TMM 3102: Protein Structure, Function and Disease

- Integrative Structural Biology: Membrane Proteins
  (October 14th, 2021)

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Lecture Outline

1. Why studying membrane proteins?

2. Challenges in membrane protein structural biology

3. Ways to study membrane protein structures
   - X-ray crystallography ✔
   - Electron microscopy ✔
   - Fluorescence ✔
   - Magnetic resonance ✔
   - Computer simulation, NMR, mass spec, cross-linking, ...

4. Strategies in structural determination of membrane proteins
1. Why studying membrane proteins?

- Encoded by some 20-30% genes in typical genome.
- Major components of the mosaic lipid bilayers in cellular membranes
- Mediate cell-to-cell communication and signaling events.
- Disruptions or mutations in humans have been implicated in diseases, such as cardiovascular and metabolic diseases, cancer, rare genetic diseases, ...
Membrane proteome (human)

(Almén et al, BMC Biol, 2009)
Half drug targets are membrane proteins.

(Yildirim et al, Nat Biotech, 2007)
Available atomic/near-atomic models of membrane proteins (2021-9-29)

PDB Statistics: Overall Growth of Released Structures Per Year

[Bar chart showing the growth of released structures per year from 1963 to 2021. The x-axis represents the year, and the y-axis represents the number of entries. The bars show the number of structures released annually and the total number of entries available.]
Available atomic/near-atomic models of membrane proteins (2021-9-29)

PDB Statistics:

Stephen White Lab at UC Irvine
https://blanco.biomol.uci.edu/mpstruc

Wed, 29 Sept 2021
2. Challenges in membrane protein structural biology

• Naturally occurred proteins exist in **low abundance**, with only a few exceptions (e.g., bacteriorhodopsin or aquaporin), and form complexes.

• *E. coli* is often not suitable for producing recombinant membrane proteins of **eukaryotic** origins.

• **No so-called standard protocol** of protein extraction, largely due to the complexity of protein-lipid interaction.

• Protocols of purification, crystallization, and *in vitro* reconstitution remain **empirical** for individual cases.
Choosing the appropriate expression hosts for recombinant proteins

(Zorman et al, Curr Opin Struct Biol, 2015)
Things to consider for membrane protein extraction and purification

- Cell disruption
- Solubilization agent
  - Detergents
  - Polymers
- