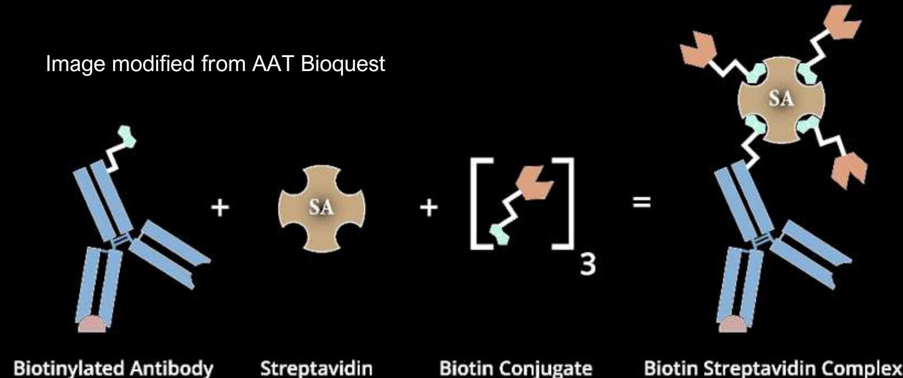


Image modified from Photometrics

- **Biotinylation** is the covalently process of attaching biotin to a protein, nucleic acid, or other molecule. It has high affinity for its interaction partners avidin and streptavidin, which can be used to tag fluorescent proteins.

Image modified from AAT Bioquest



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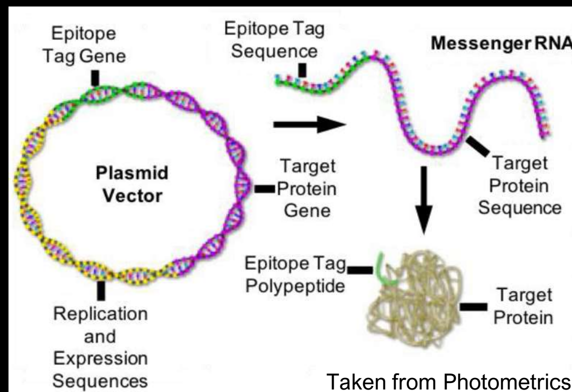
- **Epitope Tags** are useful when antibodies are not available. Those can be encoded into a plasmid vector attached to the protein of interest.

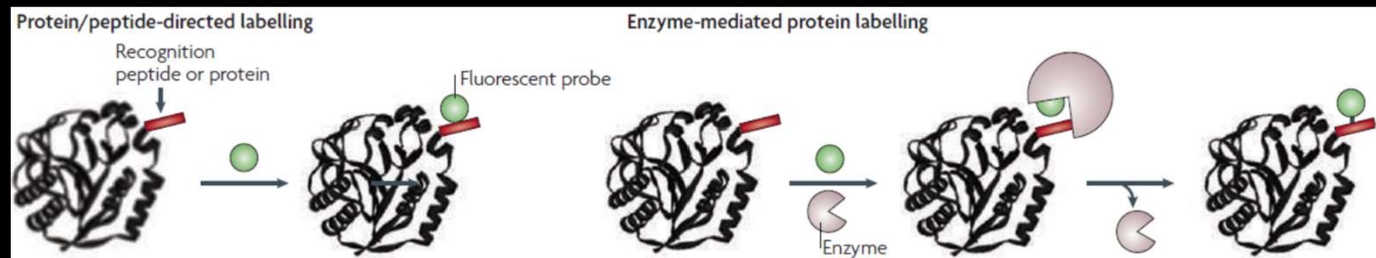


Taken from Photometrics

Examples:

- **Flag-Tag** (AA-Sequence: DYKDDDDK)
- **HA-Tag** (AA-Sequence: YPYDVPDYA)
- **V5-Tag** (AA-sequence: GKPIPNPLLGLDST)
- **Myc-Tag** (AA-Sequence: EQKLISEEDL)

- **Small molecules probes** are recruited by a peptide or protein recognition sequence that is fused to the target protein.



Taken from Fernández-Suárez & Tang, 2008

Examples:

- Direct Labeling: PolyAsp, HaloTag, SNAPTag, CLIPTag
- Enzyme Mediated Labeling: SorTag, Qtag, AB, & LAP

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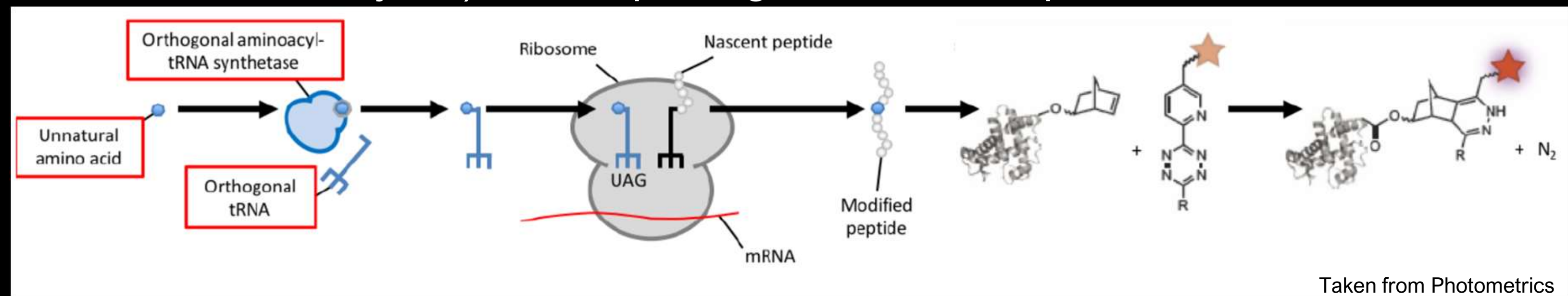
➢ **Bioortogonal Labeling** occurs between two functional groups (e.g., tetrazines & strained alkenes/alkynes), and requires genetic code expansion.

