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

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Fluorescent Labeling Techniques used in single-cell Research

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?
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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.

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

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.

- **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.
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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.

Examples:
- Flag-Tag (AA-Sequence: DYKDDDDK)
- HA-Tag (AA-Sequence: YPYDVPDYA)
- V5-Tag (AA-sequence: GKIPNPPLLGLDST)
- Myc-Tag (AA-Sequence: EQKLISEEDL)

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

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

Taken from Photometrics

Taken from Fernández-Suárez & Tang, 2008
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- **Bioorthogonal 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.
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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.  

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

  Osamu Shimomura  

  [Image: Jellyfish Aequorea Victoria]

  What made them glow?

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

  [Image: William W. Ward and Douglas Prasher]

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

  [Image: Martin Chalfie]

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

  [Image: Roger Tsien]
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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 microscopy (Protein folding, protein transport, RNA dynamics, among others)

![Spectra of GFP variants](Taken from Olympus confocal web site.)

The Nobel Prize in Chemistry 2008

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.

![Bioluminescence reaction](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.

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

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Taken from Immunocytochemistry Handbook by Novus Biologicals
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Example of immunolabeling

Example of Parallel application of targeting methods & fluorophores

Taken from Giepmans et al. Science Review 2006.
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)

Munsky, et al, *PNAS*, 2018
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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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One-layer probes

48 (20bp) probes/mRNA

Tetramethylrhodamine (TMR)

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

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

Neuert, Munsky, et al, Science 2013
Munsky, et al, PNAS, 2018
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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).

- 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
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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?
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To learn more about the basis and techniques discussed in this lecture visit the following sites:
- [https://www.labxchange.org/library/pathway/lx-pathway:ad7fbf7e-9fee-4989-b8c6-e5737d21cc91](https://www.labxchange.org/library/pathway/lx-pathway:ad7fbf7e-9fee-4989-b8c6-e5737d21cc91)

Sources: