

## StarCellBio Exercise 2 Answer Key – Orientation of Transmembrane Proteins

### Goal

In this exercise, you will use StarCellBio, a cell and molecular experiment simulator, to examine the orientation of transmembrane proteins in the plasma membrane using western blotting and flow cytometry.

### Learning Objectives

After completing this exercise, you will be able to:

1. Use StarCellBio to perform simulated western blot and flow cytometry experiments.
2. Design and implement experiments in StarCellBio using the appropriate experimental conditions and the relevant positive and/or negative controls.
3. Analyze western blot and flow cytometry results to determine the orientation of transmembrane proteins in the plasma membrane.
4. Evaluate the advantages and disadvantages of interrogating a scientific question using complementary approaches.
5. Synthesize results obtained from western blotting and flow cytometry analyses into a coherent, logical conclusion.

### Accessing StarCellBio

To begin:

1. Using **Google Chrome**, navigate to: <http://starcellbio.mit.edu>.
2. Sign in to your StarCellBio student account. If you need to set up a student account, use the course code **SCB\_SampleExercises**. Note: while you can complete these exercises as a guest by clicking on **Try an Experiment** on the right side of the StarCellBio homepage, your work will not be saved.
3. Select “**Exercise 2**” from the **Assignments** window.

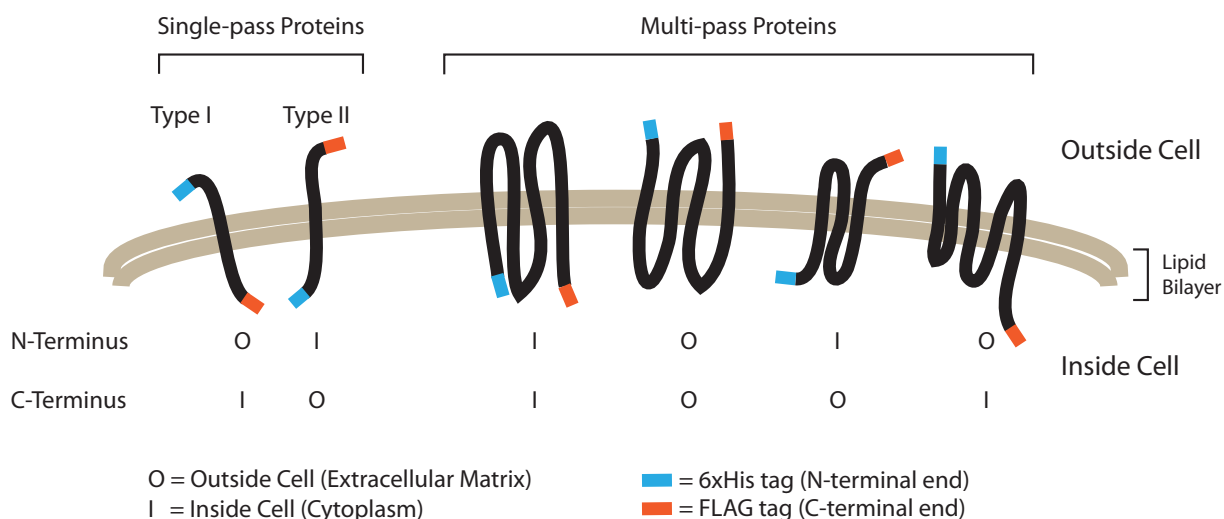
### Introduction

Your summer undergraduate research project is to determine the orientation of two newly discovered transmembrane proteins, Protein X and Protein Y, in mammalian cells to better understand how these proteins respond to extracellular and intracellular signals.

As shown in Figure 1 below, transmembrane proteins can span the plasma membrane once (single-pass proteins) or multiple times (multi-pass proteins). Depending on the number of times a protein spans the plasma membrane and the protein's orientation in the plasma membrane, a protein's N-terminal and C-terminal ends could be found either inside or outside the cell. The orientation of a protein within the plasma membrane will dictate which amino acid residues are accessible or respond to extracellular or intracellular signals, allowing a better understanding of their function within signaling transduction pathways.

To determine the orientation of Proteins X and Protein Y in the plasma membrane with respect to the location of their N- and C-terminal ends, you decide to produce two mammalian cell lines stably expressing

epitope-tagged copies of Protein X and Y, called His-ProX-FLAG and His-ProY-FLAG, respectively. The epitope-tagged versions of Proteins X and Y have a 6xHis tag (a tag containing six copies of the amino acid histidine) on their N-termini and a FLAG tag (a short peptide consisting of a specific 8 amino acid sequence) on their C-termini. Both the 6xHis and FLAG tags are detectable by specific antibodies. If the N-terminus is intracellular, then the 6xHis tag will be located on the inside of the cell as shown in Figure 1, and if the N-terminus is extracellular, then the 6xHis tag will be located outside of the cell. Similarly, the C-terminus can be intracellular, in which case the FLAG tag will be inside the cell, or extracellular, in which case the FLAG tag will be outside the cell.



**Figure 1. Transmembrane protein orientation and classification.** Proteins can be arranged with various orientations in the plasma membrane. Both the N-terminus and the C-terminus can be either intracellular or extracellular. Proteins can be single-pass, meaning that they contain a single transmembrane segment and pass through the membrane once, or multi-pass, meaning that they contain multiple transmembrane segments and span the membrane more than one time. Single-pass proteins may further be characterized as Type I proteins or Type II proteins based on their orientation in the membrane. In this figure, the multi-pass proteins were drawn with 3, 4 or 5 transmembrane segments, but within cells, multi-pass proteins may span the membrane various numbers of times.

To determine the orientation of Protein X and Protein Y in the plasma membrane you decide to use two different experimental techniques, western blotting and flow cytometry.

## Background Information

### Cell Lines

You are provided with the following cell lines:

Strain	Description
NoTags	A mammalian cell line expressing only wild-type Protein X and Protein Y and no copies of the 6xHis and FLAG tagged proteins.
ProX-Null	A mammalian cell line in which the gene encoding Protein X has been knocked out. This cell line does not express any Protein X.

ProY-Null	A mammalian cell line in which the gene encoding Protein Y has been knocked out. This cell line does not express any Protein Y.
His-ProX-FLAG	A mammalian cell line stably expressing an epitope-tagged copy of Protein X with a 6xHis tag on the N-terminus and a FLAG tag on the C-terminus.
His-ProY-FLAG	A mammalian cell line stably expressing an epitope-tagged copy of Protein Y with a 6xHis tag on the N-terminus and a FLAG tag on the C-terminus.

### Treatments

You are provided with the following treatments:

Treatment	Description
Growth media + buffer	Cells grown in growth media are collected. Intact cells are incubated with buffer only.
Growth media + ProK	Cells grown in growth media are collected. Intact cells are incubated with the Proteinase K (ProK) enzyme and appropriate buffer to digest any peptides outside the cell <sup>1</sup> .

### Western Blotting

You are provided with the following antibodies for western blotting experiments:

Antibody	Description	Expected Molecular Weight (kDa)
Mouse anti-Protein X	Primary antibody recognizing both the wild-type and epitope tagged forms of Protein X. Note: This antibody recognizes a region of Protein X known to be <u>extracellular</u> .	82 (wild type) 84 (His-ProX-Flag)
Rabbit anti-Protein Y	Primary antibody recognizing both the wild-type and epitope tagged forms of Protein Y. Note: This antibody recognizes a region of Protein Y known to be <u>extracellular</u> .	230 (wild type) 232 (His-ProY-Flag)
Mouse anti-6xHis	Primary antibody recognizing the 6xHis epitope tag.	Varies, depending on the size of the 6xHis tagged protein. The 6xHis tag adds about 1 kDa to the molecular weight of the tagged protein.
Rabbit anti-FLAG	Primary antibody recognizing the FLAG epitope tag.	Varies, depending on the size of the FLAG tagged protein. The FLAG tag adds about 1 kDa to the molecular weight of the tagged protein.
Mouse anti-PGK1	Primary antibody recognizing PGK1, a housekeeping protein expressed in all cell types at relatively equal levels.	44
Rabbit anti-mouse HRP	Secondary antibody recognizing mouse primary antibodies, conjugated to horseradish peroxidase (HRP) <sup>2</sup> .	Varies, depending on primary antibody used.
Goat anti-rabbit HRP	Secondary antibody recognizing rabbit primary antibodies, conjugated to horseradish peroxidase (HRP) <sup>2</sup> .	Varies, depending on primary antibody used.

## Flow Cytometry

You are provided with the following conditions for flow cytometry experiments:

Condition	Description
His A488	Fixed, intact cells incubated with mouse anti-6xHis primary antibody, followed by incubation with secondary antibody conjugated to Alexa Fluor 488 (green) <sup>3</sup> .
FLAG A590	Fixed, intact cells incubated with mouse anti-FLAG primary antibody, followed by incubation with secondary antibody conjugated to Alexa Fluor 590 (red) <sup>4</sup> .
ProX A488	Fixed, intact cells incubated with mouse anti-Protein X antibody, followed by incubation with secondary antibody conjugated to Alexa Fluor 488 (green) <sup>3</sup> . <u>Note:</u> The anti-Protein X antibody recognizes a region of Protein X known to be extracellular.
ProY A488	Fixed, intact cells incubated with mouse anti-Protein Y antibody, followed by incubation with secondary antibody conjugated to Alexa Fluor 488 (green) <sup>3</sup> . <u>Note:</u> The anti-Protein Y antibody recognizes a region of Protein Y known to be extracellular.

### Notes:

<sup>1</sup> Proteinase K is an enzyme that digests proteins. It cannot penetrate the plasma membrane when cells are intact (when the plasma membrane has not been disrupted or permeabilized). As a result, incubating intact cells with Proteinase K results in the digestion of extracellular peptides only.

<sup>2</sup>The provided secondary antibodies are conjugated to horseradish peroxidase (HRP). Horseradish peroxidase catalyzes a reaction that produces light as a by-product, which is detected using photographic film. The intensity and location of the light emission captured on the film indicates the relative amount and location of the bound secondary antibody on the western blot.

<sup>3</sup>This secondary antibody is conjugated to a fluorescent molecule, or fluorophore, called Alexa Fluor 488 (A488), which fluoresces or emits green light when excited by light with a wavelength of 488 nm.

<sup>4</sup>This secondary antibody is conjugated to a fluorescent molecule, or fluorophore, called Alexa Fluor 590 (A590), which fluoresces or emits red light when excited by light with a wavelength of 590 nm.

## Question 1

Your graduate student advisor suggests you first determine the orientation of Protein X and Protein Y in the plasma membrane using western blotting, an experimental technique that allows for the detection of a specific protein or peptide after cells are lysed and proteins are isolated from the rest of the cell's components. The intensity of the band on the western blot is proportional to the amount of the protein of interest expressed in the cells.

Before performing the western blotting procedure, you collect His-ProX-FLAG or His-ProY-FLAG cells and incubate the intact cells with Proteinase K. Because Proteinase K cannot penetrate the membrane of intact, unpermeabilized cells, only intracellular proteins and peptides will remain undigested after Proteinase K treatment. After Proteinase K digestion, you lyse the cells and perform a western blot analysis to determine the presence or absence of each epitope tag using the appropriate primary and secondary antibody combinations, while ensuring to include any relevant controls.

Based on your western blotting experimental results, what are the orientations of Protein X and Protein Y in the His-ProX-FLAG and His-ProY-FLAG cell lines, respectively, with respect to the location of their N- and C-terminal ends in the plasma membrane? Explain how you arrived at your answer. In your explanation include the results you obtained from any control experiments you performed and why they were important.

### Answer:

The following controls are important, however, they only reflect several of the possible controls that can be included in the experimental setup.

**Negative control experiments** using the ProX-Null, ProY-Null, and/or NoTags cell lines with or without ProK treatment would ensure that the anti-His and anti-FLAG primary antibodies used to detect the epitope tagged constructs are specific. No bands should be detected with these cell lines for either treatment.

**Positive control experiments** must be performed with all samples using the His and FLAG primary antibodies *without* Proteinase K treatment. Bands should be visible in all samples at the proper molecular weight, as outlined in the table in the background information (84 kDa for tagged Protein X and 232 kDa for tagged Protein Y). This control ensures that the absence of bands observed using these antibodies after Proteinase K treatment is due to digestion of extracellular epitopes and not the absence of these proteins in the plasma membrane or other sources of experimental error. Also, a loading control probing for PGK1 is necessary to ensure equal amounts of total protein are in all sample lysates (the bands on this western blot should ideally be all the same intensity, or else adjustments may need to be made in interpreting the results).

#### **Protein X:**

When cells expressing the tagged version of Protein X are treated with Proteinase K, there is a visible band at ~40 kDa on the western blot when probed with the anti-6xHis antibody but NOT when probed with the anti-FLAG antibody. This means that the FLAG epitope was digested during the Proteinase K treatment (outside the cell) while the 6xHis epitope was not digested (inside the cell). We can conclude that this protein is oriented with its C-terminal end (FLAG tagged) on the outside of the cell and its N-terminal end (6xHis tagged) on the inside of the cell. 40 kDa is the approximate molecular weight of the His-tagged N-terminal fragment remaining after Proteinase K digestion. The entire protein (non-digested) is 82 kDa, and a band of this size will be seen when the sample is not treated with Proteinase K.

#### **Protein Y:**

When cells expressing the tagged version of Protein Y are treated with Proteinase K, there are no visible bands on the western blot when probed with either the anti-6x His or anti-FLAG antibodies. This means that both the His and FLAG epitopes were digested during the Proteinase K treatment (they were both located outside the cell). We can conclude that this protein is oriented with both the N- and C-terminal ends on the outside of the cell. The entire protein (non-digested) is 230 kDa, and a band of this size will be seen when the sample is not treated with Proteinase K.

## **Question 2**

Your advisor then suggests using a different experimental technique, flow cytometry, to determine the orientation of Protein X and Y in the plasma membrane. Flow cytometry allows for the detection and quantification of proteins or peptides in cells. In flow cytometry, the amount of fluorescence signal emitted corresponds to the relative amount of bound secondary antibody and therefore, to the relative amount of protein/peptide present.

In this flow cytometry experiment, you collect and fix intact cells from the His-ProX-FLAG and His-ProY-FLAG cell lines. Then, you incubate the cells with either the anti-FLAG or anti-6xHis primary antibody, followed by incubation with the appropriate fluorescently-labeled secondary antibody. Because antibodies cannot penetrate intact, unpermeabilized cells, the anti-6xHis and anti-FLAG antibodies can only bind to extracellular 6xHis or FLAG epitopes. After secondary antibody incubation, you perform flow cytometry analysis to quantify the amount of emitted fluorescence while ensuring the relevant controls are included.

Based on your flow cytometry experimental results, what are the orientations of Protein X and Protein Y in the His-ProX-FLAG and His-ProY-FLAG cell lines, respectively, with respect to the location of their N- and C-

terminal ends in the plasma membrane? Explain how you arrived at your answer. In your explanation include the results you obtained from any control experiments you performed and why they were important.

**Answer:**

The following controls are important, however, they only reflect several of the possible controls that can be included in the experimental setup.

**Negative control experiments** using the ProX-Null, ProY-Null and/or NoTags cell lines with the His A488 and FLAG A595 antibody conditions should only produce background fluorescence signals ( $\sim 10^1$  fluorescence units). This ensures that the anti-His and anti-FLAG primary antibodies used to detect the epitope tagged constructs are specific.

**Positive control experiments** using the ProX A488 and ProY A595 antibody conditions with the His-ProX-FLAG and His-ProY-FLAG cell lines should produce a positive fluorescence signal. This control ensures that the absence of signal in other experiments with the tagged cell lines is due to the inability of the antibody combination used to bind to an extracellular epitope of the protein and not the absence of these proteins in the plasma membrane or other sources of experimental error.

**Protein X:**

When cells expressing the tagged version of Protein X are incubated with anti-FLAG primary antibody followed by the fluorescently-labeled secondary antibody, a red fluorescent signal (averaging  $10^3$  fluorescence units) is observed. This means that the FLAG epitope (C-terminal end) is extracellular since it can bind its corresponding primary and secondary (red) antibodies. When these cells are incubated with anti-6xHis primary antibody followed by the corresponding fluorescently labeled secondary antibody, no green fluorescent signal is detected (the signal averages  $10^1$  fluorescence units, which is equivalent to background). This means that the 6xHis epitope (N-terminal end) is intracellular since it cannot bind its corresponding primary and secondary antibodies. We know that  $10^1$  units is background fluorescence due to the negative control.

**Protein Y:**

When cells expressing the tagged version of Protein Y are incubated with anti-FLAG primary antibody followed by the fluorescently-labeled secondary antibody, a red fluorescent signal (averaging  $10^3$  fluorescence units) is observed. This means that the FLAG epitope (C-terminal end) is extracellular since it can bind its corresponding primary and secondary (red) antibodies. When these cells are incubated with anti-6xHis primary antibody followed by the fluorescently-labeled secondary antibody, a green fluorescent signal is observed (the signal averages  $10^3$  fluorescence units). This means that the 6xHis epitope (N-terminal end) is also extracellular since it can bind its corresponding primary and secondary (green) antibodies. Therefore, both the C- and N-termini are extracellular.

### Question 3

Do the conclusions for Questions 1 and 2 agree? Justify your answer.

**Yes. The two different analyses lead to the same set of conclusions: (1) Protein X's N-terminus is intracellular and its C-terminus is extracellular, and (2) Protein Y's N- and C-termini are both extracellular. The reasoning behind these conclusions is explained in the answers to Questions 1 and 2.**

### Question 4

a) Compare and contrast the information you obtain regarding the location of a protein's termini (inside or outside of the cell) through western blotting versus flow cytometry approaches.

**Answer:**

**Proteinase K treatment followed by western blotting analysis allows for the identification of a protein's intracellular termini. If no band is detected on the western blot, the terminus recognized by the primary antibody is located outside the cell; however, this is an inference, as it cannot be explicitly detected by this western blot approach. The flow cytometry approach, on the other hand, identifies a protein's extracellular termini. In intact cells, antibodies can only bind to extracellular termini. If the fluorescence signal does not exceed background levels, the terminus recognized by the primary antibody is intracellular; however, this is an inference, as it cannot be directly detected by this flow cytometry approach.**

b) What are the advantages and disadvantages of examining transmembrane protein orientation using both of these techniques versus only one of these specific approaches?

**Answer:**

**These techniques complement each other, which allows you to be more confident about your conclusions. The termini you infer to be outside the cell using the western blotting approach can be confirmed directly by flow cytometry experiments, and the termini you infer to be inside the cell using flow cytometry can be confirmed directly using the western blotting approach. A disadvantage of using more than one method/approach is that it is associated with an increase in time, reagents, and cost.**

## **Question 5**

a) What is the number of transmembrane segments that a transmembrane protein can have if:

- (i) both terminal ends are extracellular?
- (ii) one terminal end is extracellular and the other is intracellular?
- (iii) both terminal ends are intracellular?

Explain your answers. If for any given scenarios there is more than one possibility, explain what rules or restrictions determine the number of segments.

**Answer:**

**In scenarios (i) and (iii) where both terminal ends of the protein are extracellular, the protein will need to have an even number of transmembrane segments (2, 4, 6, etc.). In scenario (ii) where one terminal end is extracellular and the other intracellular, the protein must have an odd number of segments (1, 3, 5, etc.).**

b) Which scenarios (i, ii, and/or iii) can apply to single-pass and multi-pass proteins?

**Answer:**

**Since single-pass proteins only have 1 transmembrane segment, they can only have one extracellular and one intracellular end (scenario ii). Multi-pass proteins can belong to any of the 3 scenarios, depending on their number of transmembrane segments.**

## **Question 6**

Based on the results from all of your experiments, what can you conclude about the number of transmembrane segments in Protein X and Protein Y? Can Protein X and Protein Y be single-pass and/or multi-pass proteins? Explain your answers.

**Answer:**

**Protein X's terminal ends are on opposite sides of the plasma membrane (N-terminus is extracellular and C-terminus is intracellular). As a result, I can conclude that Protein X must have**

an odd number of transmembrane segments, but it can be either a multi-pass or single-pass protein. Protein Y has both terminal ends on the same side of the plasma membrane (the extracellular side). As a result, I can conclude that Protein Y cannot be a single-pass protein and should have an even number of transmembrane segments.