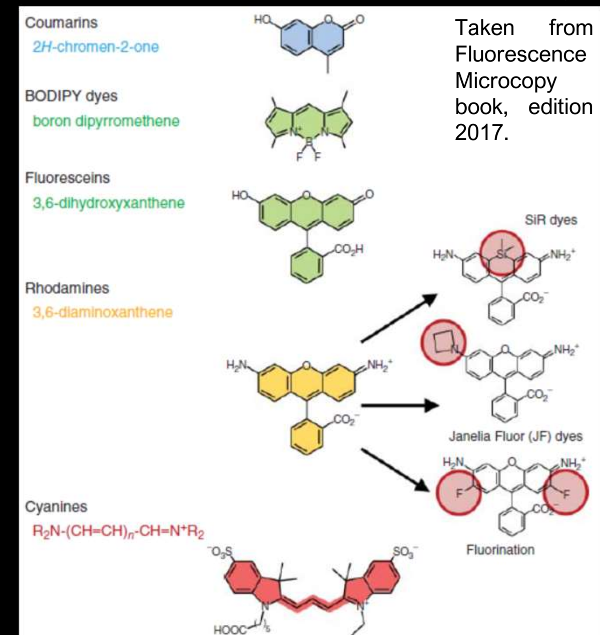


❑ **Organic dyes** are generally planar molecules of about 1-2 nm. These dyes belong to different families based on their core scaffolds.

Examples of commercial organic dyes:

❖ Alexa Fluor posses the largest spectral variety available. Alexa molecules are negatively charged and hydrophilic fluorescent.

❖ Cyanines (Cy2, Cy3, Cy5, & Cy7), can be linked to nucleic acids or proteins through their interactive groups.



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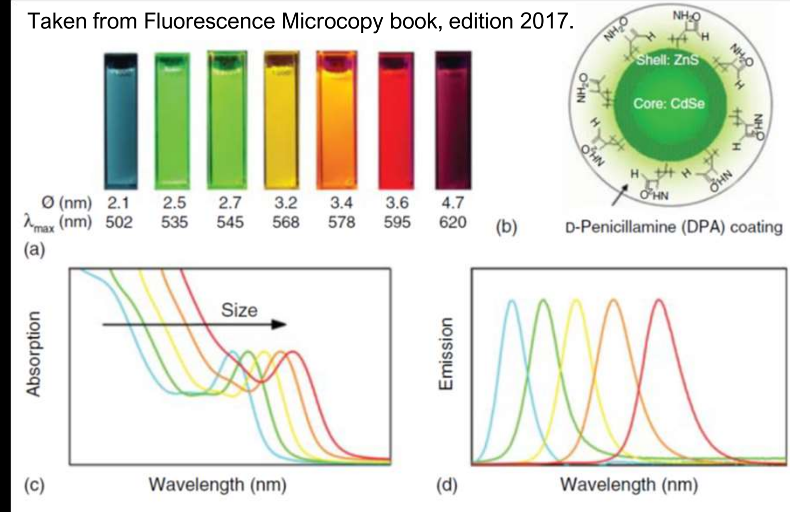
- **Quantum dots** are inorganic semiconductor nanocrystals, typically composed of a cadmium selenide core and a zinc sulphide shell. For biological applications, these are coated with a layer that improves solubility, and then conjugated to targeting biomolecules, such as antibodies or streptavidin.



- **Minor groove binding dyes** bind tightly to DNA in the minor groove region. There are about 50 molecules that bind DNA, but they also bind RNA. The only ones that have low affinity for RNA are DAPI and Hoechst. Thus, these are commonly used as a nuclear stain.

Taken from Fluorescence Microcopy book, edition 2013.

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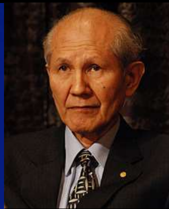
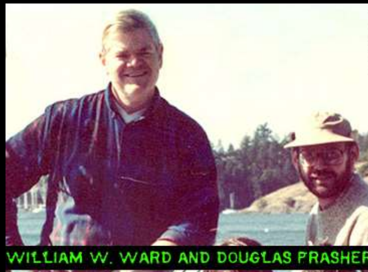
- **Fluorescent proteins** are very small and specific, and genetically encoded into the protein of interest. Green Fluorescent Protein (GFP), was the 1st fluorescent protein to be reported.

Jellyfish Aequorea Victoria

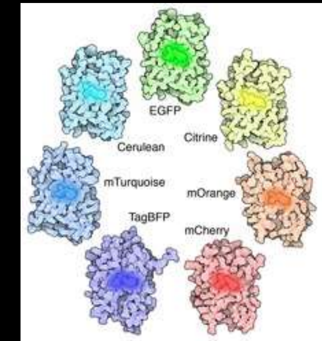

Osamu Shimomura

1960: Green protein was purified from Jellyfish by Shimomura in Japan


WILLIAM W. WARD AND DOUGLAS PRASHER

1992: Douglas Prasher reported the cloning and nucleotide sequence for *wt*-GFP in gene

Roger Tsien



1994-2016: Roger Tsien mainly contributed to much of our understanding of GFP works and for developing new techniques and mutants of GFP

Martin Chalfie

Green Fluorescent Protein as a Marker for Gene Expression

Martin Chalfie,* Yuan Tu, Ghia Euskirchen, William W. Ward, Douglas C. Prasher†

A complementary DNA for the *Aequorea victoria* green fluorescent protein (GFP) produces a fluorescent product when expressed in prokaryotic (*Escherichia coli*) or eukaryotic (*Caenorhabditis elegans*) cells. Because exogenous substrates and cofactors are not required for this fluorescence, GFP expression can be used to monitor gene expression and protein localization in living organisms.

Light is produced by the bioluminescent jellyfish *Aequorea victoria* when calcium binds to the photoprotein aequorin (*1*). Although activation of aequorin in vitro or in heterologous cells produces blue light, the jellyfish produces green light. This light is the result of a second protein in *A. victoria* that derives its excitation energy from aequorin (*2*), the green fluorescent protein (GFP).

Purified GFP, a protein of 238 amino acids (*3*), absorbs blue light (maximally at 395 nm with a minor peak at 470 nm) and emits green light (peak emission at 509 nm with a shoulder at 540 nm) (*2*, *4*). This fluorescence is very stable, and virtually no

1994: The coding sequence of fluorescent GFP is expressed in heterologous cells of E. Coli and C. elegans by the lab of Martin Chalfie

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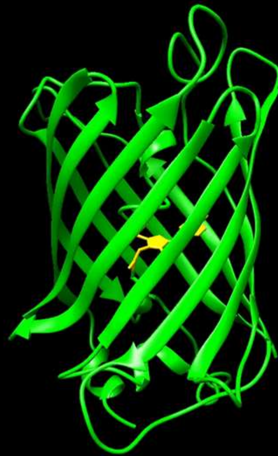
❑ **Fluorescent proteins** (GFP)

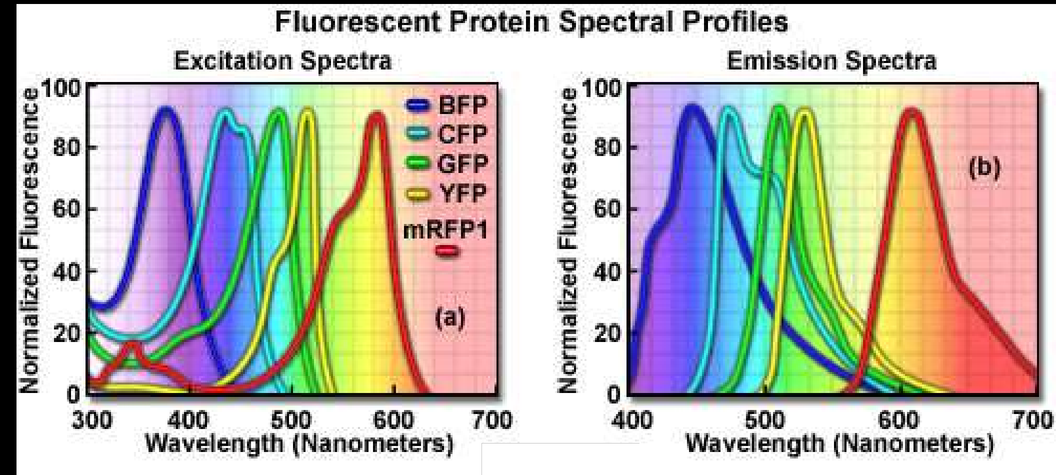


Movie of the GFP structure created by Erik A. Rodriguez with UCSF chimera in memory of Roger Tsien

Most common applications:

- Reporter assay (GFP as a reporter gene)
- Fluorescence microcopy (Protein folding, protein transport, RNA dynamics, among others)

Spectra of GFP variants



Taken from Olympus confocal web site.

The Nobel Prize in Chemistry 2008

Photo: U. Montan
Osamu Shimomura
Prize share: 1/3

Photo: U. Montan
Martin Chalfie
Prize share: 1/3

Photo: U. Montan
Roger Y. Tsien
Prize share: 1/3

Discovery, expression and development of GFP

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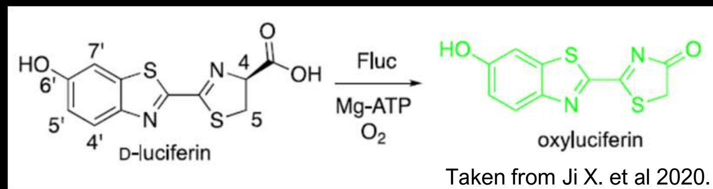
- **Bioluminescence** is the production and emission of light by a living organism on its own. It uses energy from adenosine triphosphate (ATP), but does not require light.

The principal chemical reaction in bioluminescence involves a light-emitting molecule and an enzyme, called luciferin and luciferase, respectively.

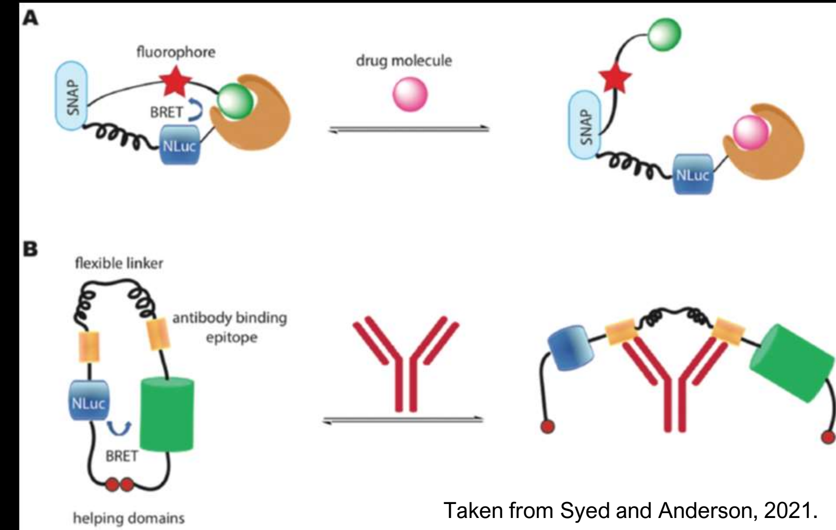


Taken from Ji X. et al 2020.

Several applications, the most common ones:

- Fluc and Rluc bioluminescence is their use as reporter genes for the study of gene expression in prokaryotic and eukaryotic cells and systems.
- Sensors of pH, metal ions, ROS species, enzymes, drug molecules, among others.
- Protein-Protein interaction.
- *In vivo* imaging.

Examples of Luciferase based sensors



Taken from Syed and Anderson, 2021.

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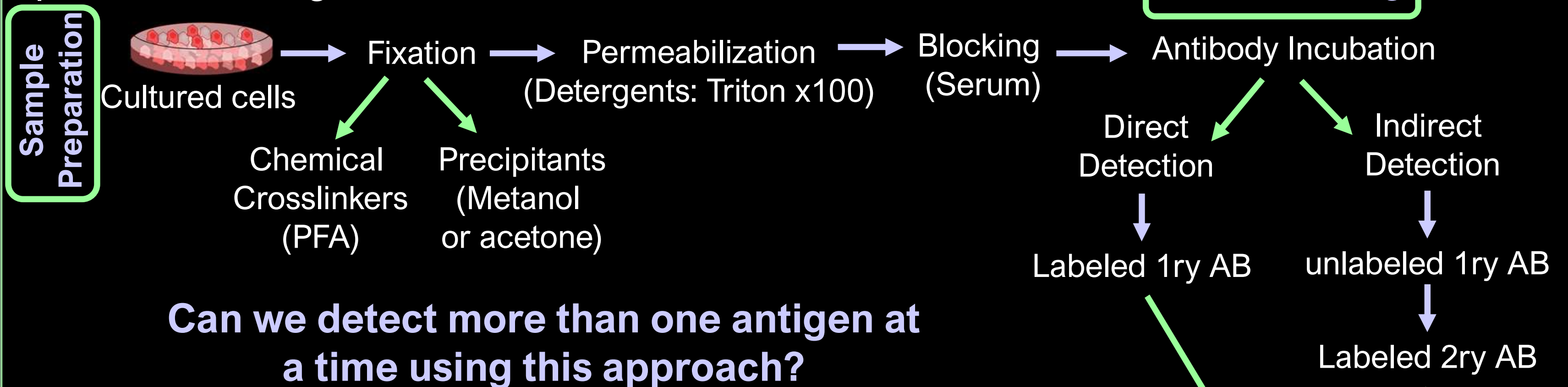
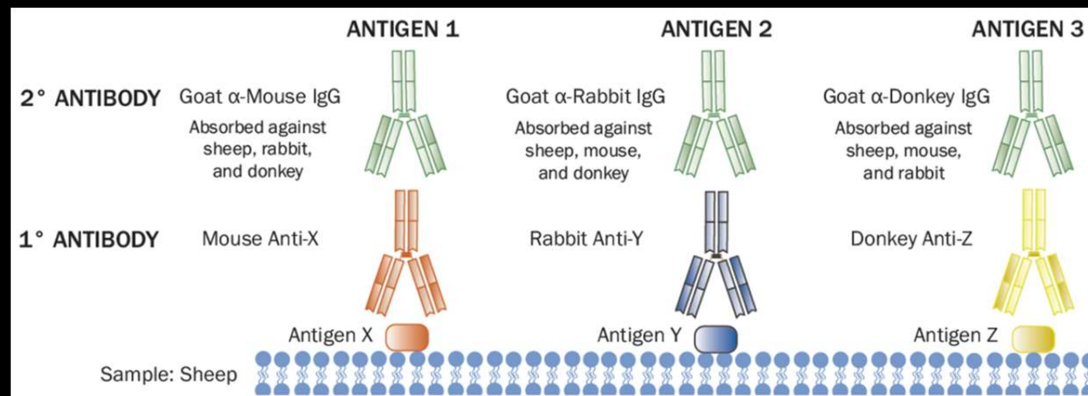
a. Immunolabeling is a biochemical method that allows the detection and localization of an antigen in a cell, tissue or organ, the antigen is usually a protein, and the detection is performed using antibodies.



Can we detect more than one antigen at a time using this approach?

Nuclear counterstaining (DAPI, Hoechst)
↓
Mounting
↓
Fluorescence Microscopy



Taken from Immunocytochemistry Handbook by Novus Biologicals

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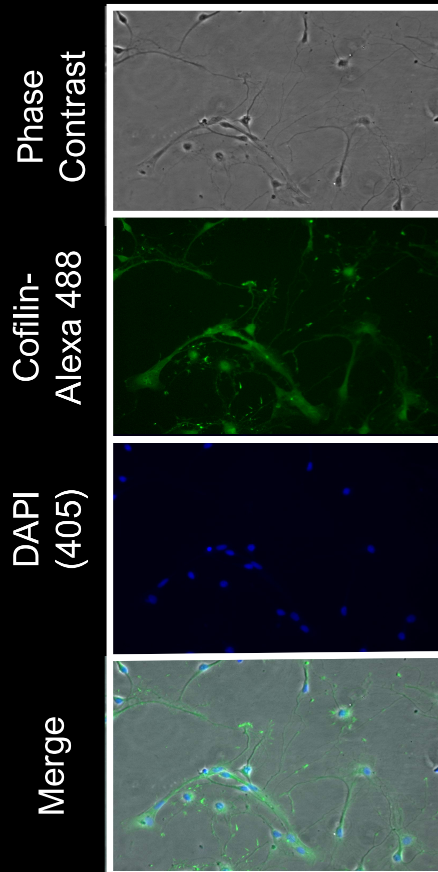
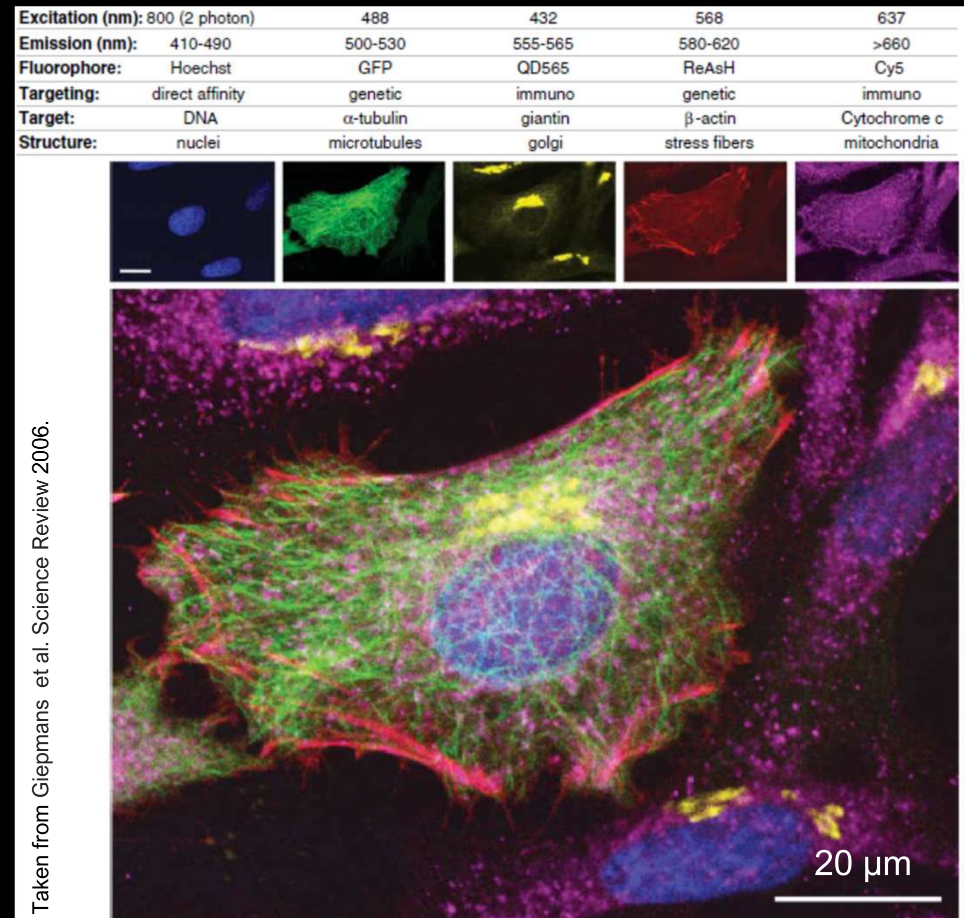
Example of immunolabeling



Example of Parallel application of targeting methods & fluorophores



Excitation (nm):	800 (2 photon)	488	432	568	637
Emission (nm):	410-490	500-530	555-565	580-620	>660
Fluorophore:	Hoechst	GFP	QD565	ReAsH	Cy5
Targeting:	direct affinity	genetic	immuno	genetic	immuno
Target:	DNA	α-tubulin	giantin	β-actin	Cytochrome c
Structure:	nuclei	microtubules	golgi	stress fibers	mitochondria

Taken from Giepmans et al. Science Review 2006.

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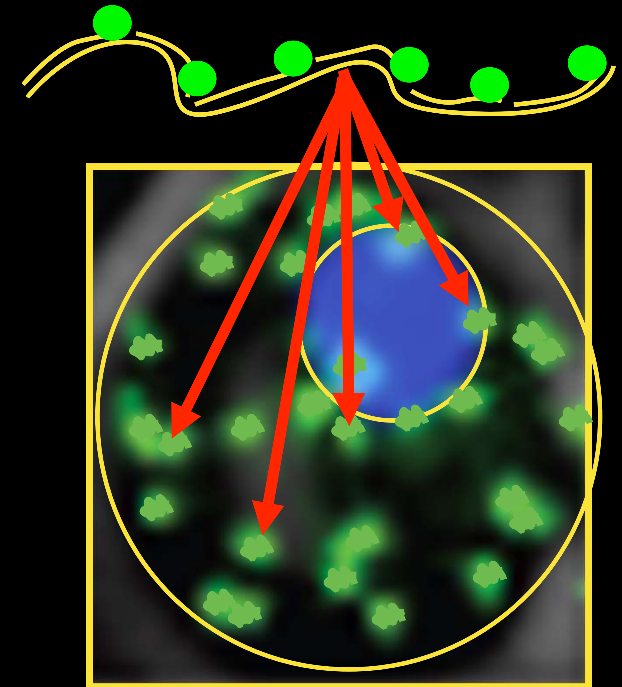
b. Single-Molecule Fluorescence In situ Hybridization "smFISH" (Femino, Singer, 1998) allows the quantification of endogenous transcription response:

- **Number** of individual mRNA per cell,
- **3D Location** of individual mRNA,
- **DNA transcription** site activity,

One-layer probes

48 (20bp) probes/mRNA

Tetramethylrhodamine (TMR)



Neuert, Munsky, et al, *Science* 2013
Munsky, et al, *PNAS*, 2018

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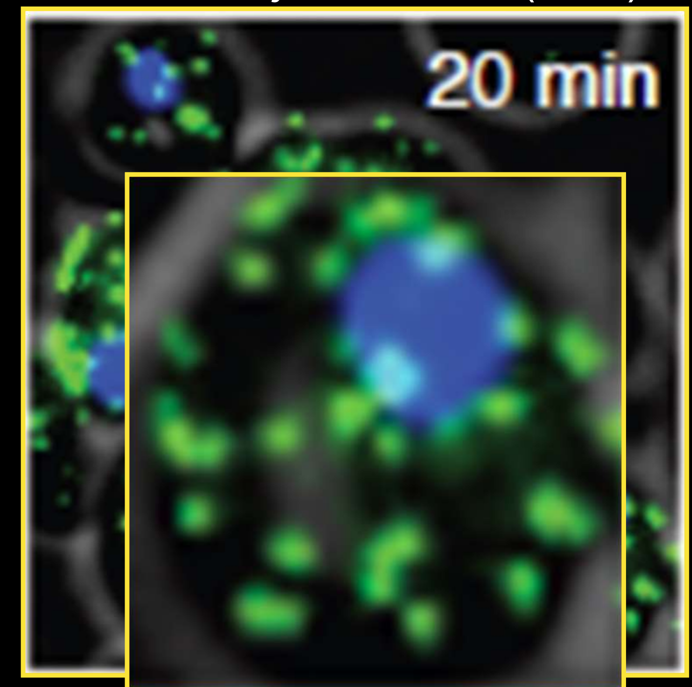

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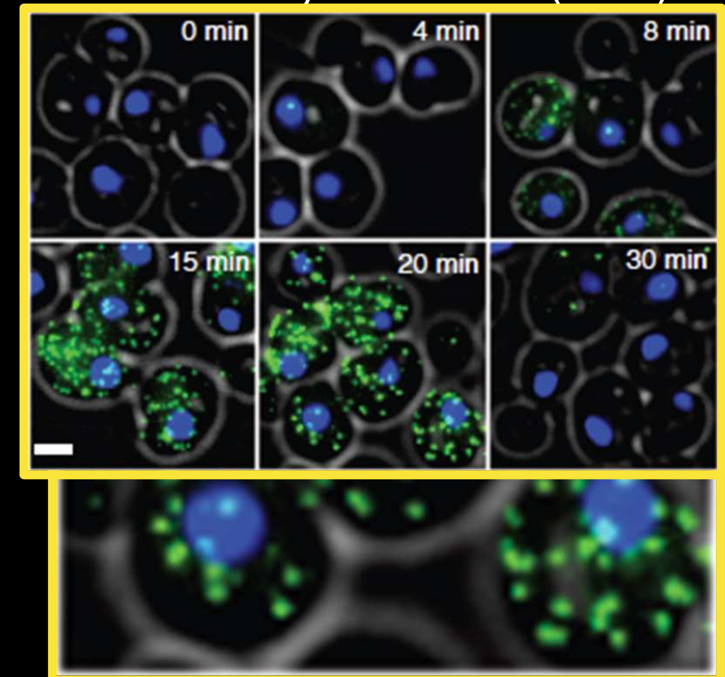
- Fast (1-2 minute) time resolution,
- 100s or 1000s of cells per time point, or condition.

