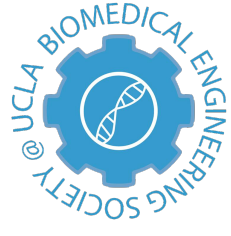
Module 3: Immunostaining

BMES Cell Team

Fall 2020



Outline

- Review of Modules 1 and 2
- Introduction to Immunostaining
- Antibody-Antigen Reaction
- Overview of the Protocol

Serial Dilutions for Western Blotting

- When you're dealing with changes in **concentration**, the two most important equations are:

1. **Beer's Law**

$$A = \varepsilon \ell C \qquad (1)$$

2. **The Dilution Equation**

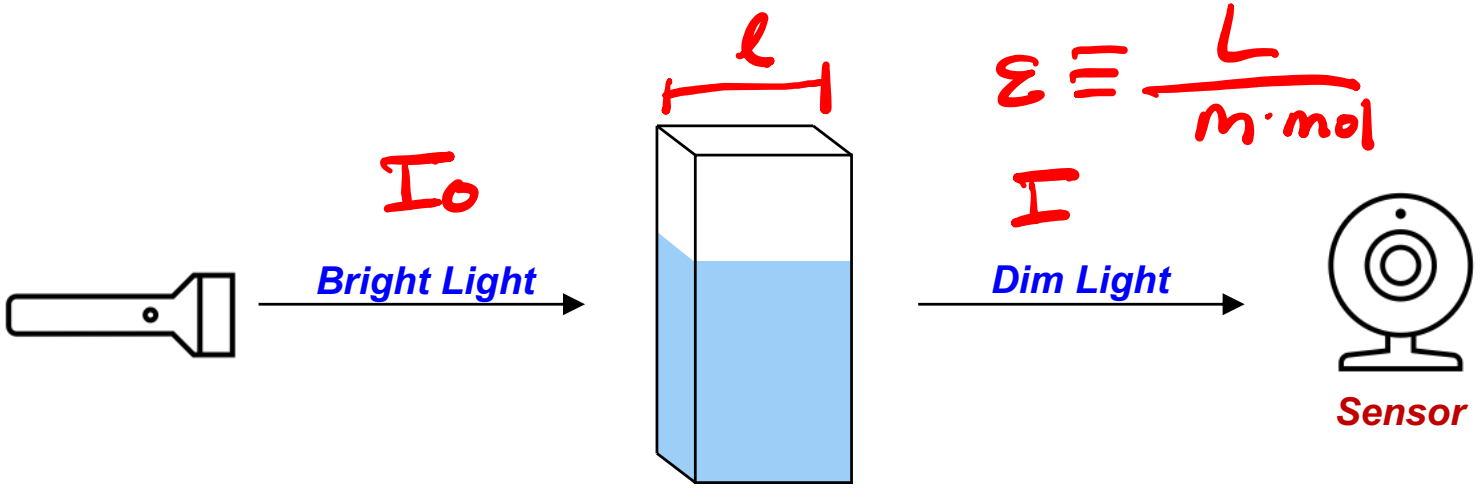
$$C_1 V_1 = C_2 V_2 \qquad (2)$$

Beer's Law

$$A = \varepsilon \ell C$$

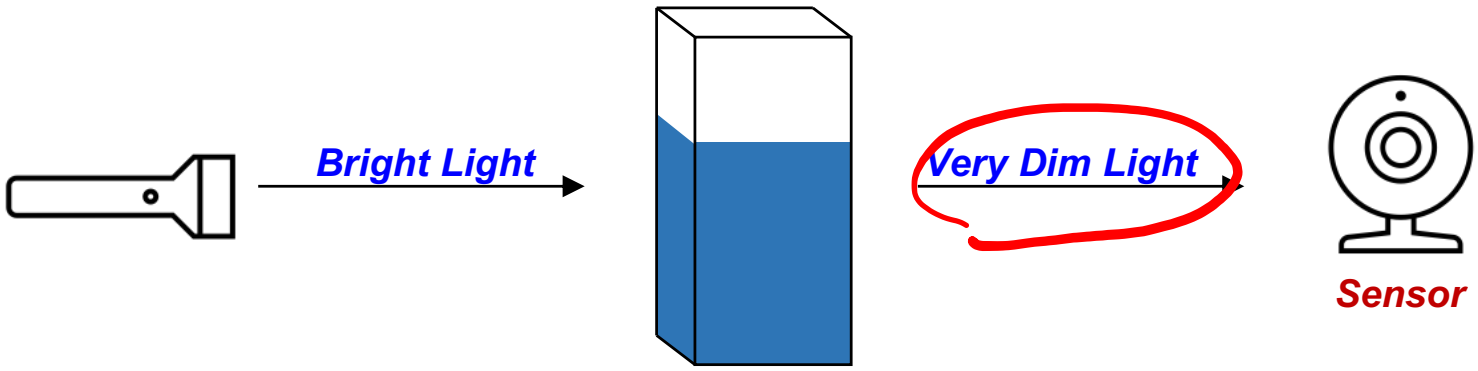
- A is the Absorbance unitless
- ε is a constant that depends on the substance we are dealing with
- ℓ is the length of the container or path m
- C is the concentration of the substance we are dealing with $M = \frac{mol}{L}$

$\varepsilon \equiv \frac{L}{m \cdot mol}$

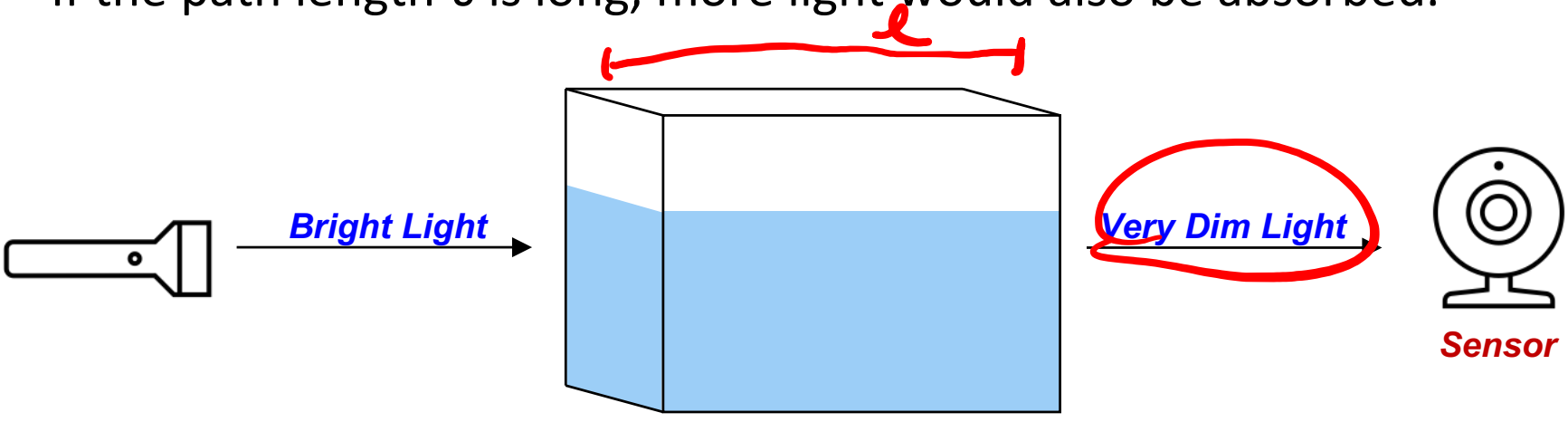


Beer's Law

- If the solution is concentrated, more light would be absorbed.

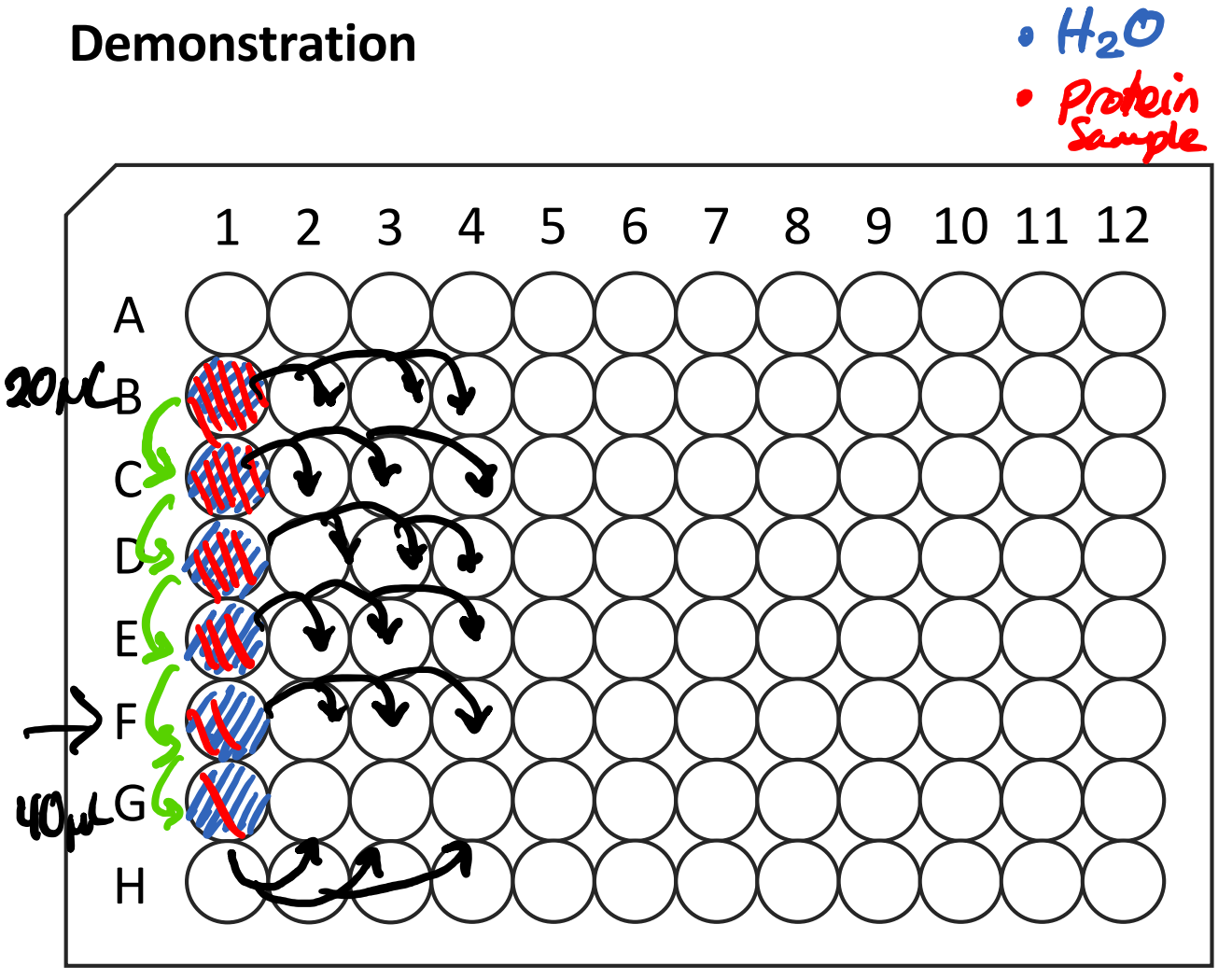


- If the path length ℓ is long, more light would also be absorbed.



Serial Dilutions for Western Blotting

Demonstration



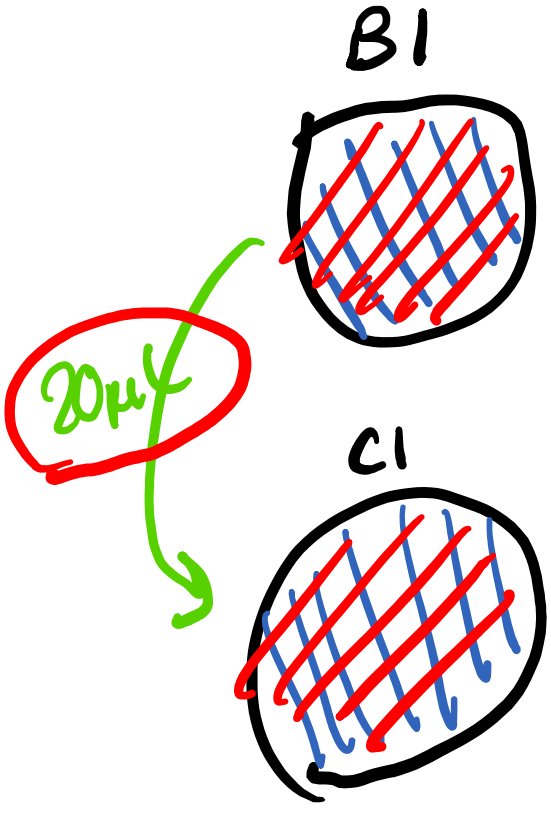
Lab Protocol

Part 1: Serial Dilutions

1. Pipet 20 µL of H_2O from B1 down to G1
2. Pipet 20 µL protein standard into B1, mix well, then bring 20 µL down into the next and mix well
3. Continue performing serial dilutions until the last well

Part 2: Creating Triplicates

1. Starting from the least concentrated standard in G1, transfer 5 µL to each of the three wells on the right
2. Continue upwards until all replicates have been made



$M_1 = \alpha \ \frac{mol}{L}$ protein standard

$M_1 V_1 = M_2 V_2$

V_1: Amount pipetted into C_1 from B_1

V_2: Total volume of C_1

$\alpha(20\mu L) = \beta(V_2)$

$M_2 = \beta \ \frac{mol}{L}$ protein standard

$\beta < \alpha$

$V_2 = \frac{20\alpha}{\beta} \ \mu L$

$$M_1 V_1 = M_2 V_2$$

$$(\alpha)(20\mu L) = M_2(20\mu\ H_2O + 20\mu L \text{ pipetted})$$

$$M_2 = \frac{1}{2}\alpha$$

Interpreting Western Blot Data

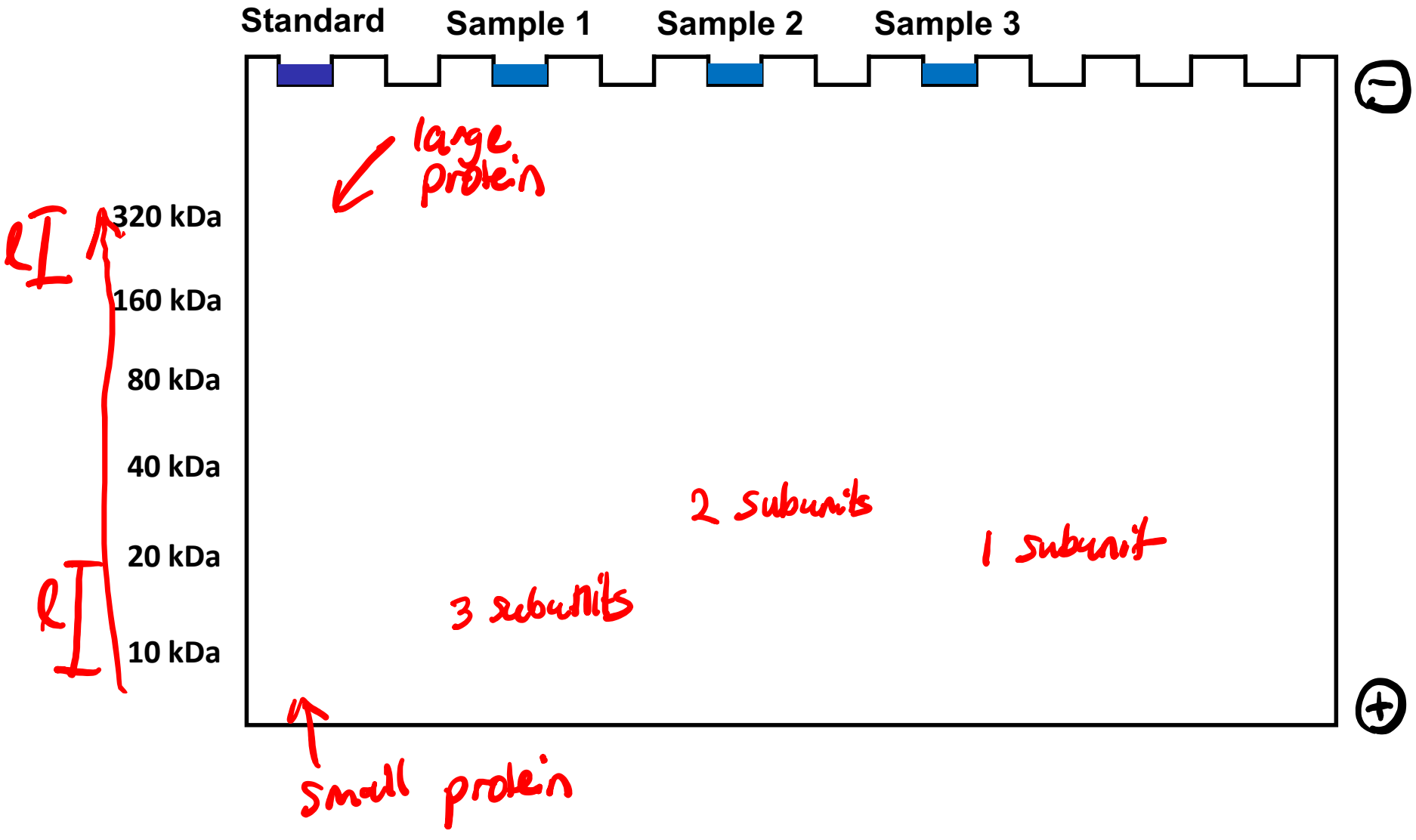
- In Module 2, we derived one of the kinematic equations to show the relationship between mass and displacement:

$$x = \frac{F}{2m}t^2 + v_0 t + x_0 \qquad (3)$$

- From this equation, we see that the mass of a protein (m) varies inversely with the displacement

- Thus, smaller proteins will travel further in Gel Electrophoresis

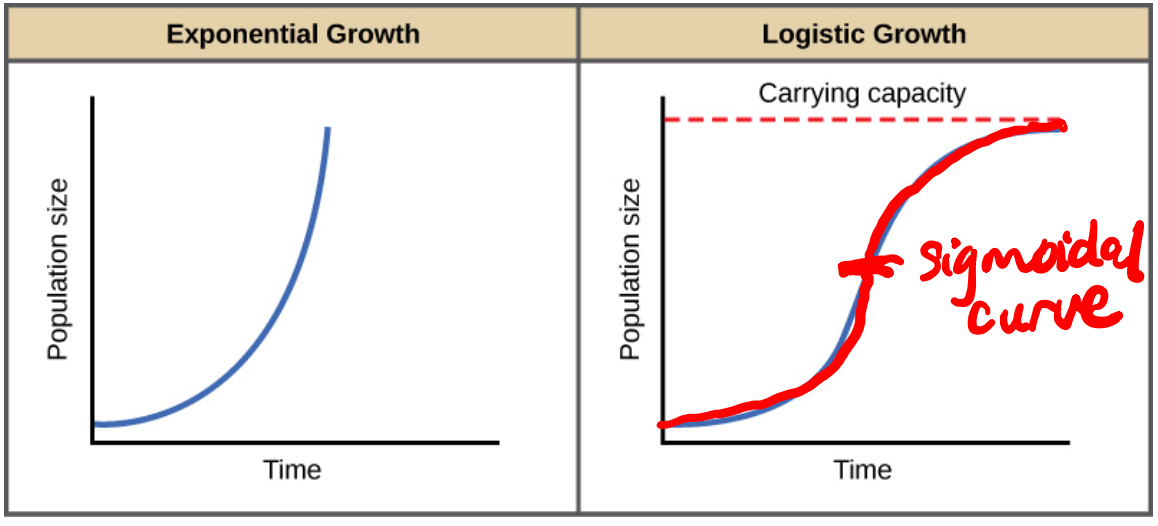
Interpreting Western Blot Data



Modelling Cell Growth with Differential Equations

- Don’t worry about knowing how to solve the differential equation since I just wanted you to know where it is mathematically derived from

- However, the point I want you to take away from this is that most microorganisms (i.e. cells, bacteria, viruses) exhibit a **logistic growth pattern**, which can be modelled thru differential equations



Introduction to Immunostaining

- **Definition:** Immunostaining is an antibody-based method used to detect the presence of a specific protein in cells and tissues.

- Note that this is a **qualitative** procedure, since we are only trying to detect the presence of a specific protein
    - This is different from Western Blotting because we don't care about the exact concentration of a protein

Introduction to Immunostaining

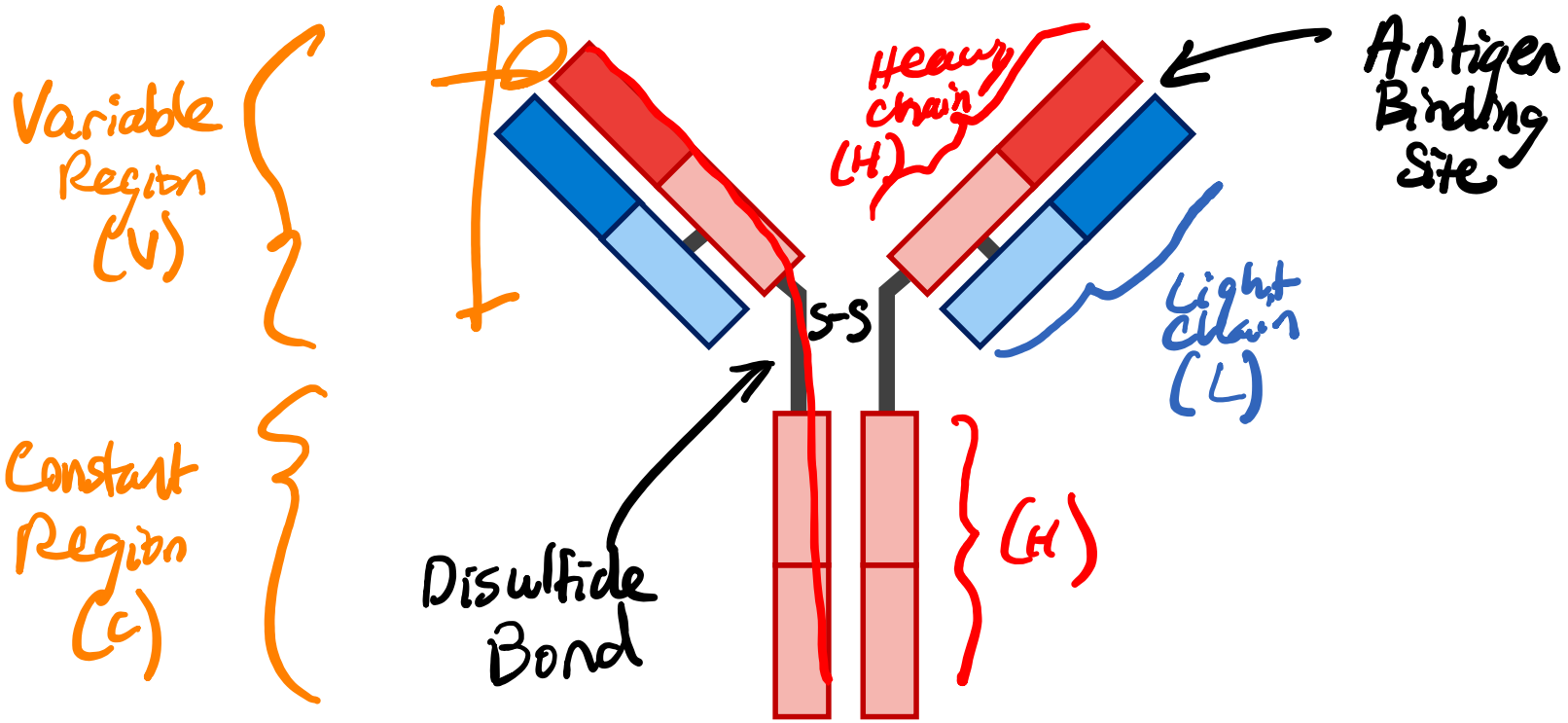
Applications of Immunostaining

oncogenes → protooncogenes

- **Cancer Detection** — We can use immunostaining to detect prognostic markers for cancer.
- **Viral Infections** — We can use antibodies specific to a virus to test whether a patient is indeed infected.
- **Genetic Testing** — If a genetic disease is widespread in your family, you can use immunostaining to determine whether you will develop that disease.

Antibody-Antigen Reaction

Parts of the Antibody — Also called Immunoglobin (Ig)



Ligand and receptors

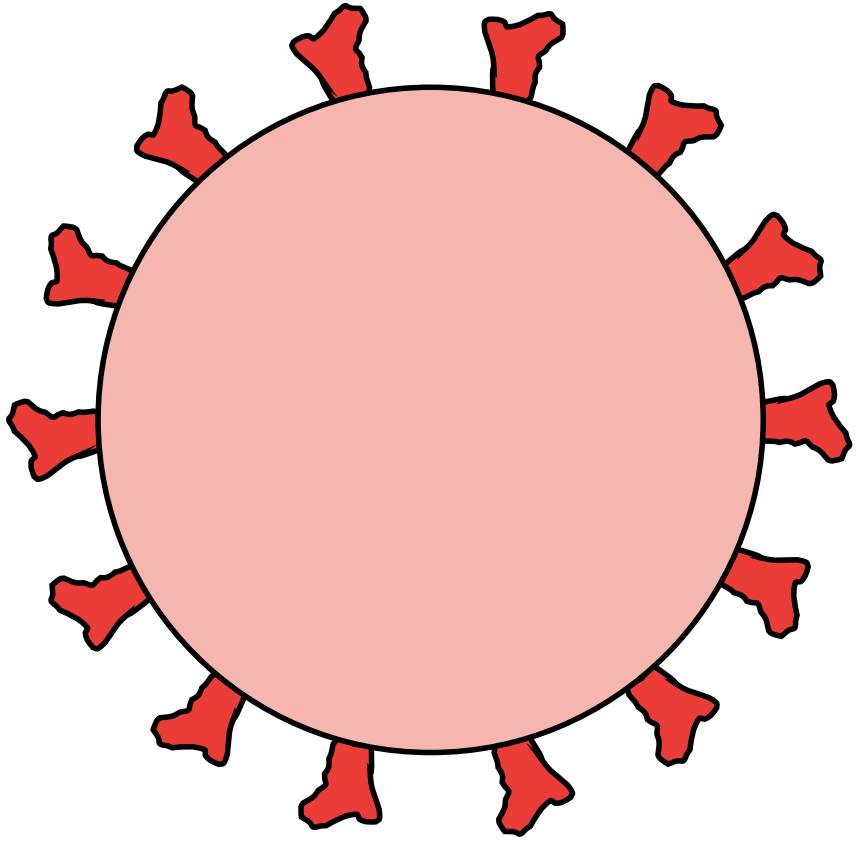
Antigens and antibodies are special proteins that bind to each other.

↳ Antigens are proteins attached to a target element such as a microorganism or virus

↳ Antibodies bind to these antigens, usually to disable or detect the target element

- Antibodies are usually produced by cells in your immune system, but they can also be genetically engineered.

Antibody-Antigen Reaction



Overview of the Protocol

1. Fix cells
2. Permeabilize the cell membrane
3. Incubate with primary antibodies
4. Incubate with secondary antibodies
5. Image cells with microscope

The Experiment

- If campus reopens this year, we will perform this experiment using the following cells:
  - **3T3 Mouse Fibroblasts** — Connective tissue
  - **Neural Stem Cells**

- Also, we will use immunostaining to detect the following proteins:
  - **Actin** — A protein found in almost all cells and makes up the cytoskeleton. It is used to drive movement and cellular transport processes. structural stability
  - **SOX2** — A transcription factor that helps maintain the *pluripotency* of stem cells.

1. Fixing Cells

What is the purpose of fixing cells?

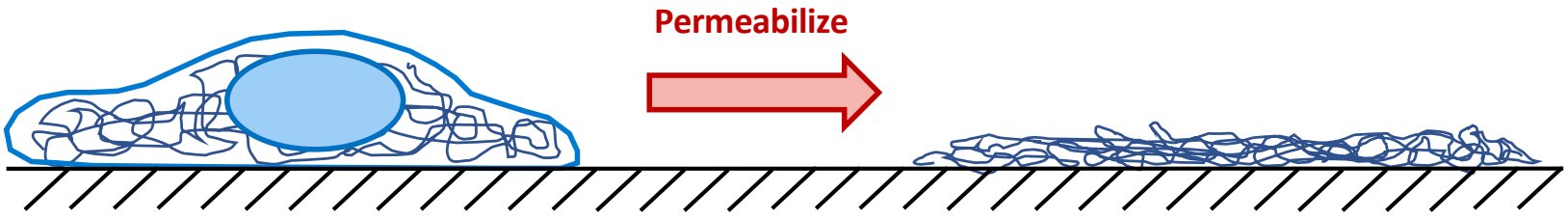
- It halts all cellular processes and immobilizes proteins
- This makes it easier for the antibodies to access and bind to proteins

How do we fix cells?

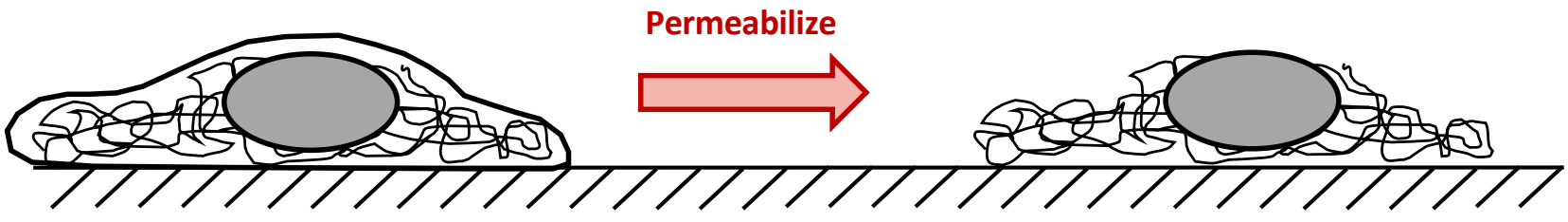
- We use a 4% solution of paraformaldehyde (PFA)
    - This reagent allows proteins to form covalent bonds with each other and their surroundings

1. Fixing Cells

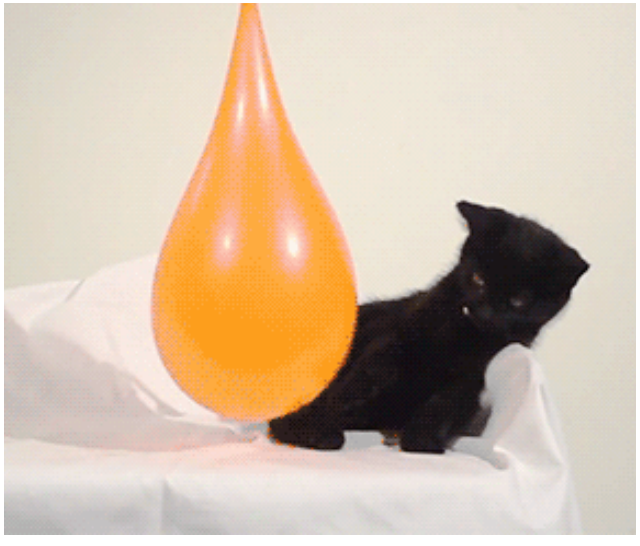
Unfixed Cell



Fixed Cell



1. Fixing Cells

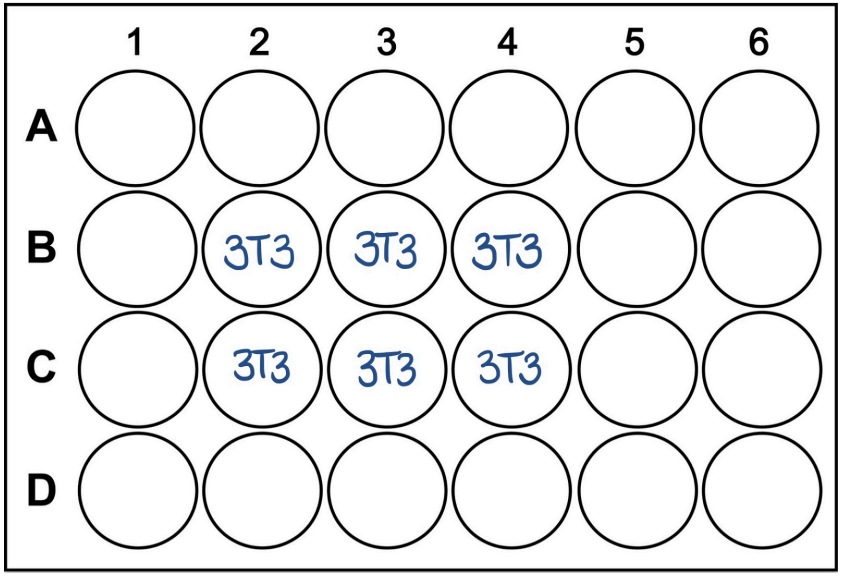


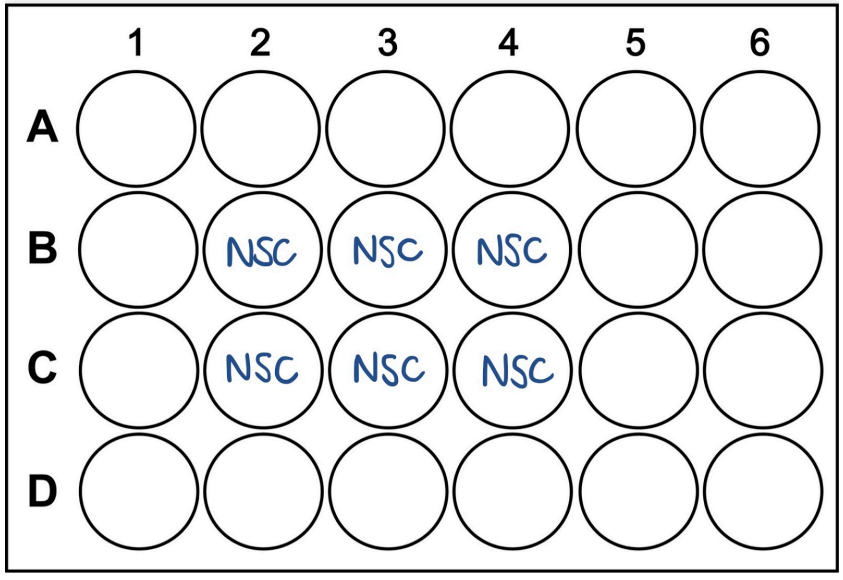
Think of it as popping a balloon filled with **water** vs. a balloon filled with **ice.**

- To make it easier to detect antibodies, we want the cell to stay intact after we lyse the membrane
    - Thus, we prefer the ice-filled balloon

1. Fixing Cells

Here is a layout of the 24-well plate that we will be using:



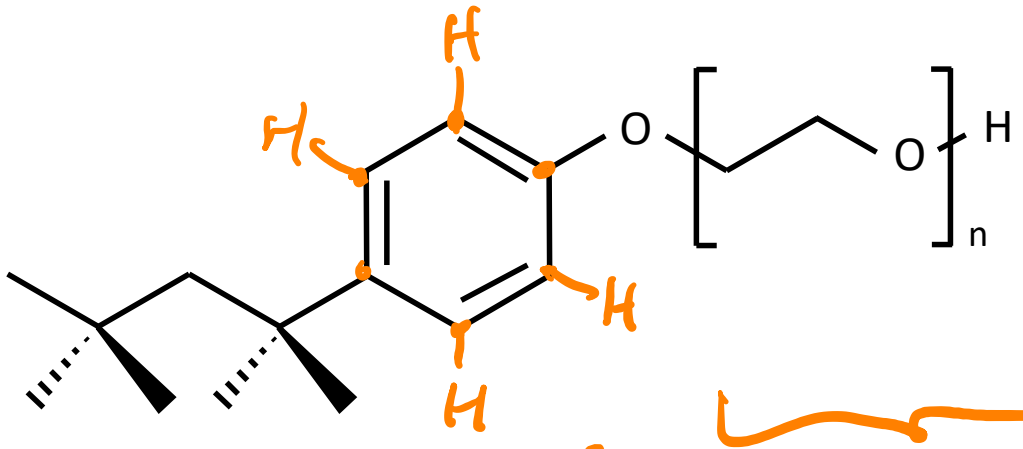


2. Permeabilization

- Intercellular vs. Membrane-Bound Proteins:
    - Based on their functions described earlier, which category do you think Actin and SOX2 fit under? Intercellular
- For *intercellular* proteins, we need to lyse the cell membrane so the antibodies can access them
- In order to accomplish this, we use a reagent called **PBS-Triton**
    - PBS-Triton removes the membrane-bound proteins, which are responsible for keeping the membrane intact
- Also, we need to wash with **PBS-Tween 20** before and after lysis
    - PBS-Tween 20 removes background noise caused by non-specific binding, thus helping you obtain more accurate results

2. Permeabilization

- Let's take a closer look at PBS-Triton:



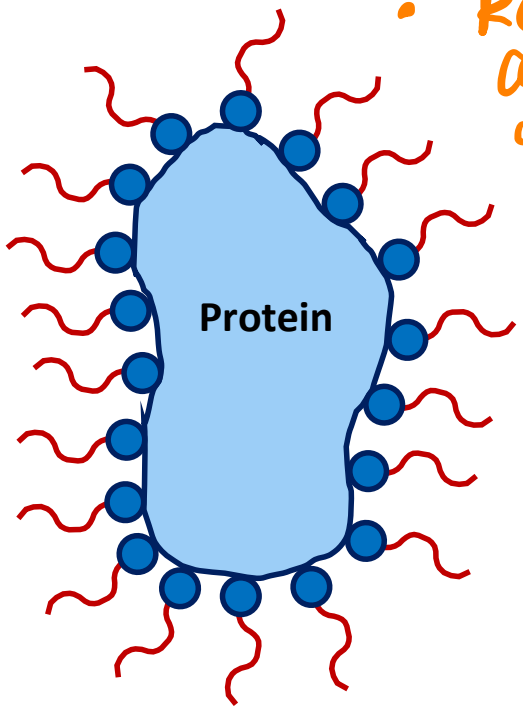
Hydrocarbons (C,H)
nonpolar

Heteroatoms
(O)
polar

amphipathic

2. Permeabilization

Protein-Surfactant Complex



- Remember that a cell membrane contains membrane bound proteins

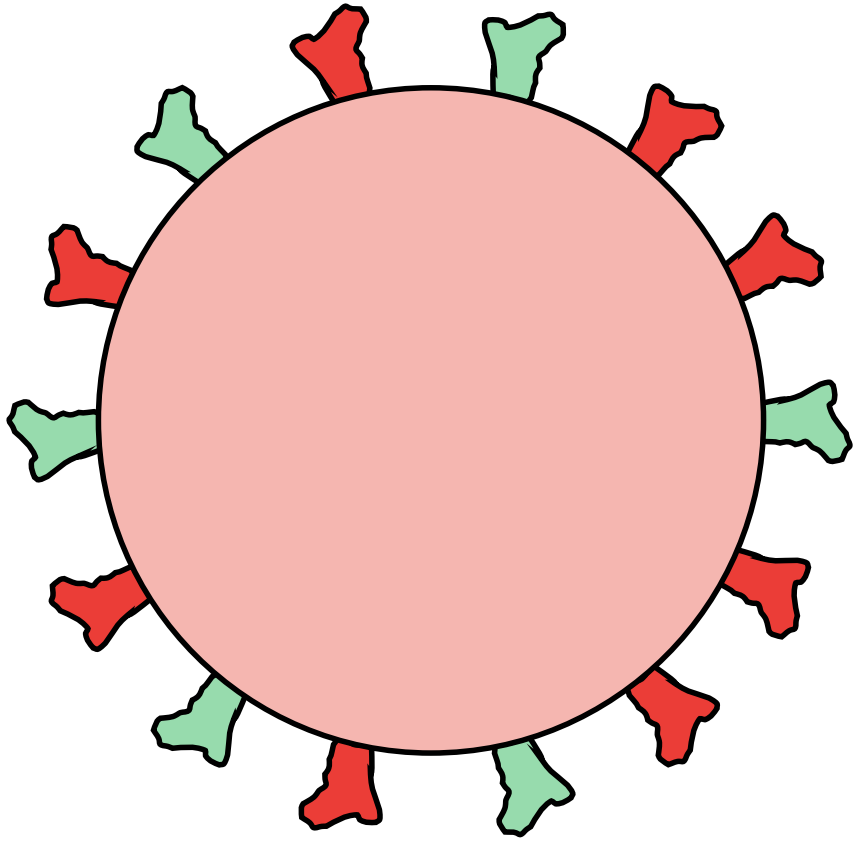
- These proteins are hydrophillic, so the head of the surfactant is attached to it

Surfactant
(PBS-Triton)

2. Permeabilization

- Now, let's see why we need to add PBS-Tween 20:

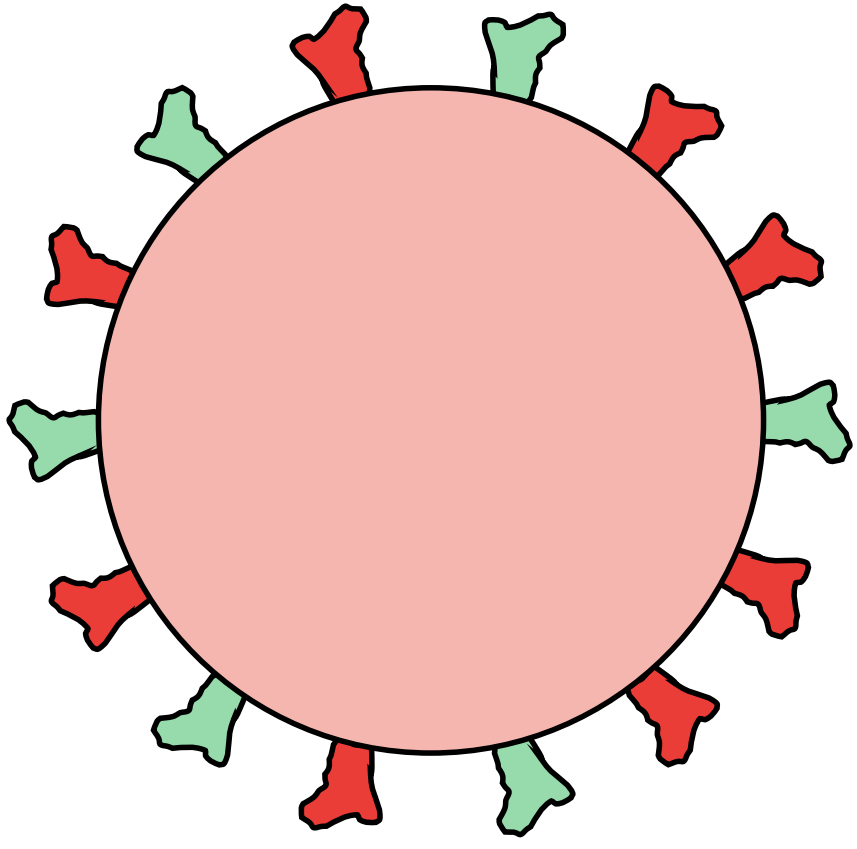
Specific Binding



2. Permeabilization

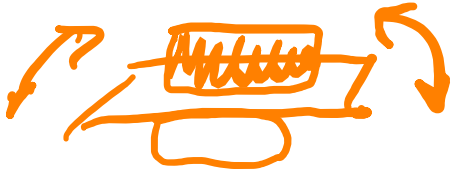
- Now, let's see why we need to add PBS-Tween 20:

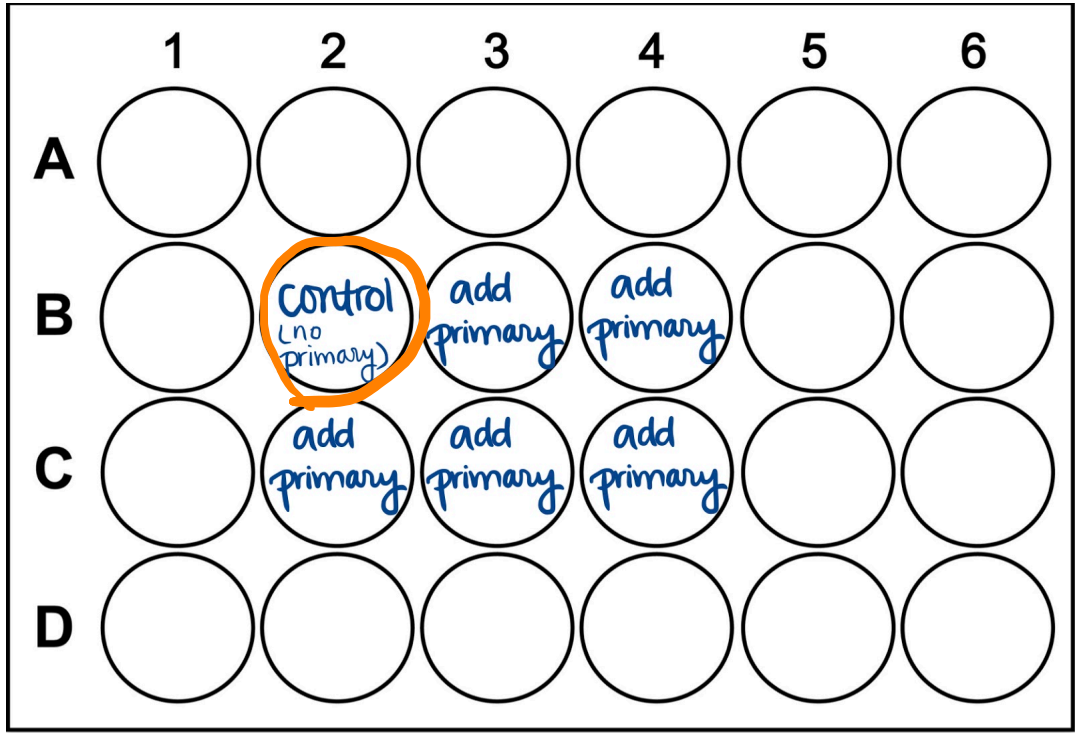
Non-Specific Binding



3. Primary Antibody Incubation

- The primary antibody we will use is **Rabbit anti-SOX2**
    - This should only bind to NSCs and not 3T3s
- Incubate overnight





3. Primary Antibody Incubation

- We need to add 500 µL of antibody solution to each well, and we have a total of ten wells. What is the total volume of solution we need?

$$(500\ \mu L) \times (10\ \text{wells}) = 5\ mL = 5000\ \mu L$$

- The SOX2 solution should be diluted at a factor of 1:200. To prepare this solution with 5 mL of PBS-Tween 20, how much SOX2 should we add?

25 µL SOX2

Method 1 (Estimation)

Since the solution will be diluted by 200x, we know the antibody portion will be insignificant.

Set up a ratio:

$$\frac{1}{200} = \frac{x\ \mu L \quad \text{(volume of solute)}}{5000\ \mu L \quad \text{(PBS-Tw.20)}}$$

Because $5000\ \mu L \gg x\ \mu L$

$$x = 25\ \mu L$$

Method 2 (Exact

$$\frac{1}{200} = \frac{X\ \mu L}{5000\mu L + X\mu L}$$

(50X2 + PBS-Tween 20)

$$\frac{1}{200}(5000 + X) = X$$

$$X = 25.13\mu L \approx 25$$

4. Secondary Antibody Incubation

- Again, we want to block with PBS-Tween 20 to prevent nonspecific binding
- All secondary reagents will be wrapped in foil to avoid photobleaching
    - If reagents are exposed to light, they won't be able to fluoresce when we image it later
- The secondary antibodies used will be:
    - **Hoechst —** Used as a control to stain all nuclei to fluoresce blue
    - **Goat anti-Rabbit —** Binds to Rabbit anti-SOX2 to fluoresce green
    - **Phalloidin —** Binds to Actin in the cytoskeleton to fluoresce red
- We want to incubate for one hour

4. Secondary Antibody Incubation

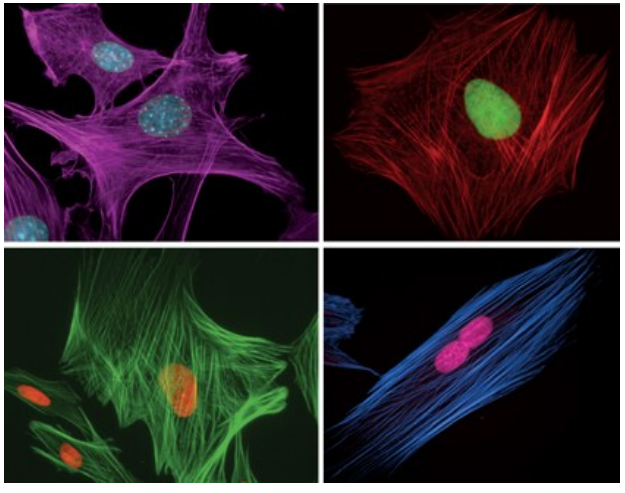
- Again, we need to add 500 µL of antibody solution to each well, and we have a total of ten wells.
    - Thus, we need 5 mL of total solution

- The Hoechst and Goat anti-Rabbit solution should both be diluted at 1:1000. Phalloidin should be diluted at 1:500. How much antibody is required to prepare each solution if you start out with 5 mL of PBS-Tween 20?

5 µL Hoechst
5 µL Goat anti−Rabbit
10 µL Phalloidin

5. Imaging

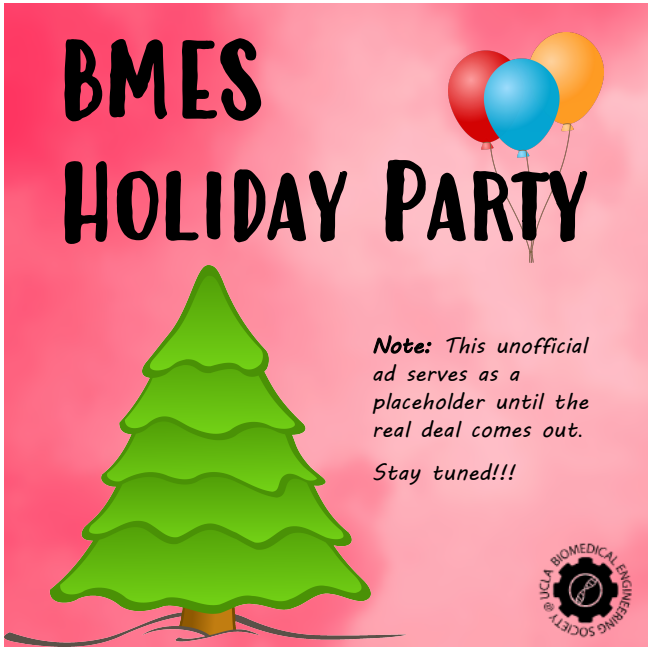
- Again, turn off the lights to avoid photobleaching
- Use a transmitted channel at 4x and 10x objective to view the cells
- Use a fluorescent channel to view the secondary antibodies:
    - For **Hoechst**, use DAPI at 10x
    - For **SOX2**, use GFP at 10x
    - For **Phalloidin**, use RFP at 40x



Reminders and Announcements

BMES Events

- **Holiday Party**



December 2 (6–8pm PT)

Come celebrate the holidays and end of fall quarter with your favorite bioengineers! Although food can't be provided this year, the games and memories will be extra fun this year!

More details coming soon...

Reminders and Announcements

Cell Team Events

- **Journal Club 3** on Thursday 11/19 at 6:00pm PT
- **Module 4** on Monday 11/23 at 7:00pm PT