3rd Annual Undergraduate Quantitative Biology Summer School

uq-bio

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

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Central Dogma of Molecular Biology

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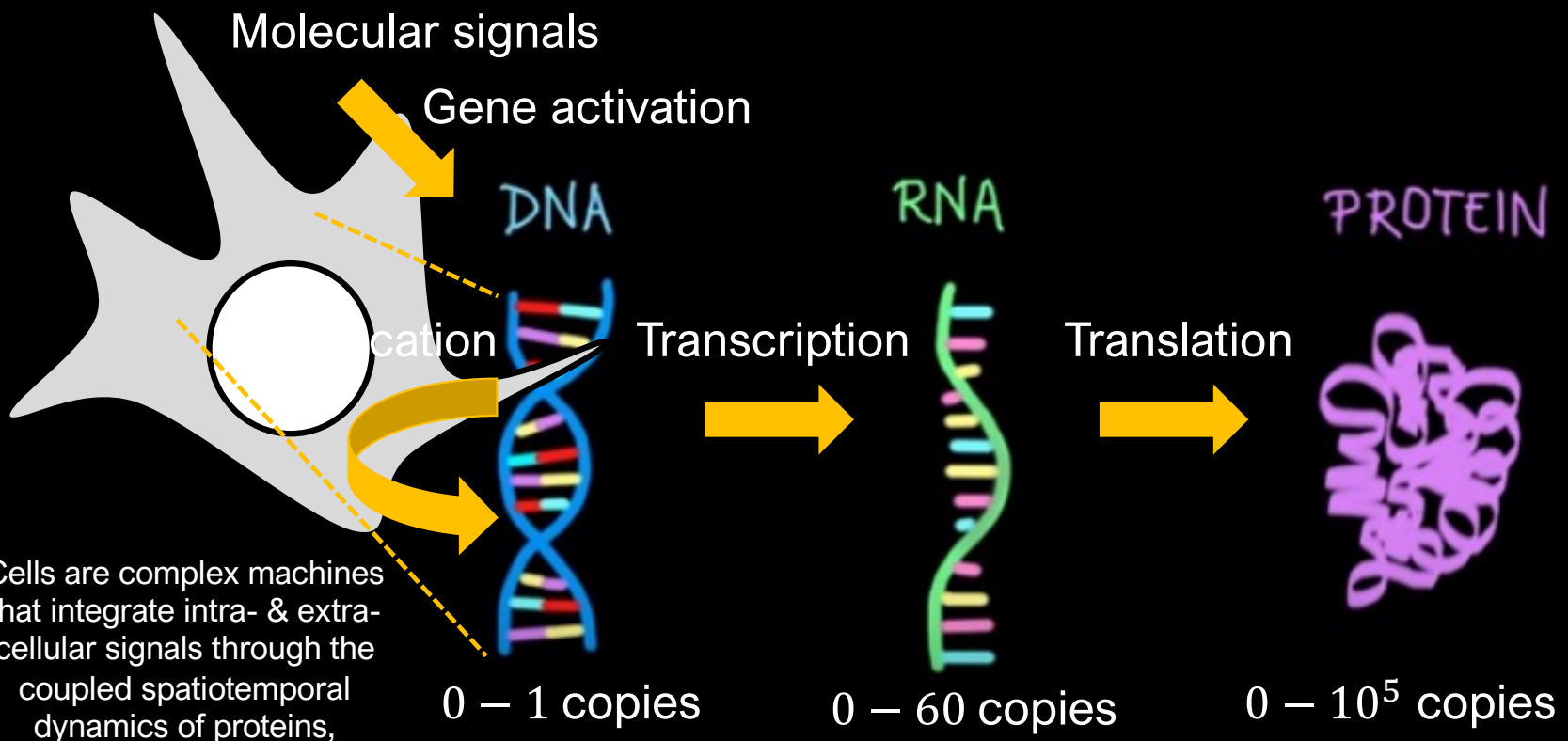
Outline-Part 1:

1. The Central Dogma of Molecular Biology

2. Labeling Proteins for Single-Molecule Imaging
a. Methods for labeling proteins
b. Fluorophores
c. Bioluminescence

3. Labeling Techniques Employed in Fixed cells
a. Immunolabeling
b. Single-Molecule Fluorescence In situ Hybridization (smFISH)



Cells are complex machines that integrate intra- & extra-cellular signals through the coupled spatiotemporal dynamics of proteins, metabolites, nuclei acids, among others.

How can we visualize these processes?

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

Fluorescence Microscopy

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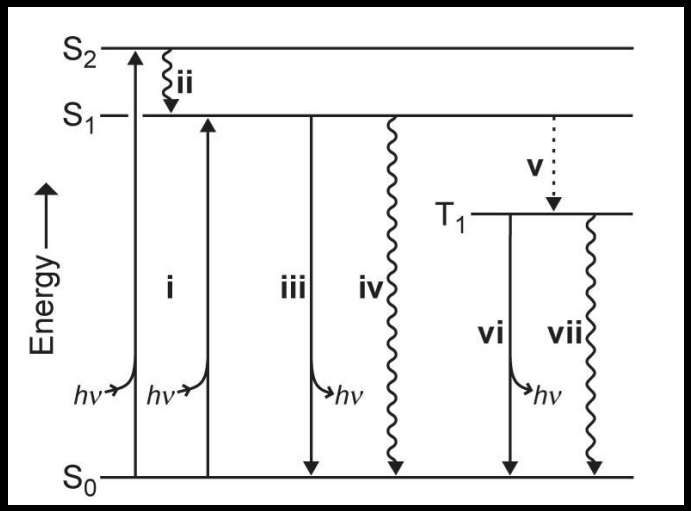
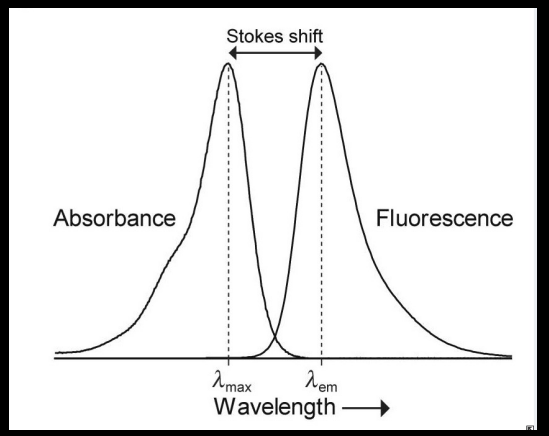
Allows biological processes to be studied as they occur in space and time, at the cellular and molecular levels.

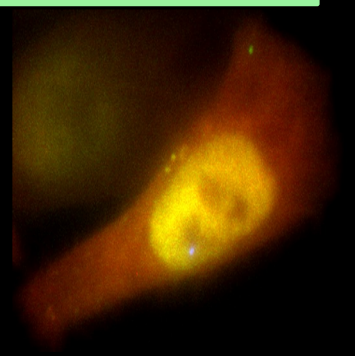


Jablonski diagram, displaying the energy levels and possible routes of excitation & de-excitation of a fluorophore.

How does fluorescence occur?



Example of fluorescence **excitation** and **emission** spectra of a single fluorophore.



Lavis et al. ACS Chem Biol. 2008 Mar 20;3(3):142-55.

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

Fluorescent Labeling Techniques

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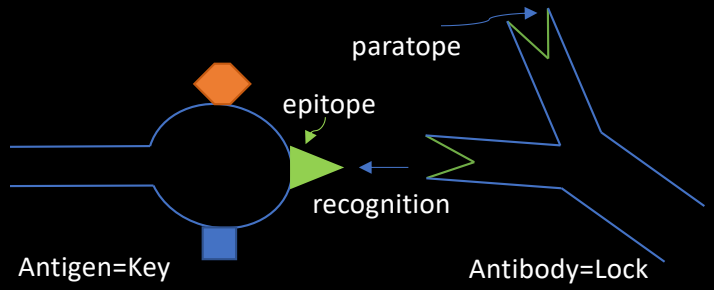
➢ Conventionally, they



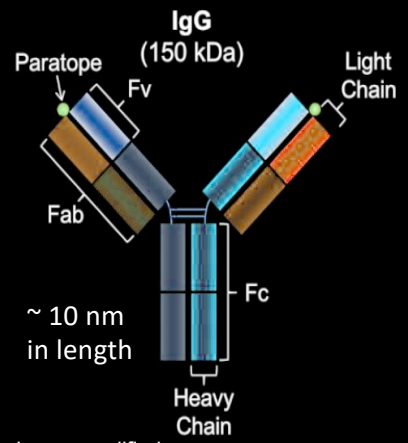
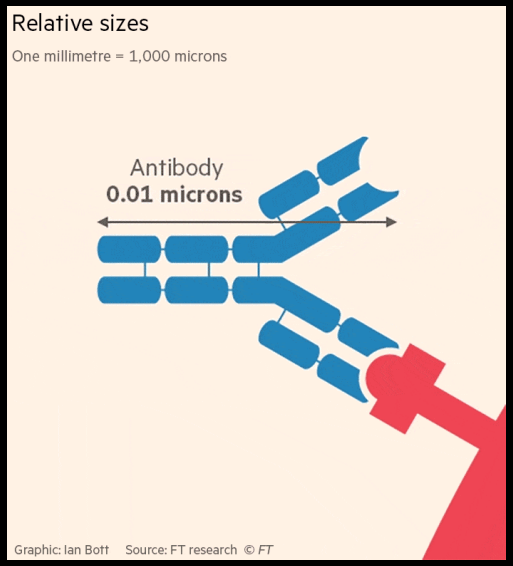


Image modified from Photometrics



Advantages:
1. It is more specific.
2. Can access inner protein domains (e.g., posttranslational modifications).

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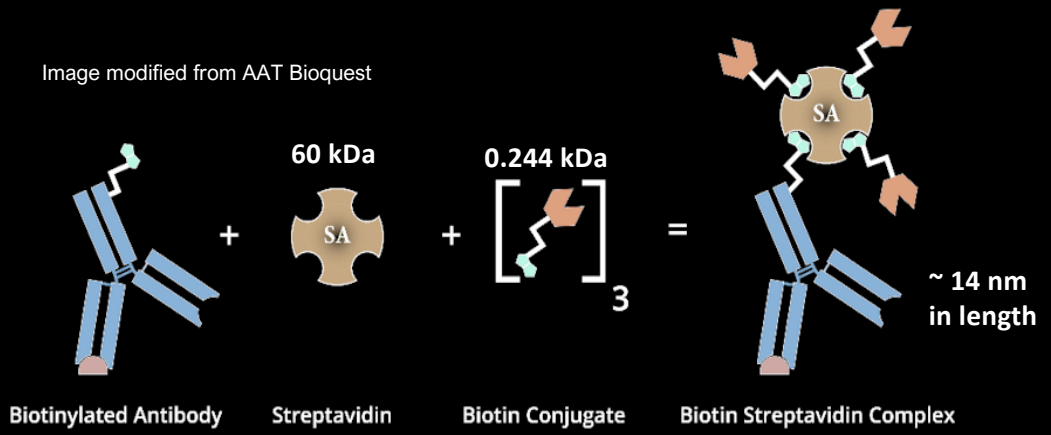
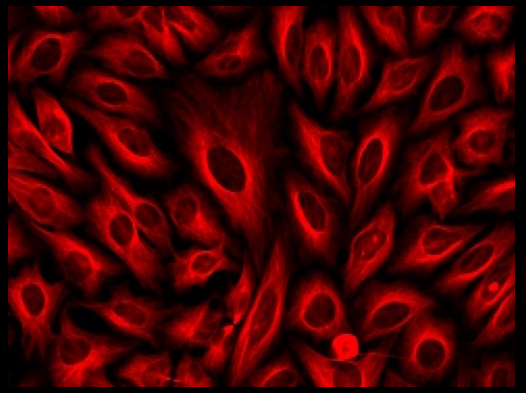
- **Biotinylation** is the covalently process of attaching biotin to a protein, nucleic acid, or other molecule. It has high affinity for its interaction partners avidin and streptavidin, which can be used to tag fluorescent proteins.

Image modified from AAT Bioquest





Immunofluorescent stain of U+03b1-tubulin in HeLas. (Rb Anti-tubulin 1ry AB + biotinylated gt anti-rb IgG, and iFluor 555-Streptavidin conjugate.

Advantages:

1. Size: Extensive binding to biologically active macromolecules e.g., antibodies.
2. Specificity & binding strength between biotin and streptavidin $K_d \sim 10^{-14} mol/L$. (rapid, resistant to pH, T, organic solvents, and denaturing reagents).
3. Does not interfere with physiological processes enabling live cell imaging.

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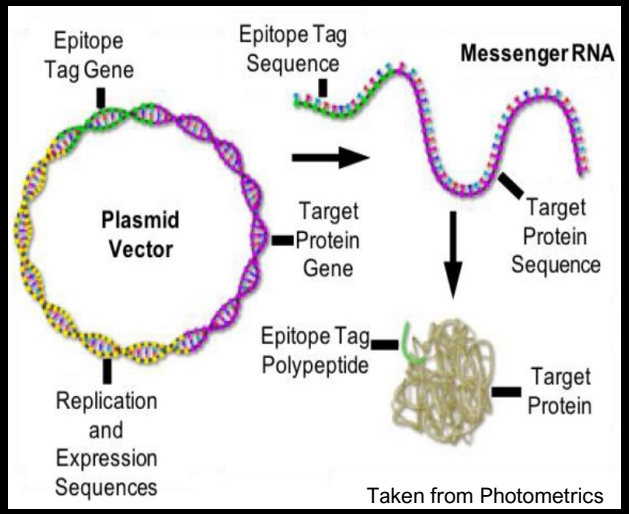
- **Epitope Tags** are useful when antibodies are not available. Those can be encoded into a plasmid vector attached to the protein of interest.



Taken from Photometrics

Examples:

- **Flag-Tag** (AA-Sequence: DYKDDDDK), ~1.0 kDa
- **HA-Tag** (AA-Sequence: YPYDVPDYA) , ~1.1 kDa
- **V5-Tag** (AA-sequence: GKPIPNPLLGLDST) , ~1.4 kDa
- **Myc-Tag** (AA-Sequence: EQKLISEEDL), ~1.2 kDa

Advantages:

1. Live-cell imaging, e.g., translation processes in real time.

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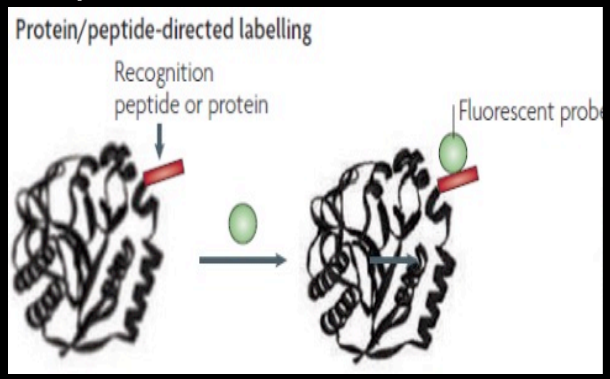
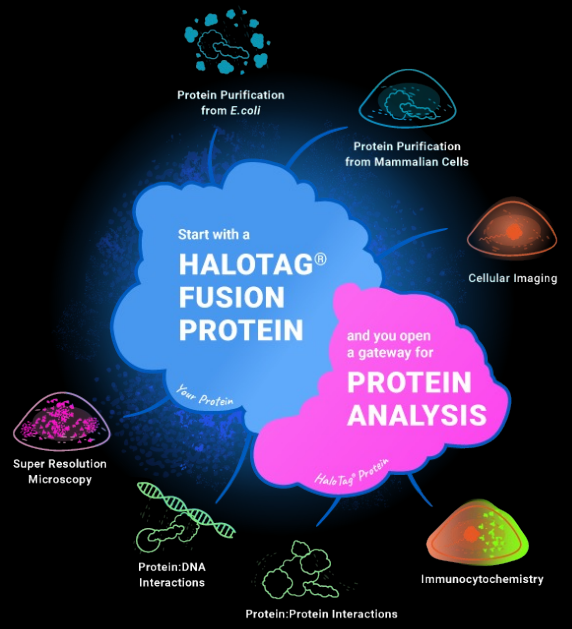
➢ **Small molecules probes** are recruited by a peptide or protein recognition sequence that is fused to the target protein.



Taken from Fernández-Suárez & Tang, 2008

Examples:

- ❖ Self or Direct Labeling: PolyAsp, HaloTag (33kDa), SNAPTag and CLIPTag (~20 kDa).
- ❖ Enzyme Mediated Labeling: SorTag & Qtag (~10 kDa), AB, & LAP



Advantages:

1. Can be used in combination with or as an alternative to antibodies.
2. Live-cell imaging, e.g., translation processes in real time.
3. Binding to a fluorescent probe is rapid and irreversible.
4. Enzyme mediated tags are smaller than self-tags, but require an enzyme to mediate labeling.

Fluorophores

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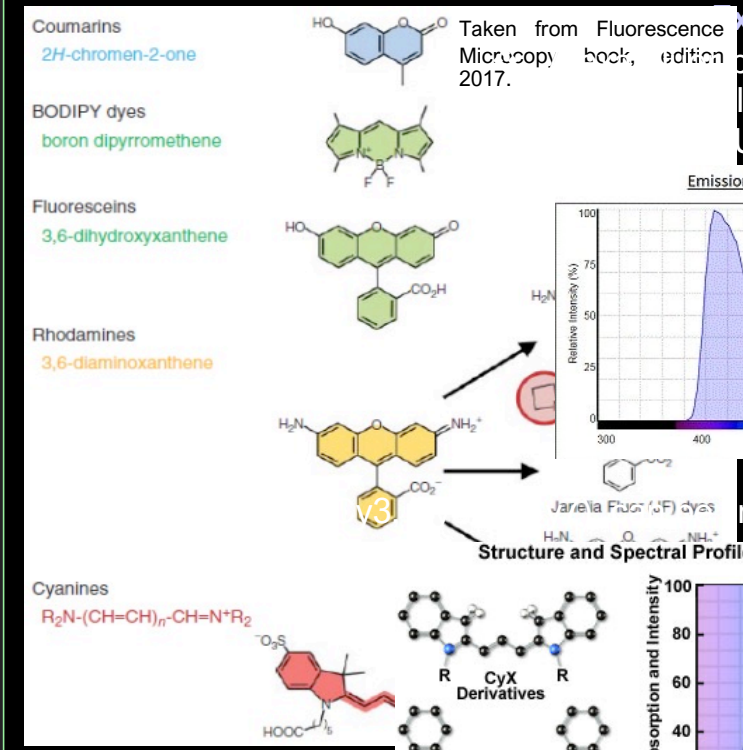
- **Organic dyes** are generally planar molecules of about **1-2 nm**. These dyes belong to different families based on their core scaffolds.



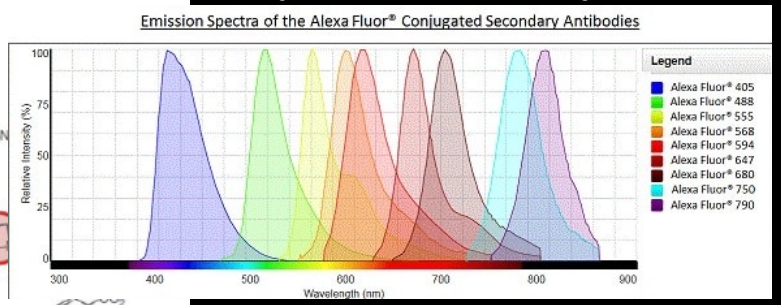
Taken from Fluorescence Microscopy book, edition 2017.

Examples of commercial organic dyes:

Possesses the largest spectral variety available (UV to near-infrared

Alexa molecules are negatively charged and hydrophilic

Usually linked to secondary AB.



Advantages

- Enhanced photostability.
- Adsorption spectra matched to common laser lines.
- pH insensitivity.
- Water soluble.

Linked to nucleic acids or proteins through their interactive groups.

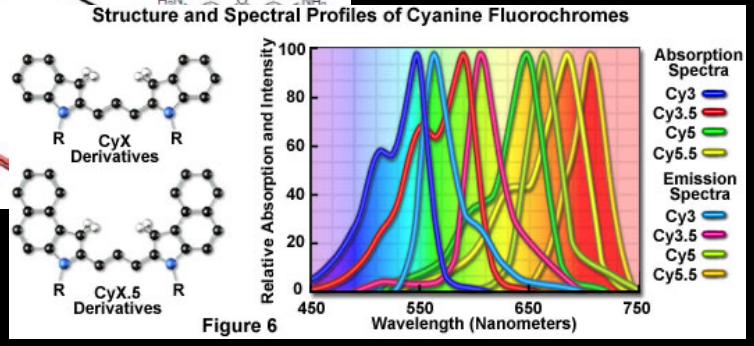


Figure 6

Advantages

- Broader adsorption spectral regions than members of Alexa Fluor family, thus more versatile when choosing the excitation source.

Taken from Olympus confocal web site.

Fluorophores

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- **Quantum dots** are inorganic semiconductor nanocrystals, typically composed of a cadmium selenide core and a zinc sulphide shell, coated with a layer to improve solubility. Its size ranges **10-15 nm.**

Advantages

- Long-term photostability.
- High fluorescent intensity levels.
- Multiple colors with single-wavelength excitation for all emission profiles emission.

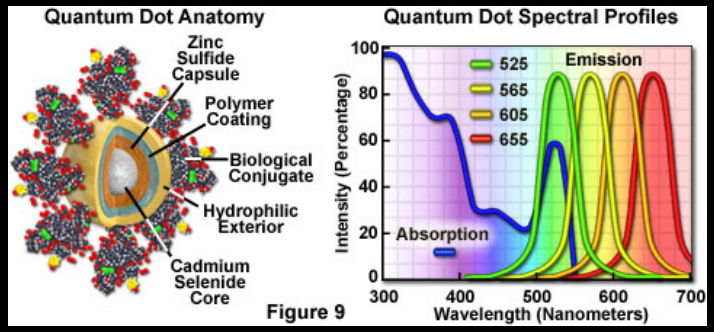
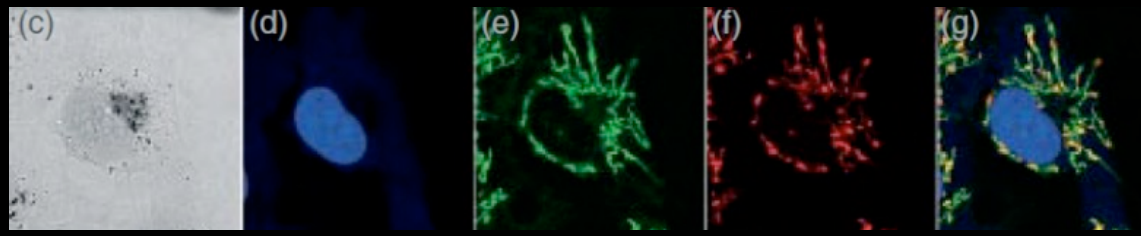


Figure 9

Taken from Olympus confocal web site.

- **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.

Fluorescent Proteins (GFP)

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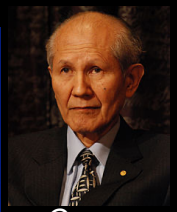
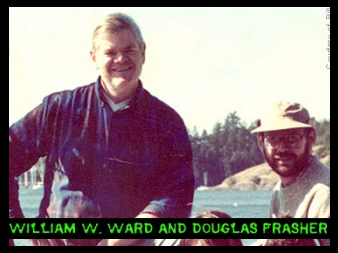
➢ **Fluorescent proteins** are found naturally, are very small, specific, and can be genetically encoded into the protein of interest. Green Fluorescent Protein (**GFP**), was the 1st fluorescent protein to be reported, its molecular weight is 28 kDa (~4 nm).

Advantages

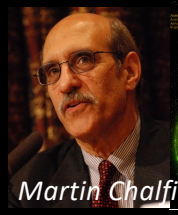
- Investigation of Intracellular process in living organisms.
- Visualize, monitor and track individual molecules with high spatiotemporal resolution.
- Respond to a wider variety of biological events and signals.
- Extremely low phototoxicity.

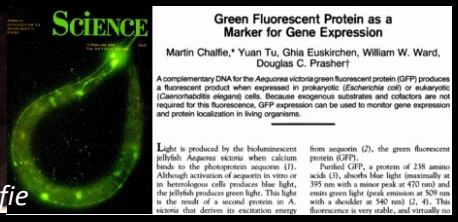

Osamu Shimomura

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



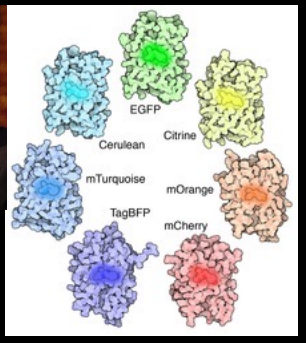
1992: Douglas Prasher reported the cloning and nucleotide sequence for *wt*-GFP in gene


Martin Chalfie



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


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

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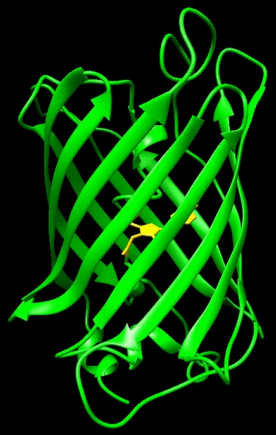
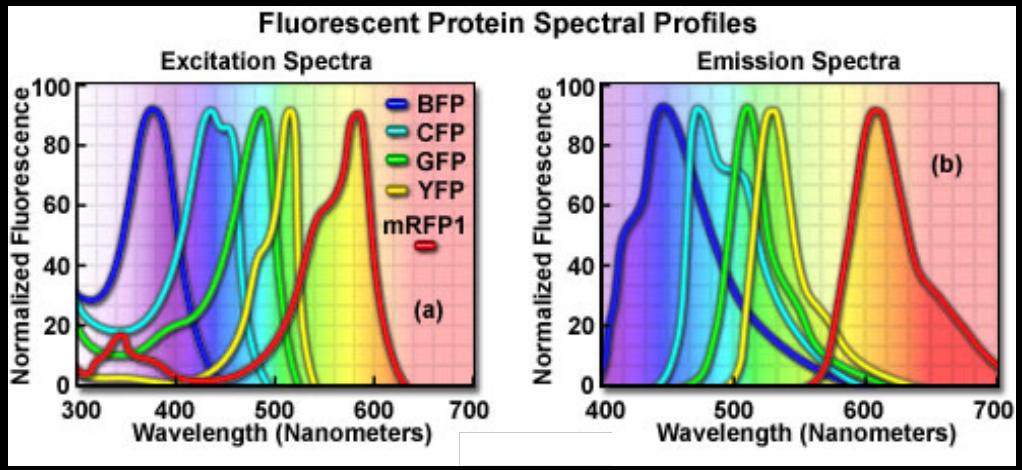
➢ **Fluorescent proteins** (GFP)



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

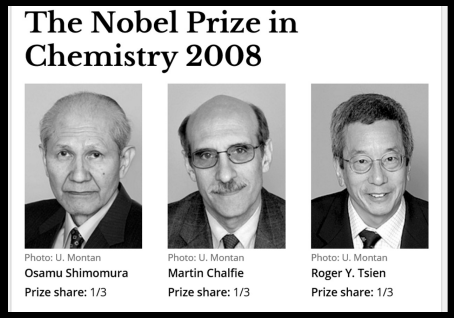
Spectra of GFP variants



Taken from Olympus confocal web site.

Most common applications:

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



Discovery, expression and development of GFP

Bioluminescence

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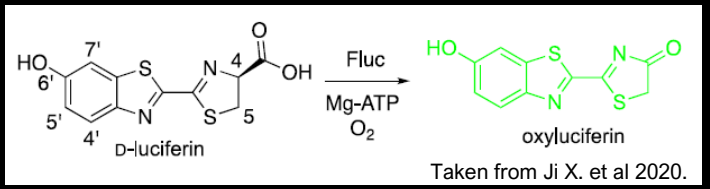
- **Bioluminescence** is the production and emission of light by a living organism on its own. It uses energy from adenosine triphosphate (ATP) but it does not require light.

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, enzymes, drug molecules, among others.
- Protein-Protein interaction.
- *In vivo* imaging.

Cell Culture

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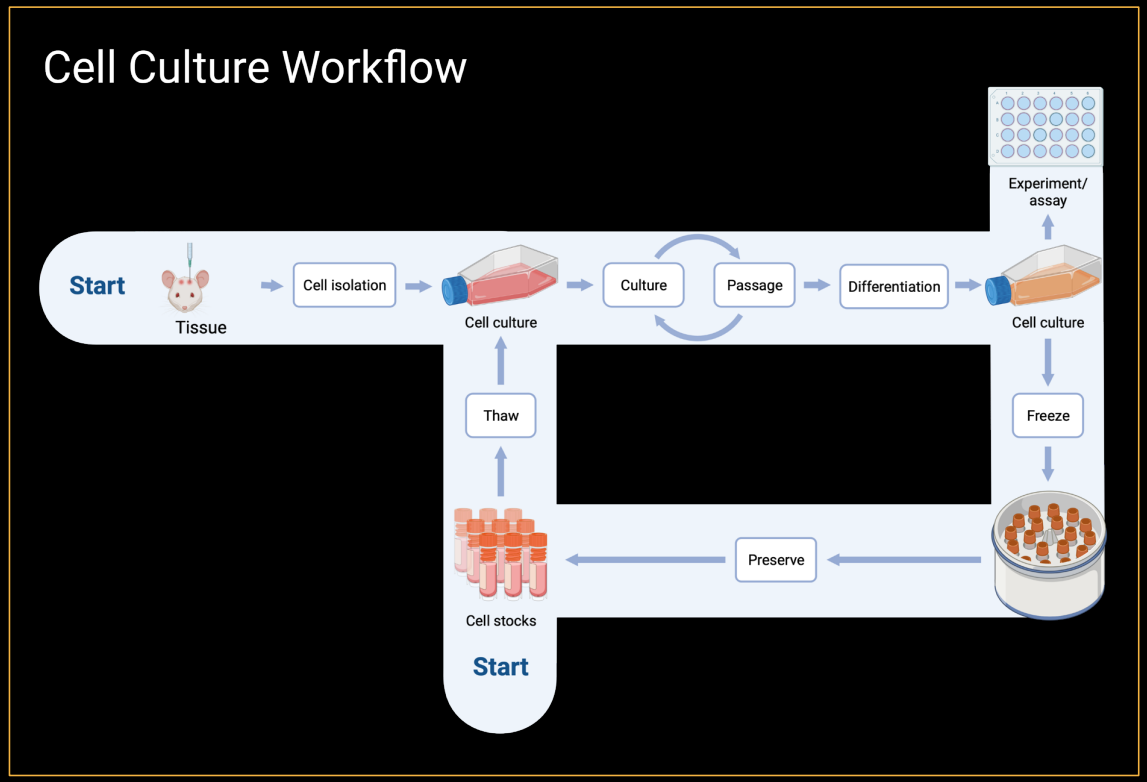
a. Cell Culture is the process of growing and maintaining cells in a controlled environment outside of their natural organism. Maintaining healthy cells, from immortal HeLa cells to delicate neurons, is crucial for studying disease mechanisms, developing new therapies, and advancing our understanding of single-cell processes



Fluorescent labeling techniques used in single-cell research

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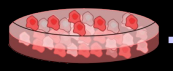
a. Immunolabeling is a biochemical method that allows the detection and localization of an antigen in a cell, tissue or organ, the antigen is usually a protein, and the detection is performed using antibodies.

Sample Preparation



Cultured cells → Fixation → Permeabilization (Detergents: Triton x100) → Blocking (Serum) → Antibody Incubation

Fixation → Chemical Crosslinkers (PFA)

Fixation → Precipitants (Methanol or acetone)

Immunolabeling

Antibody Incubation → Direct Detection → Labeled 1ry AB

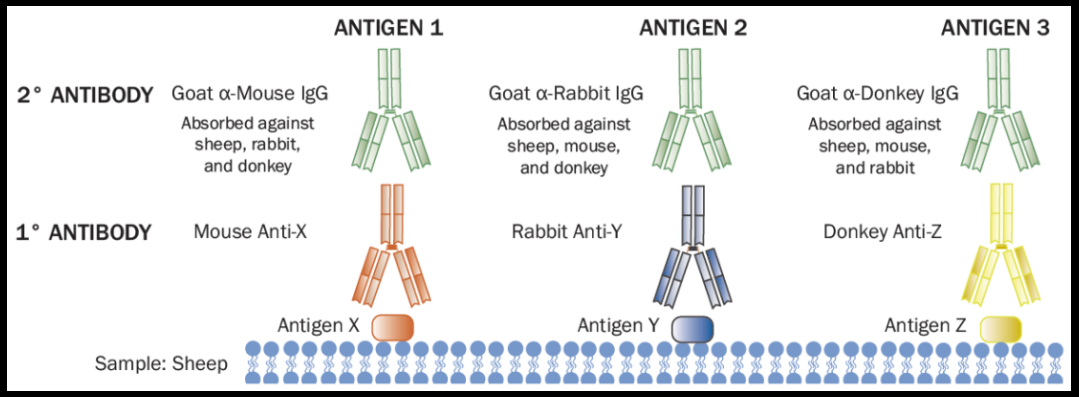
Antibody Incubation → Indirect Detection → unlabeled 1ry AB → Labeled 2ry AB

Labeled 1ry AB → Nuclear counterstaining (DAPI, Hoechst)

Labeled 2ry AB → Nuclear counterstaining (DAPI, Hoechst)

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

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



Taken from Immunocytochemistry Handbook by Novus Biologicals

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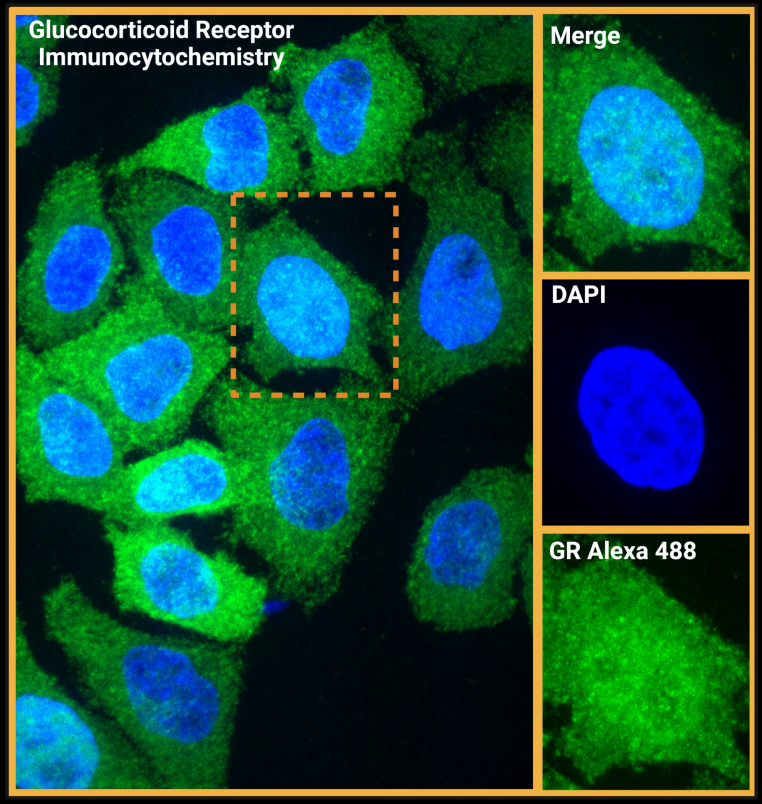
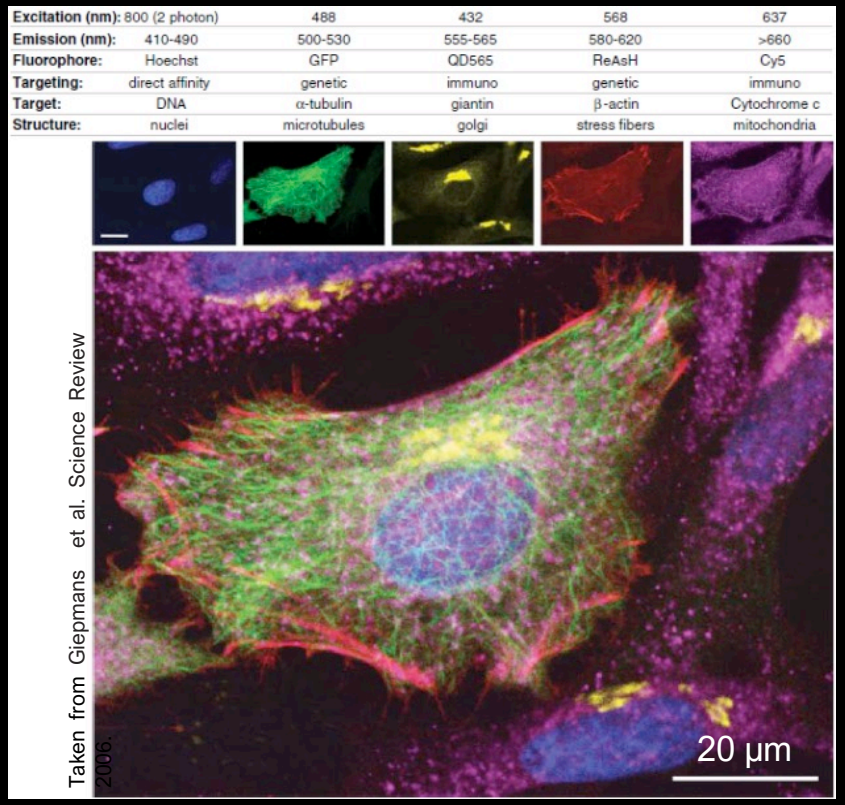
Example of a simple immunolabeling



Example of Parallel application of targeting methods & fluorophores



Taken from Giepmans et al. Science Review 2006.

Single-Molecule Fluorescence In situ Hybridization (smFISH)

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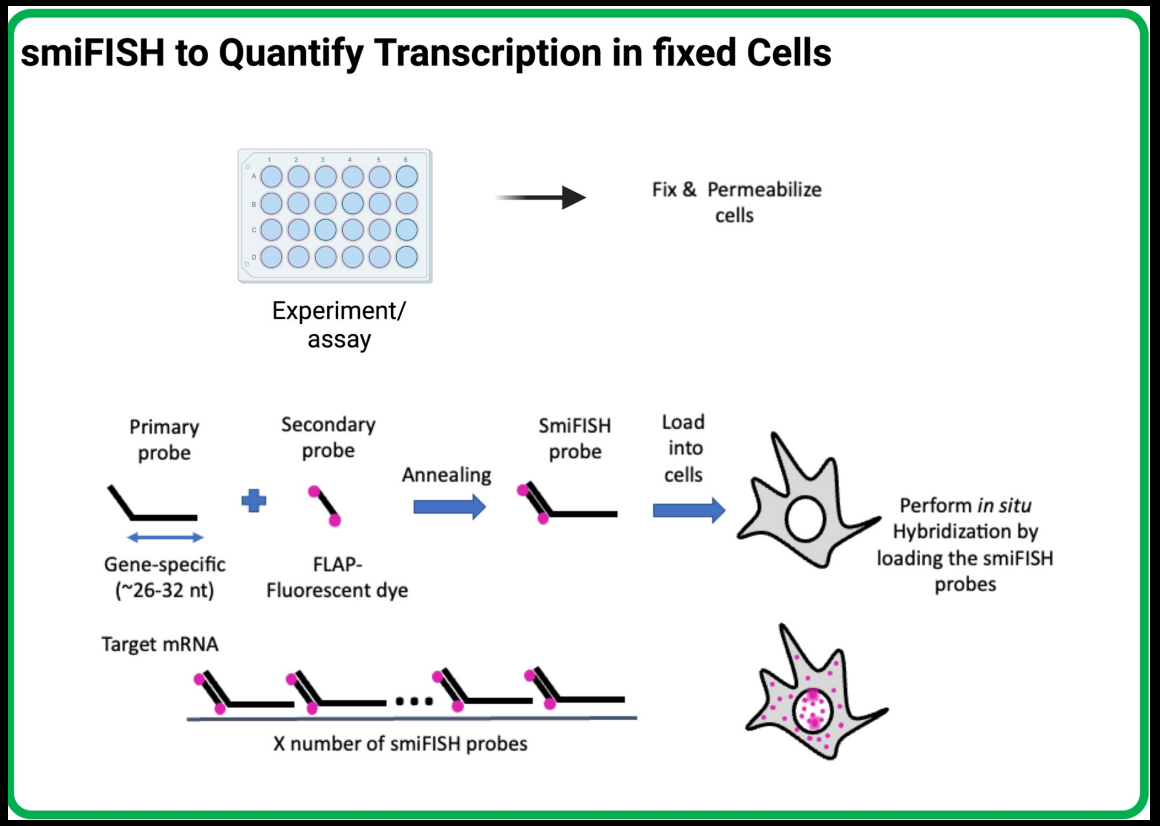
b. smiFISH: i for "Inexpensive" quantification of endogenous mRNA



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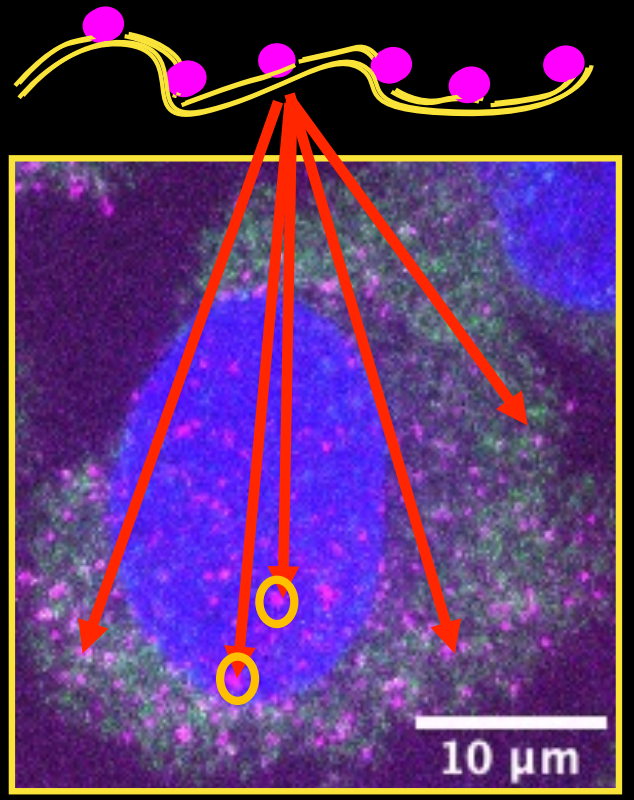
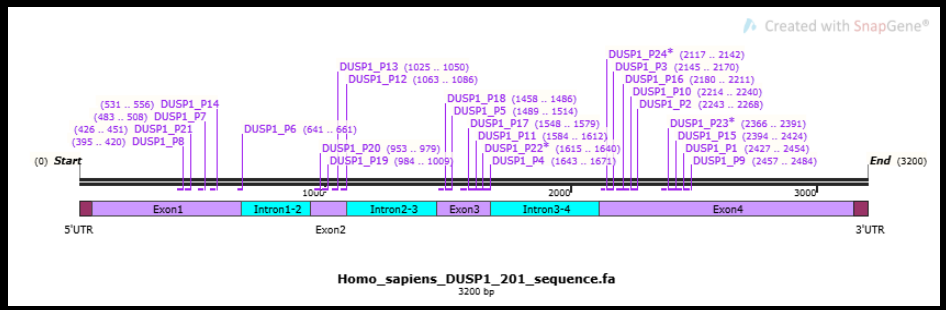
b. Single-Molecule Fluorescence In situ Hybridization "smFISH" (Femino, Singer, 1998) allows the quantification of endogenous transcription response:

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



smFISH probes
21 (20bp) probes/mRNA
Dual Specificity Phosphatase 1 (DUSP1)



Single-Molecule Fluorescence In situ Hybridization (smFISH)

uq-bio

Outline-Part 1:

1. The Central Dogma of Molecular Biology

2. Labeling Proteins for Single-Molecule Imaging
a. Methods for labeling proteins
b. Fluorophores
c. Bioluminescence

3. Labeling Techniques Employed in Fixed cells
a. Immunolabeling
b. Single-Molecule Fluorescence In situ Hybridization (smFISH)

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

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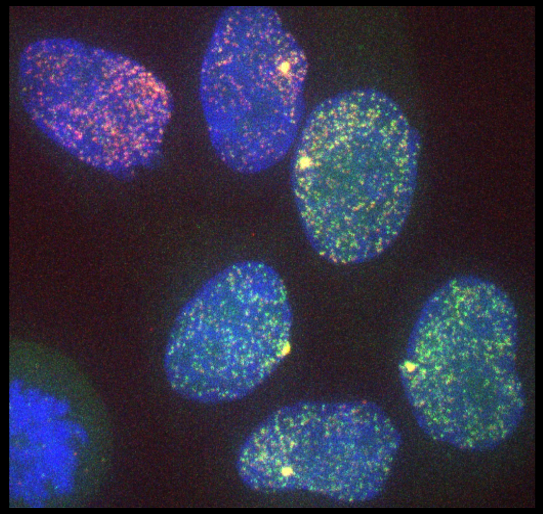

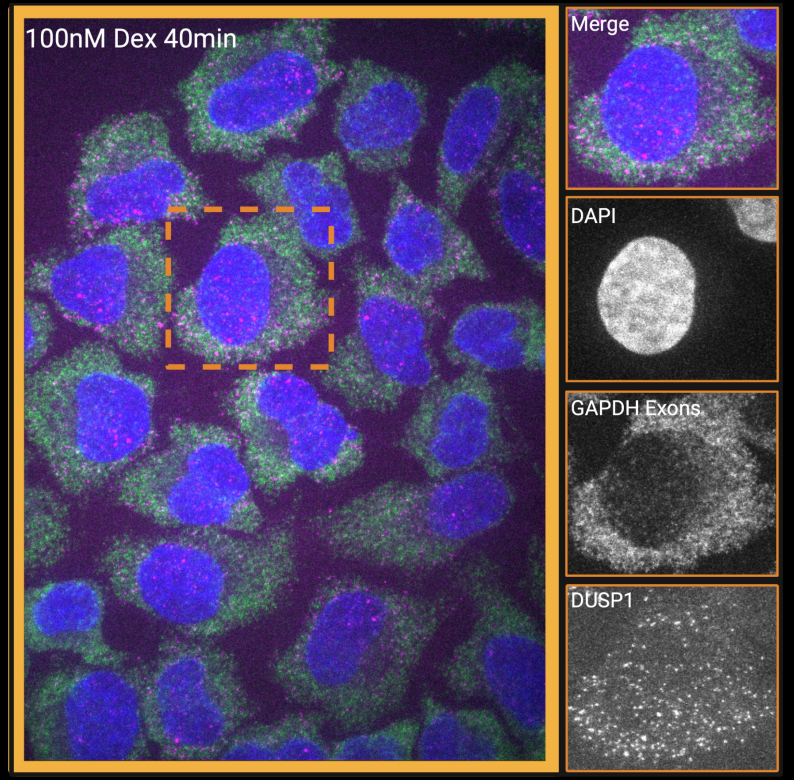

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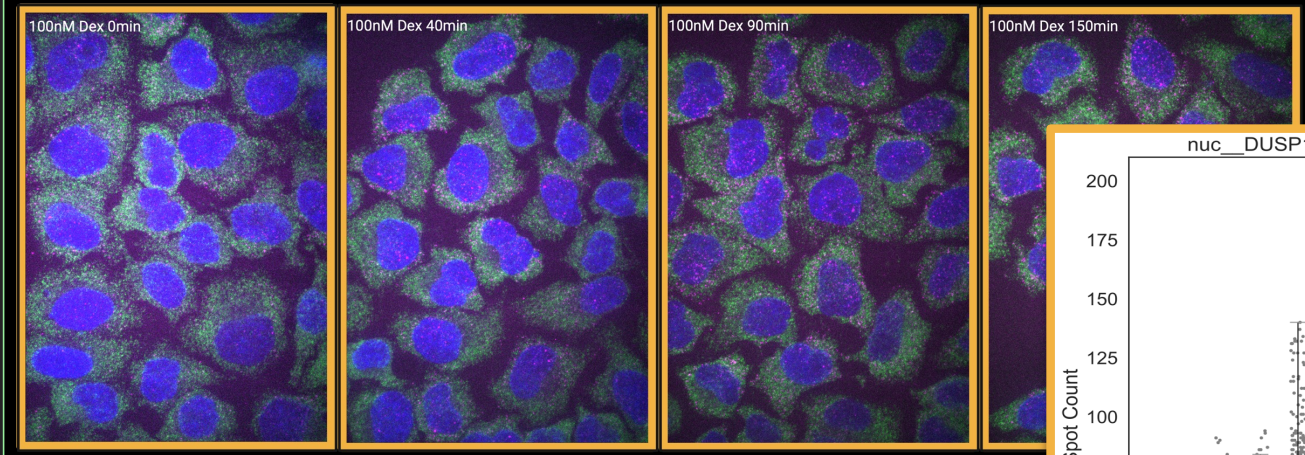

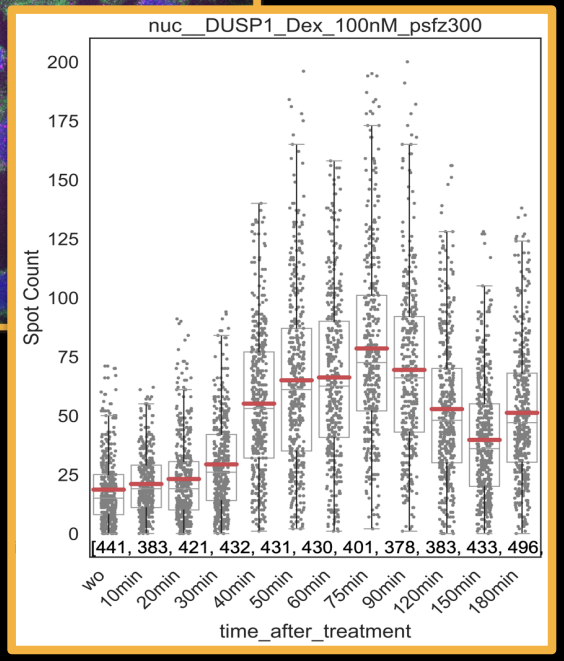

- **Number** of individual mRNA per cell,
- **3D Location** of individual mRNA,
- **DNA transcription** site activity,
- Fast (1-2 minute) time resolution,
- 100s or 1000s of cells per time point, or condition.

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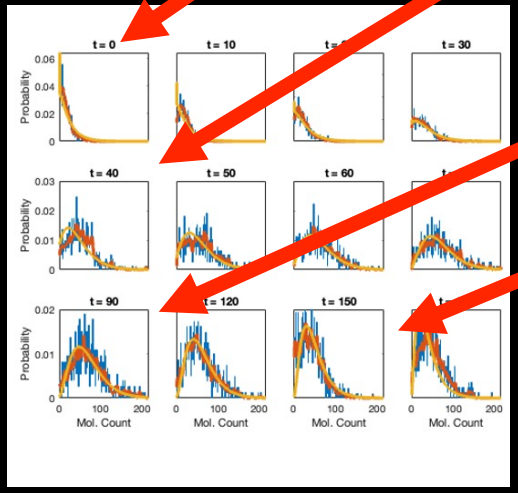
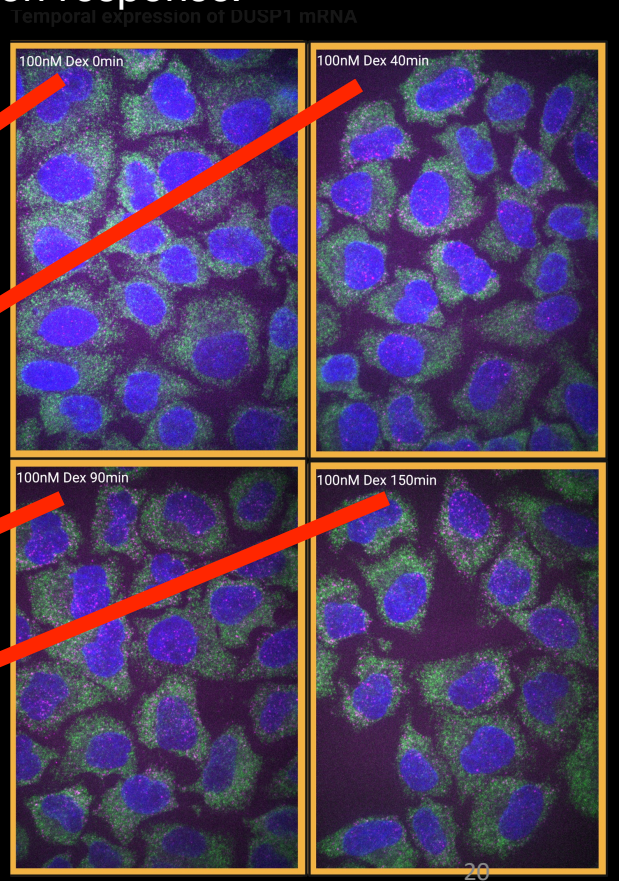
- 100s or 1000s of cells per time point, or condition.

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





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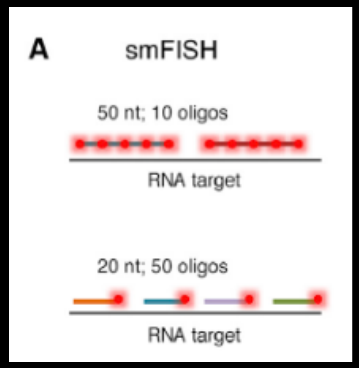
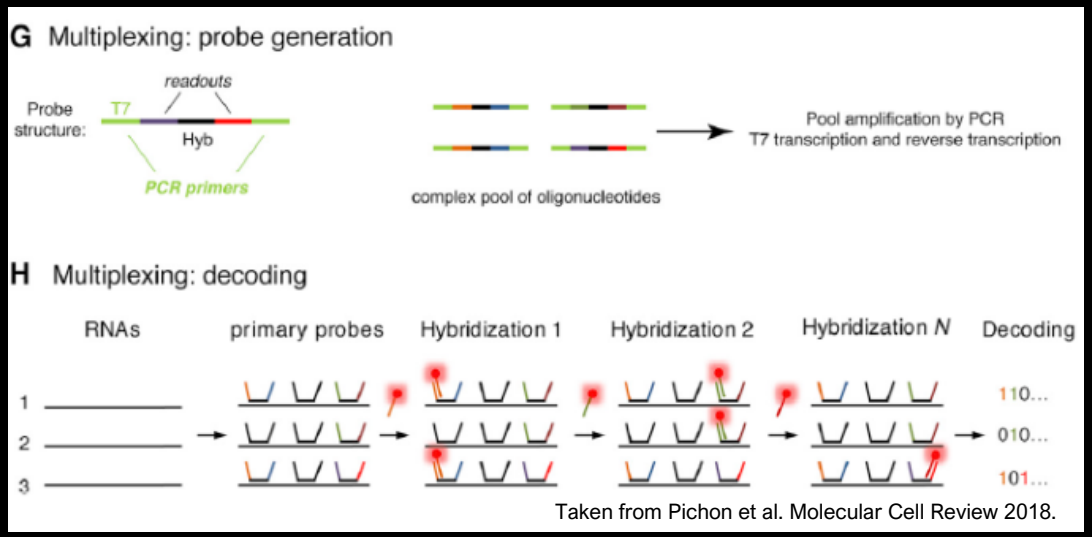
Types of smFISH based on probe design



- 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).



Taken from Pichon et al. Molecular Cell Review 2018.

- 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.

Single-Molecule Fluorescence In situ Hybridization (smFISH)

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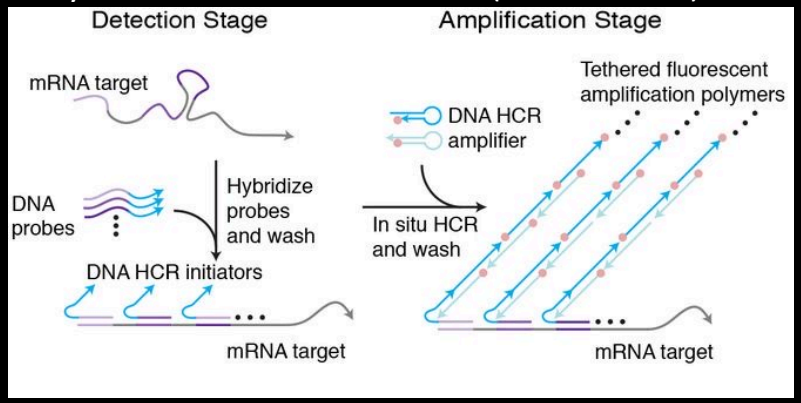
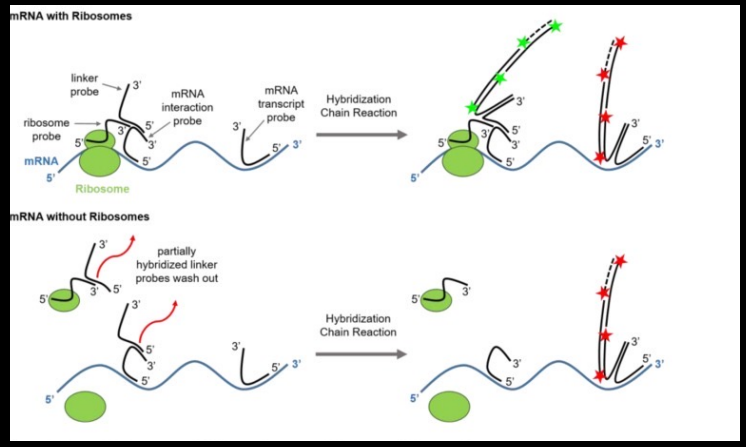
Other smFISH Methods

- Hybridization chain reaction (HCR smFISH)



- Ribosomal occupancy



smFISH examples

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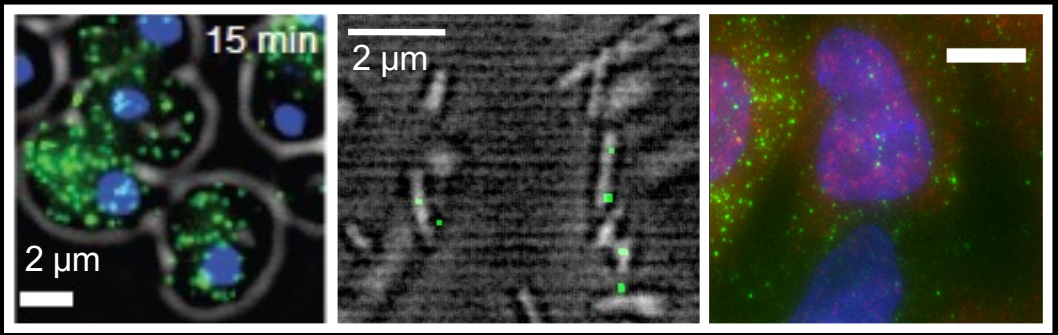
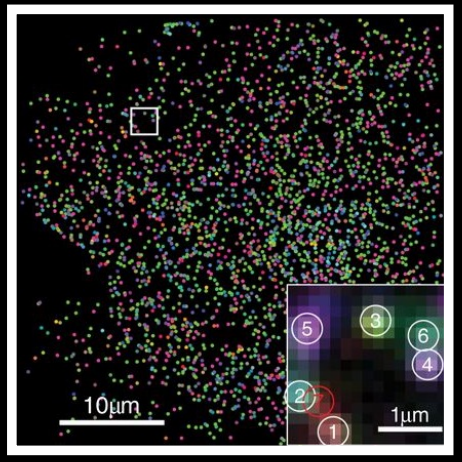
Examples of smFISH, it has been applied to many different RNA in many different organisms.



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

Labeling Techniques in live cells at the single-molecule level

Outline-Part 2:
1. Labeling Techniques Employed in live cells:
a. To visualize transcription
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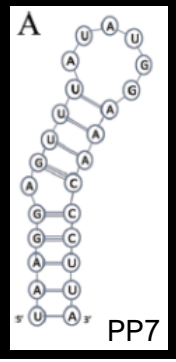
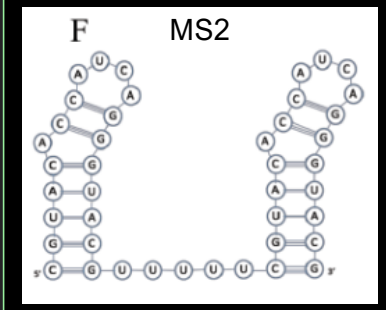
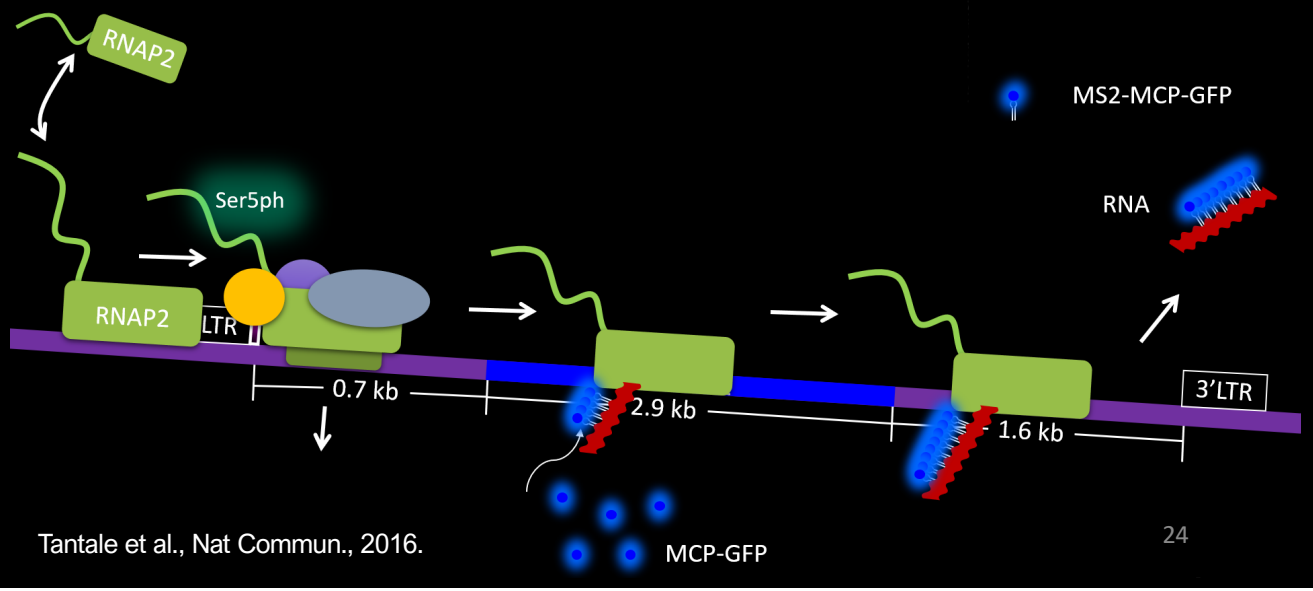
a. Phase Imaging/diffraction tomography
3. Sources

- **MS2 (Bertrand et al. 1998) or PP7 (Larson et al. 2011) tagging** are aptamers-based approaches to label RNA. This technique takes advantage of the natural interaction of MS2 or PP7 bacteriophages coat proteins (MCP or PCP) with a stem-loop structure from the phage genome.





Taken from Gemmill et al. 2020



Tantale et al., Nat Commun., 2016.

Labeling Techniques in live cells at the single-molecule level

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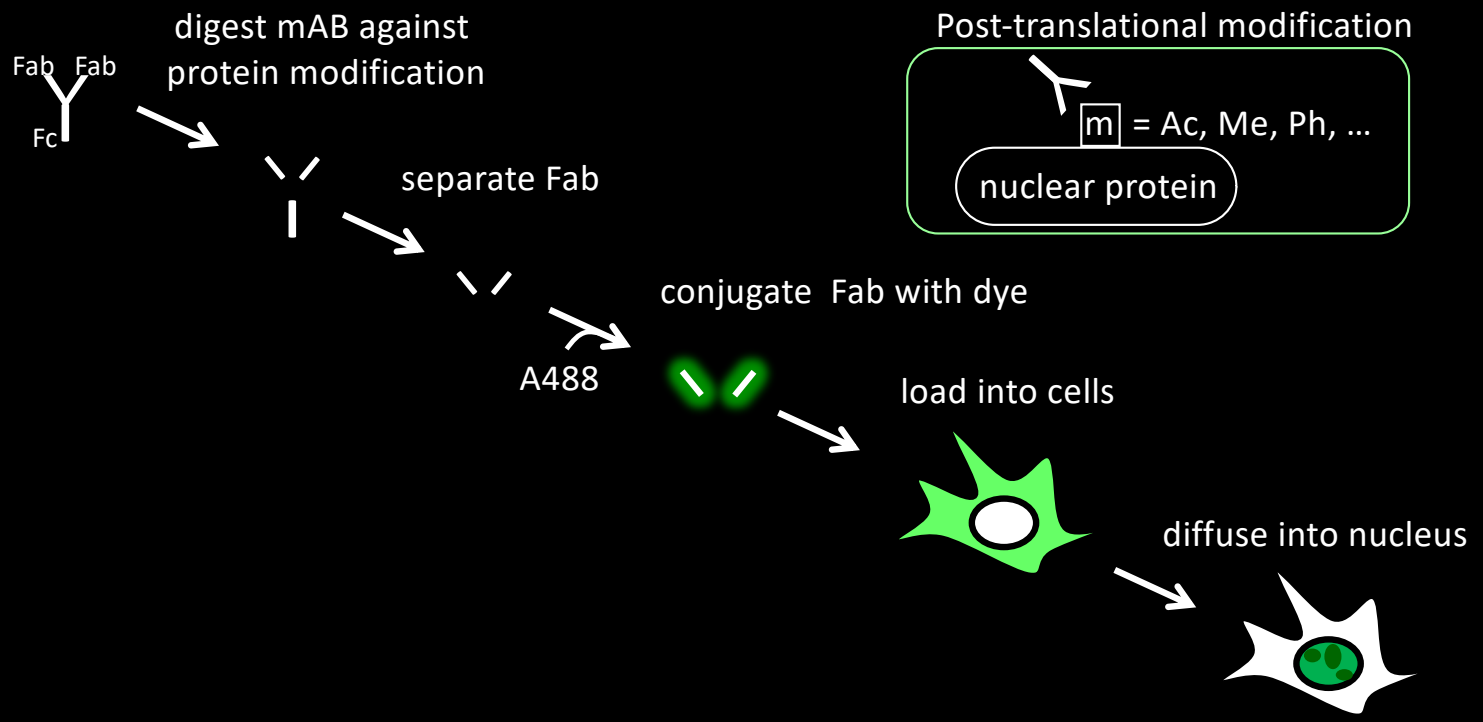
- **FabLEM** are fragmented antibodies designed to target endogenous post-translational modifications in live cells.



Hayashi-Takanaka et al, NAR, 2011

Visualizing transcription by genetically encoded probes

Outline-Part 2:
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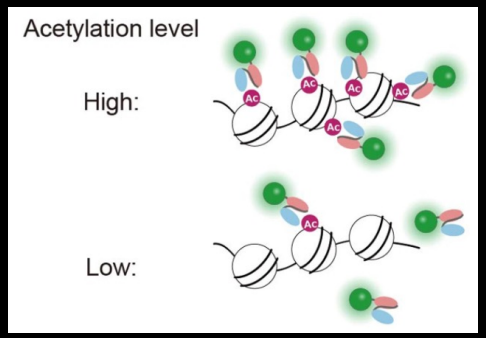
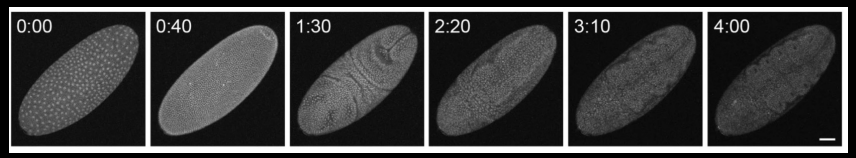
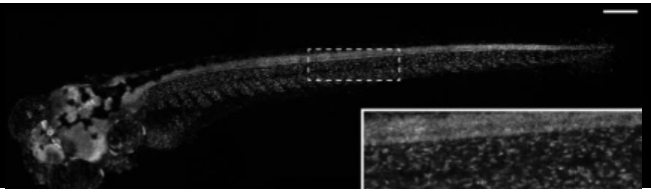
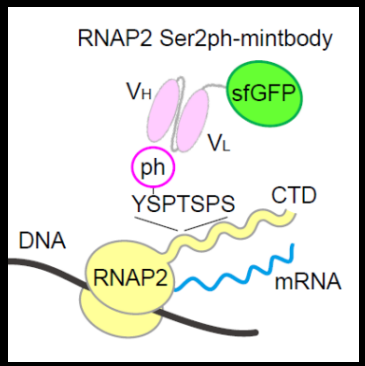
- **Genetically encoded modification-specific intracellular antibody (mintbody) probes** are designed against a specific modification (such as H3K9ac or RNAP2-Ser2ph). To generate a mintbody, the coding sequence of several antibodies heavy and light chains specific against the desired modifications are cloned and tagged with a fluorescent protein (e.g. sfGFP) and then transfected into the desired cells.

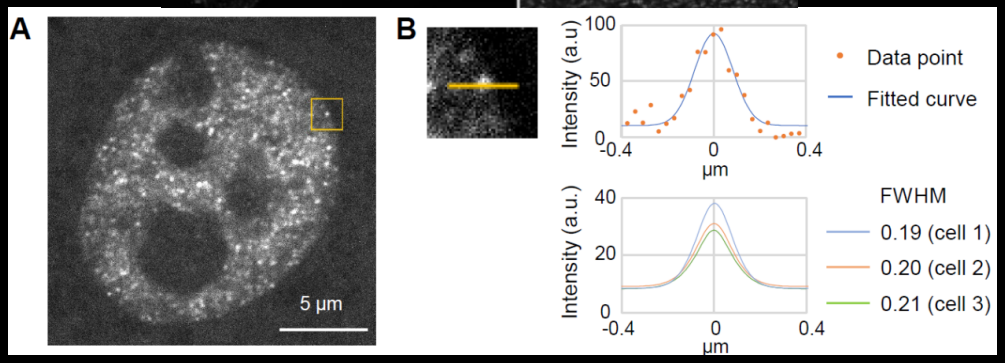






Sato et al., Sci. Rep, 2013





Uchino et al., J Cell Bio, 2022

Visualizing transcription dynamics at a gene array

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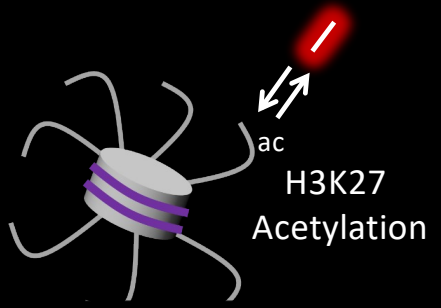
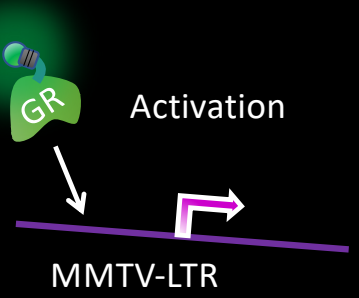
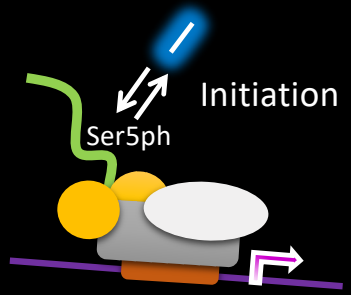
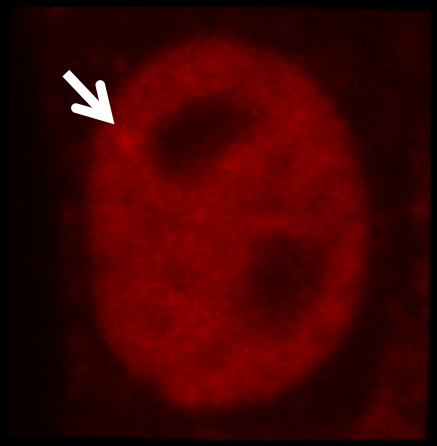
Example of imaging modifications using FabLEM at a tandem gene array

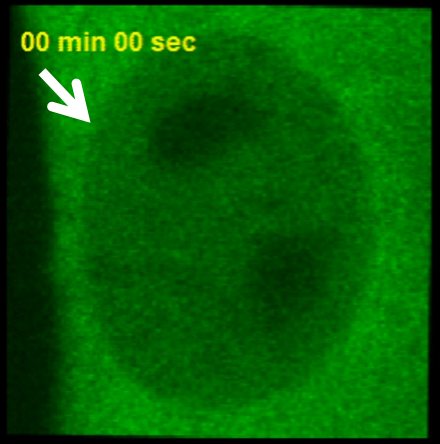




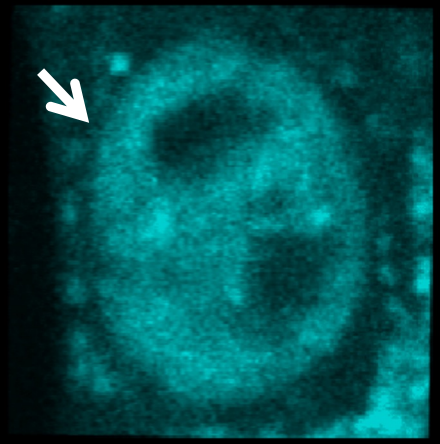




H3K27ac



GFP-GR



RNAP2 Ser 5ph

MMTV-LTR: Mouse Mammary tumor virus-Long Terminal Repeat
GR: Glucocorticoid Receptor
Stasevich et al, Nature 516, 272-275 (2014)

Visualizing the transcription cycle at a single-copy gene

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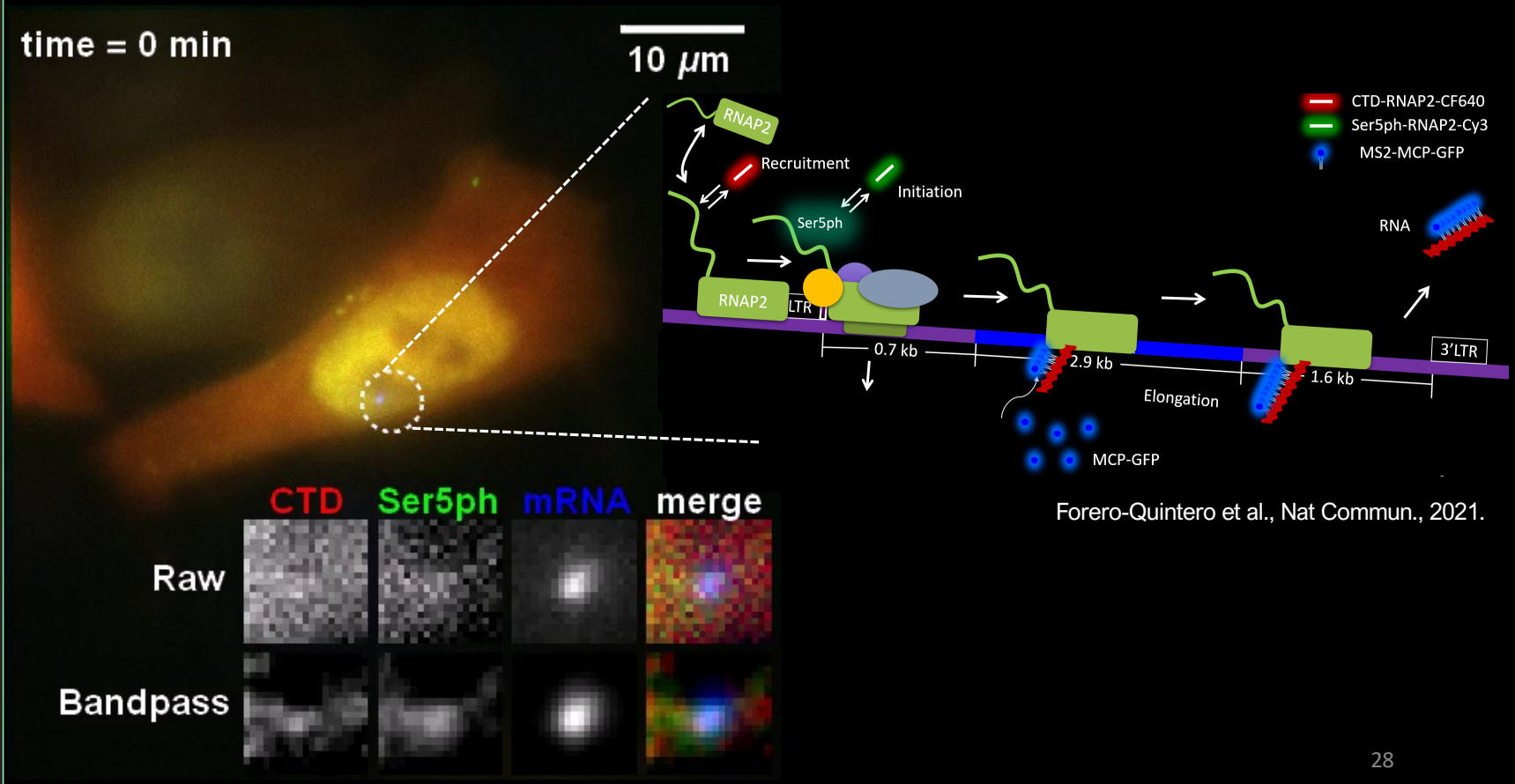
Example combining MS2 labeling and FabLEM to visualize RNAP2 phosphorylation dynamics at a single-copy gene



Forero-Quintero et al., Nat Commun., 2021.

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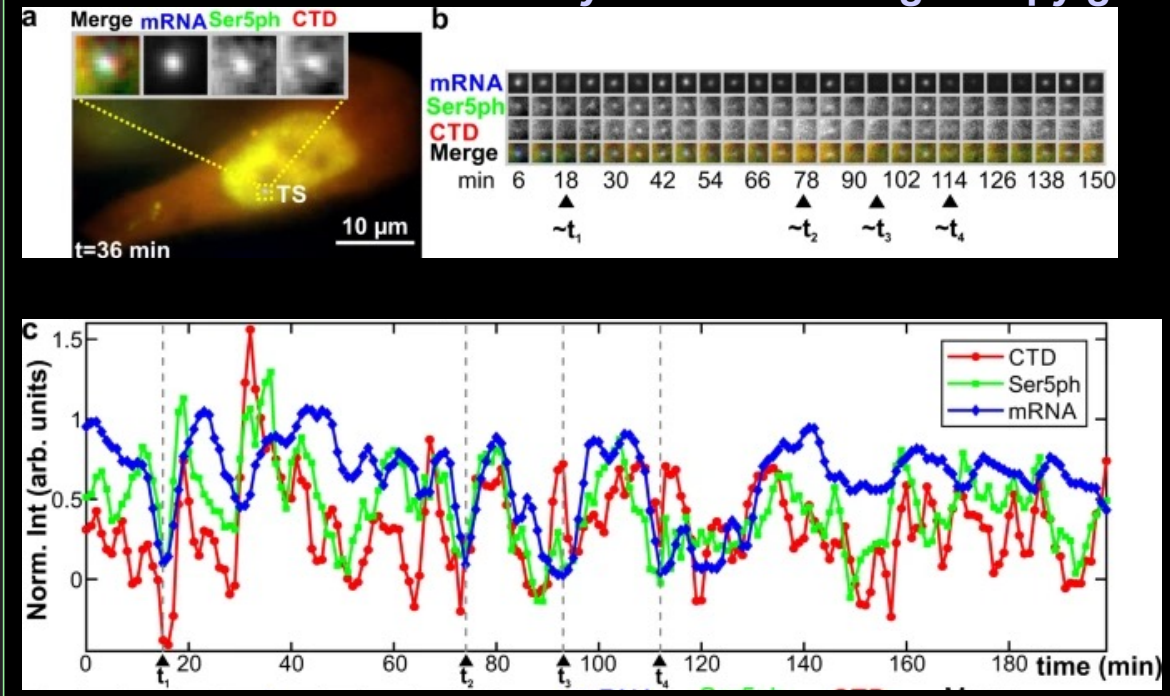

Forero-Quintero et al., Nat Commun., 2021.

Visualizing translation by Nascent Tracking Chain (NCT)

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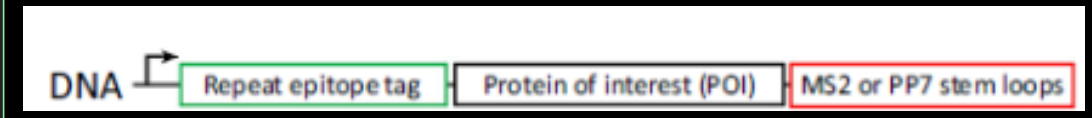
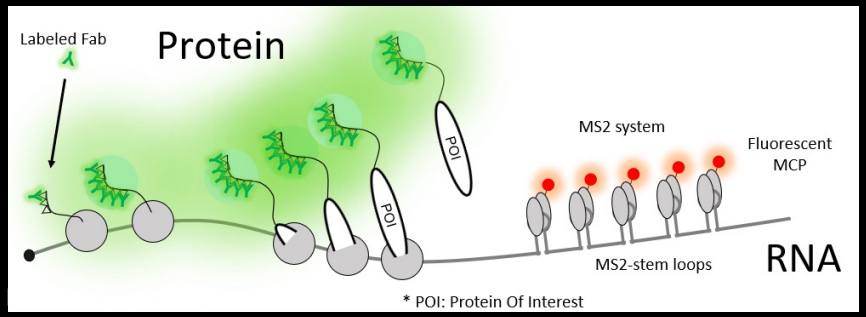
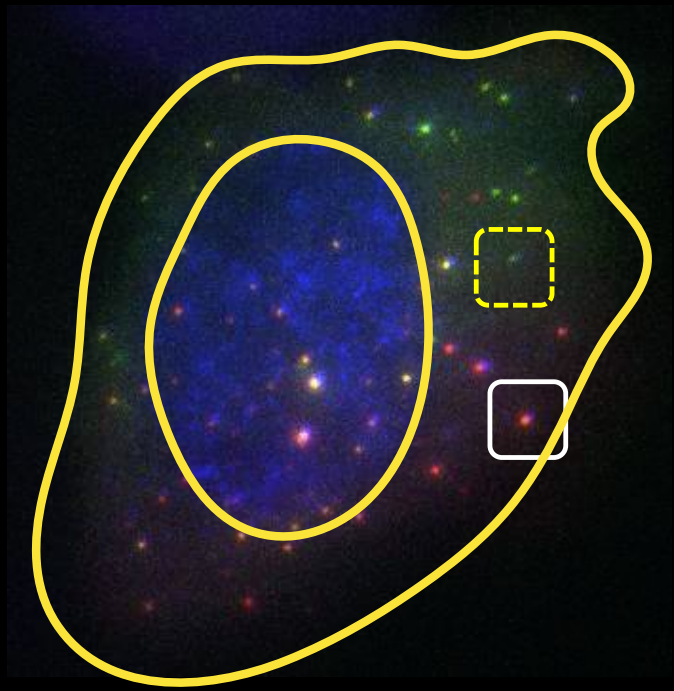
➢ **Nascent Chain Tracking (NCT)** allows of single-mRNA nascent peptide translation.



Morisaki et al., Science, 2016



Taken from Lyon and Stasevich. Trends in Genetics Rev., 2017



- ❑ RNA can be labeled in live cells using MS2/MCP system.
- ❑ Peptides can be labeled with multiple fluorescent antibody fragments.
- ❑ Quantify Nascent protein translation from a single mRNA.

Current methods for visualizing single-mRNA translation

uq-bio

Outline-Part 2:

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3. Sources

Dr. Ning Zhao

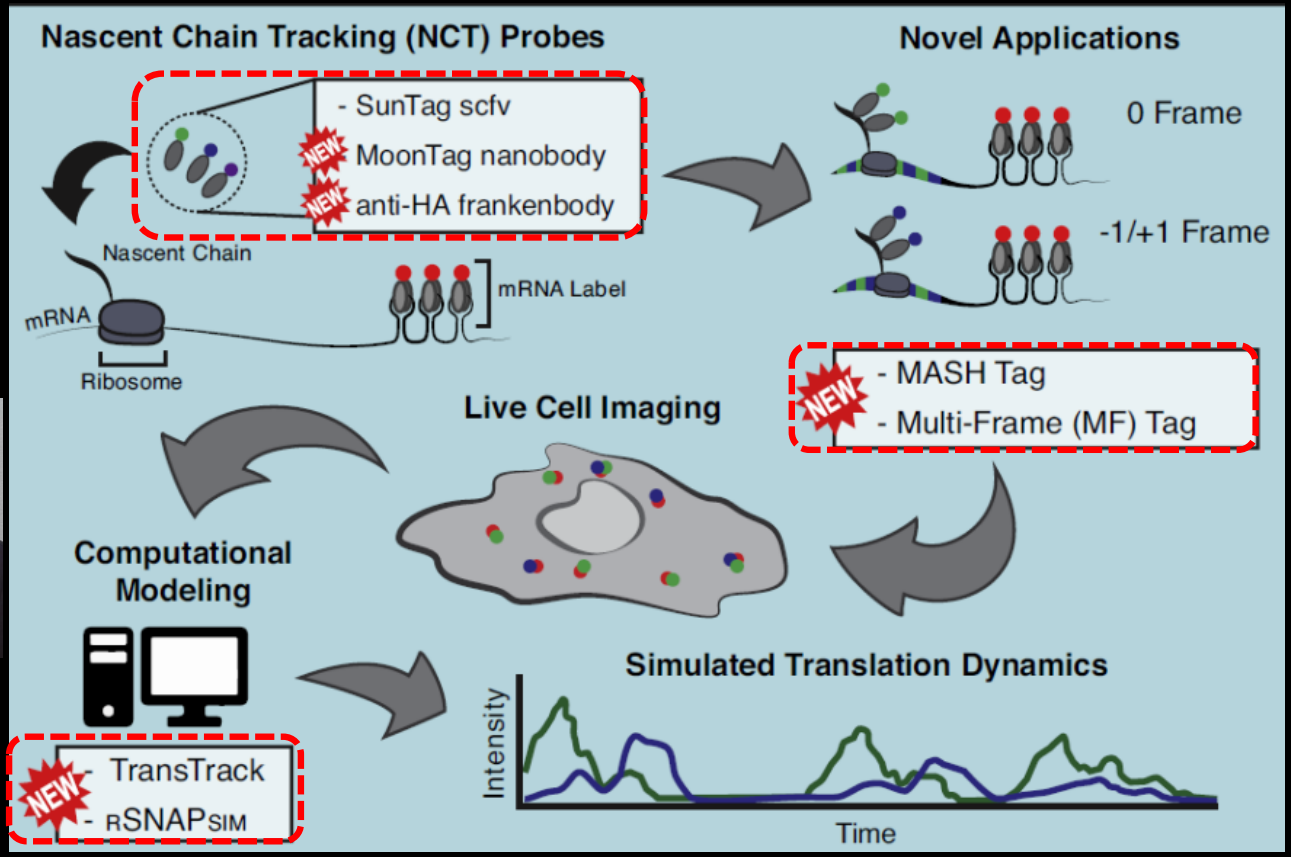
Zhao et al. Nat. Commun. **10**, 2019

Dr. Luis Aguilera

Will Raymond

PLOS Comp. Bio. 2019

Advances in single-mRNA translation imaging technology



Taken from Cialek et al. Current Opinion in Genetics & Development. 2020

NCT Applications

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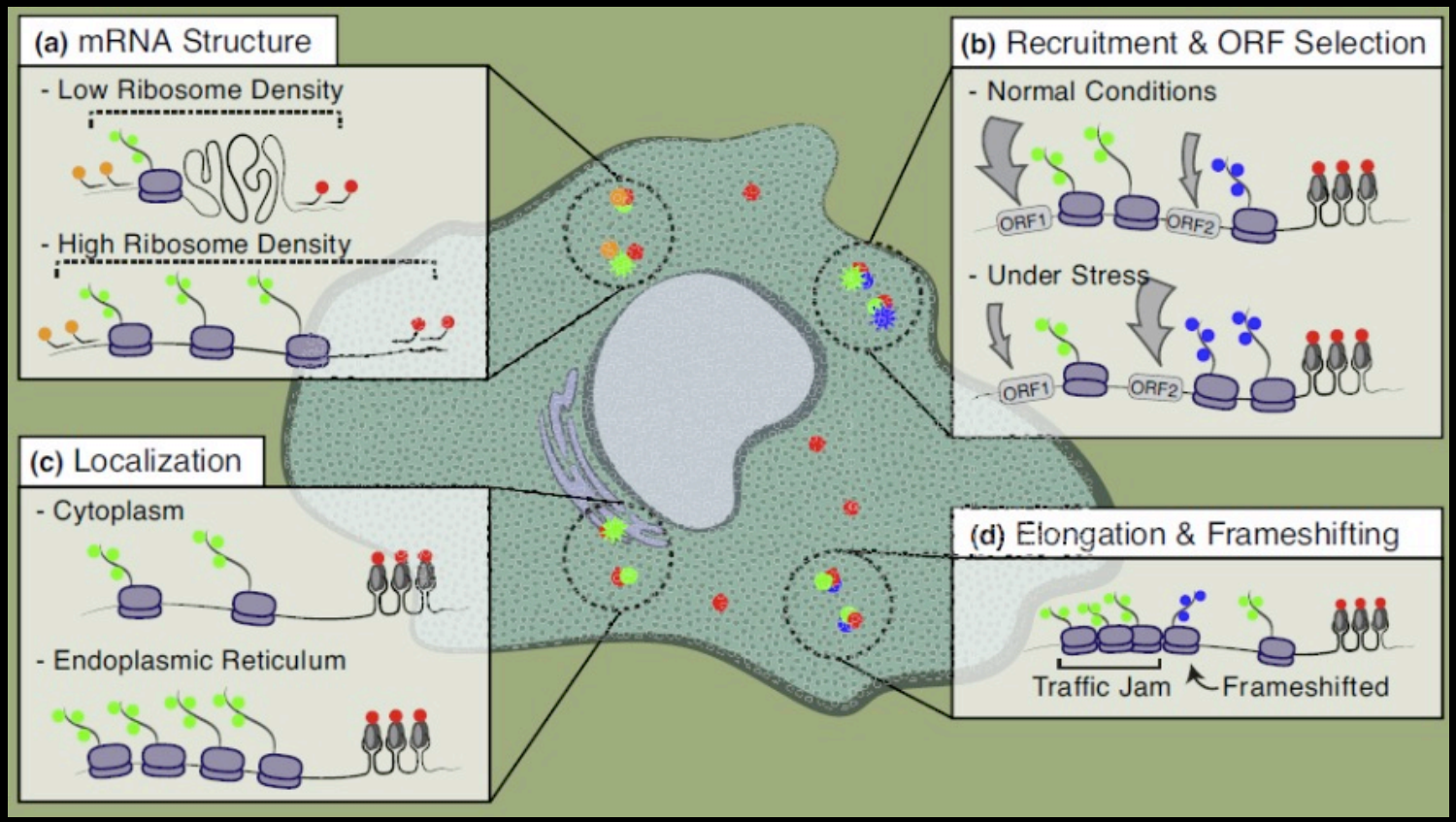
Recent applications of NCT to study active translation dynamics