One-layer probes

48 (20bp) probes/mRNA
Tetramethylrhodamine (TMR)



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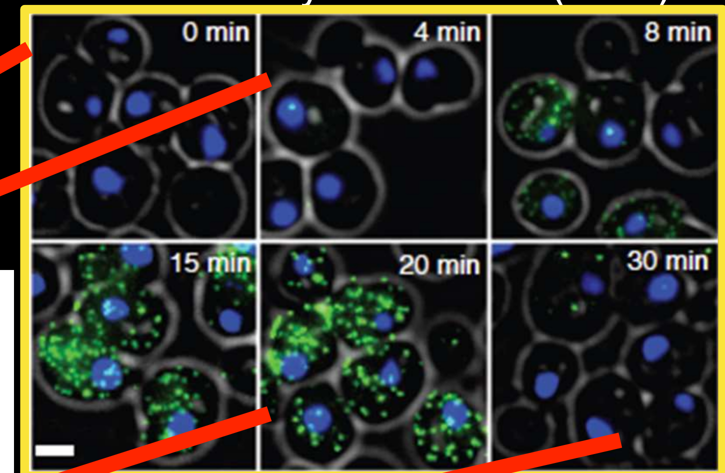
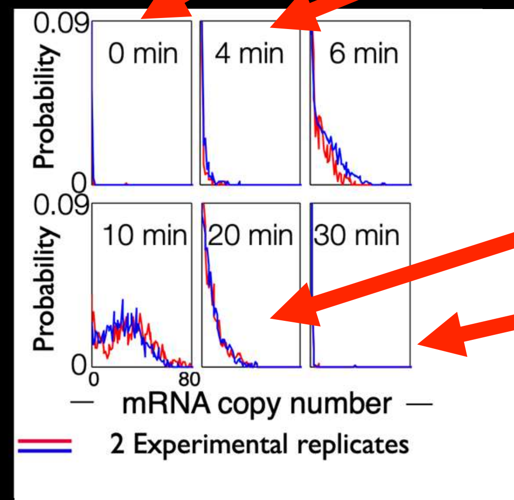


smFISH yields highly reproducible & quantitative measurements of (noisy) single-cell responses



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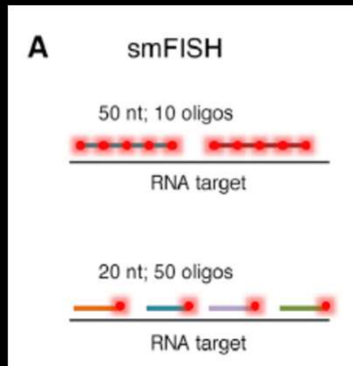
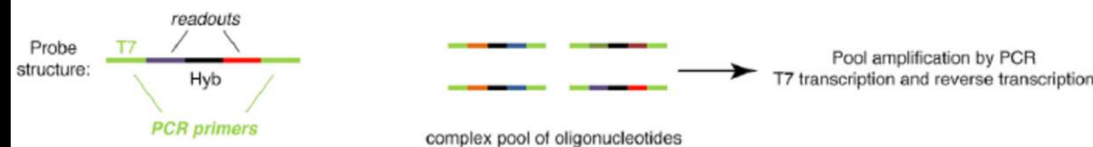
Types of smFISH based on probe design

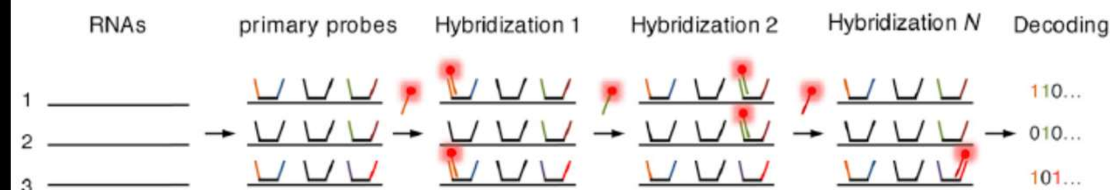


- Traditional **smFISH** directly targets RNA within a cell by using multiple oligonucleotides (10-50 per target).
- Two-layer probes smFISH (like **smiFISH**), indirectly labels the target RNA by fluorescently label a secondary structure carried in the primary probe (24 oligos per target are ideal).

G Multiplexing: probe generation



H Multiplexing: decoding



- Multiplexing **smFISH** is generally used to scale up the number of RNA targets, and it requires a parallel on-chip probe synthesis as well encoding schemes to allow the identification of bound RNAs.

Taken from Pichon et al. Molecular Cell Review 2018.