Taken from Cialek et al. Current Opinion in Genetics & Development, 2020

Visualizing transcription by dCas9 labeling

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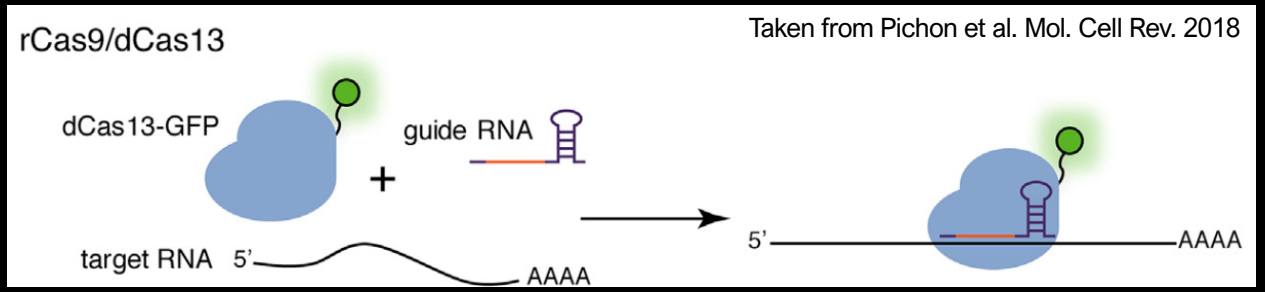
- **dCas9 labeling** reproposes the CRISPR/Cas9 system (Nobel Prize in Chemistry 2020) to bind and image RNA. The catalytically inactive Cas enzyme is fused to a fluorescent protein and binds target RNA in the presence of a guide RNA.



Taken from Pichon et al. Mol. Cell Rev. 2018

Label-free methods

Outline-Part 2:

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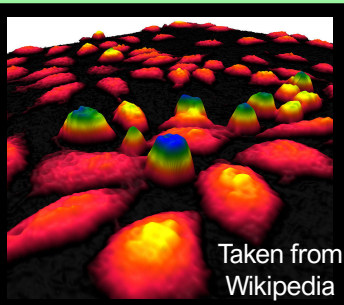
2. Label-free Methods:

a. Quantitative Phase Imaging

3. Sources

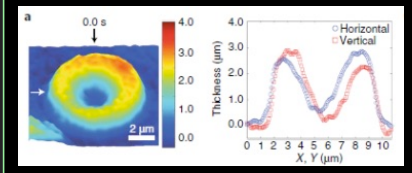
➢ **Quantitative phase imaging (QPI)** is an emerging valuable tool to visualize cells and tissues without using fluorescent labels. QPI quantifies the phase shift that occurs when light waves pass through a more optically dense object by combining qualities found in microscopy, holography and light scattering techniques.



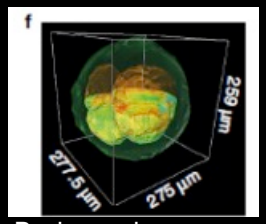
Taken from Wikipedia

Some applications

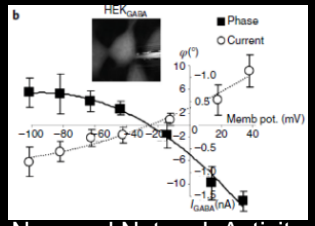
Basic Science



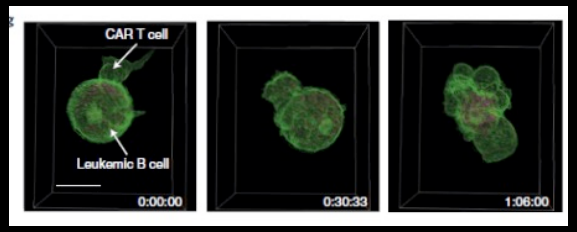
RBC structure



Bovine embryos over several days

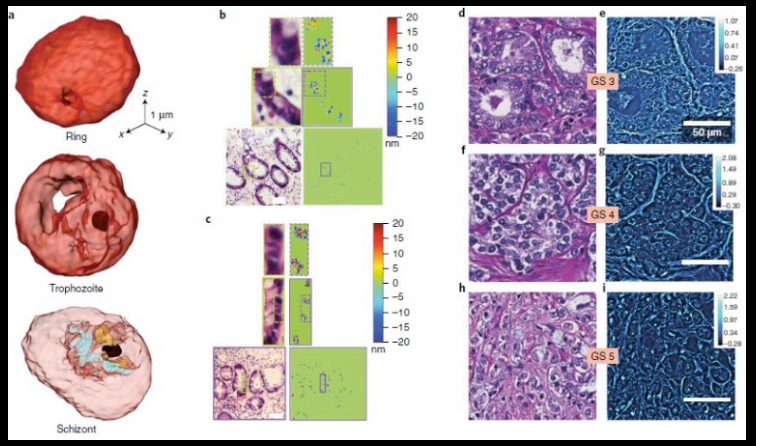


Neuronal Network Activity



3D imaging of a chimeric antigen receptor T cell killing a target cancer cell

Medical



Blood Screening & photodynamic anticancer activity

Park et al. Nat. Photonics Rev., 2018.

Lab Tour Demo

Outline-Part 2:

1. Labeling Techniques Employed in live cells:

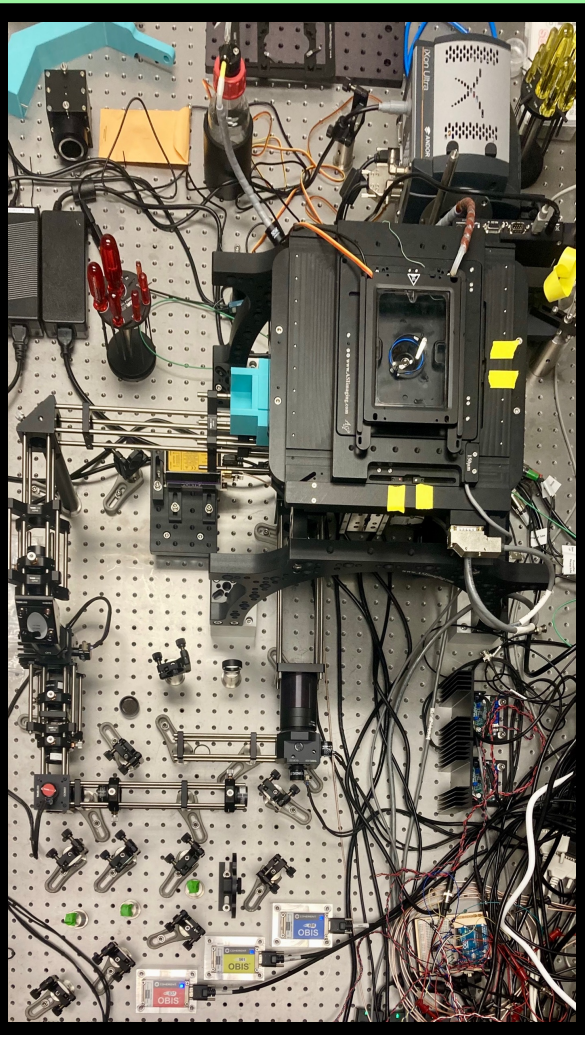
a. To visualize transcription

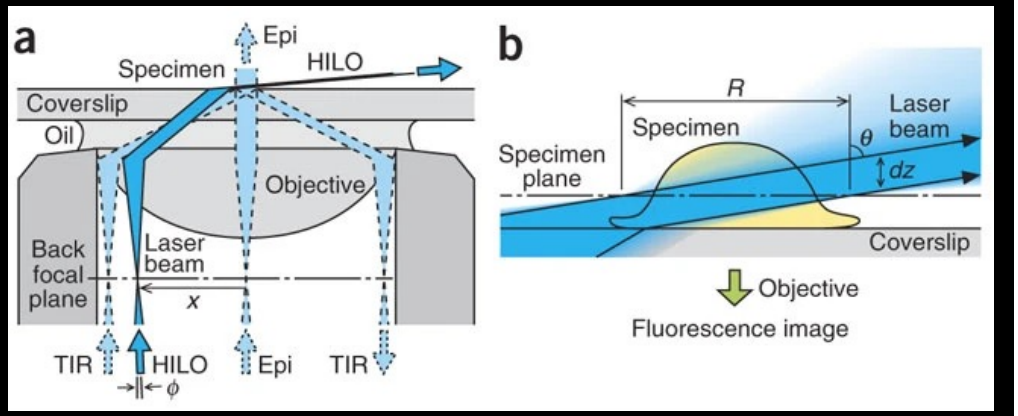
b. To visualize translation-Nascent Tracking Chain Probes

2. Label-free Methods:

a. Quantitative Phase Imaging

3. Sources





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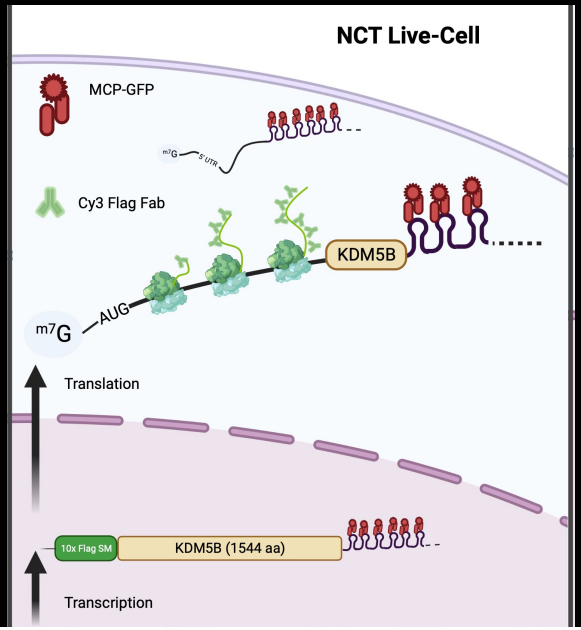

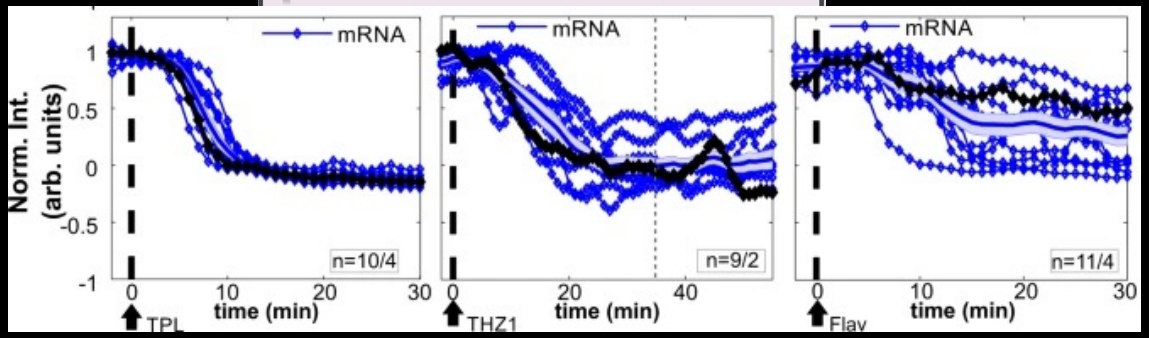

Resources

Outline-Part 2:

1. Labeling Techniques Employed in live cells:

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b. To visualize translation- Nascent Tracking Chain Probes

2. Label-free Methods:

a. Quantitative Phase Imaging

3. Sources

Sources:

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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/
- http://www.olympusconfocal.com/theory/fluorophoresintro.html