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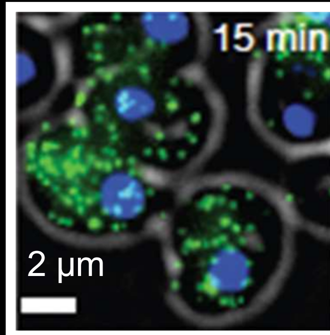
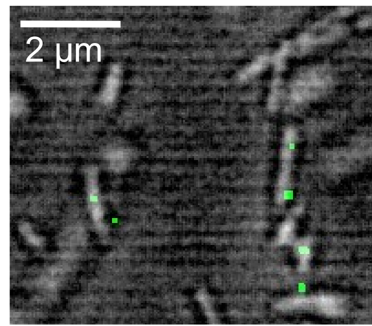
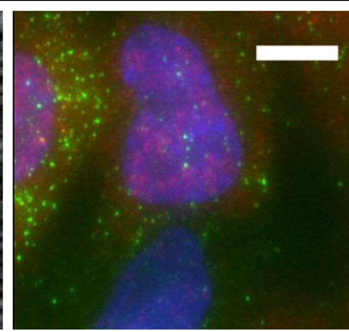
Examples of smFISH, it has been applied to many different RNA in many different organisms.



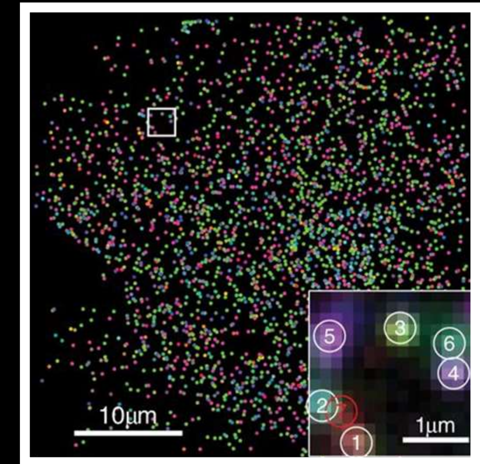
STI1 mRNA in *Saccharomyces cerevisiae* (budding yeast)
-G. Neuert (VU)



Ysr35 sRNA in *Yersinia Pseudotuberculosis* (339nt)
-D. Shepherd
(LANL / CU Denver)



c-Fos mRNA (green) and p-p38 kinase (red) in U2OS cells
-A. Senecal (CNRS)



merFISH detection of 160 different mRNA species in an IMR90 (human fetal lung) cell
-Chen et al, 2015

Fluorescent Labeling Techniques used in single-cell Research

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Outline-Part 1.1:

1. The Central Dogma of Molecular Biology
2. Labeling Proteins for Single-Molecule Imaging
a. Methods for labeling proteins
b. Fluorophores

Outline-Part 1.2:

3. Labeling Techniques Employed in Fixed cells
a. Immunolabeling
b. Fluorescence In situ Hybridization (FISH)
4. Questions & Practice
5. Supplementary Material

Exercise 1:

Imagine you need to mark simultaneously three different events in your cells. How do you choose the right fluorophores, so their emission spectra do not bleed into one another? Use the fluorescence spectra viewer below to determine which combination of fluorophores would work to that aim.

https://www.thermofisher.com/order/fluorescence-spectraviewer#!/

Question 1:

Maria is doing a rotation in Prof. Wilson lab, an expert in smFISH. She desires to study simultaneously the expression of several genes involved in a metabolic pathway. However, she is unsure about which type of smFISH to use. Based on what was discussed in this module which type smFISH will you advise Maria to try?

Fluorescent Labeling Techniques used in single-cell Research

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To learn more about the basis and techniques discussed in this lecture visit the following sites:

- https://www.khanacademy.org/science/in-in-class-12-biology-india/xc09ed98f7a9e671b:in-in-the-molecular-basis-of-inheritance
- https://www.labxchange.org/library/pathway/lx-pathway:ad7fbf7e-9fee-4989-b8c6-e5737d21cc91
- https://www.ibiology.org/online-biology-courses/microscopy-series/fluorescence-microscopy/

Sources:

1. Gerd U. Nienhaus & Karin Nienhaus. Fluorescence Labeling. Fluorescence Microscopy, from Principles to Biological Applications. Editorial Wiley, edition 2013 & 2017, chapter 4.
2. Dobrucki and Kubitscheck. Fluorescence Microscopy. From Principles to Biological Applications. Editorial Wiley, edition 2013 & 2017, chapter 3.
3. Labeling Proteins for Single-Molecule Imaging by Photometrics.
4. Fernández-Suárez & Tang, Nat. Rev. Mol. Cell Biol., 2008.
5. Ji X. et al. Bioluminescence imaging in mice with synthetic luciferin analogues. Methods Enzymol. 2020, Chapter 8.
6. Syed and Anderson. *Chem. Soc. Rev.*, 2021, 50, 5668.
7. Immunocytochemistry Handbook by Novus Biologicals.
8. Giepmans et al. Science Review 2006.
9. Neuert & Munsky Science 2013.
10. Munsky PNAS 2018.
11. Pichon et al. Molecular Cell Review 2018.
12. Chen et al, 2015.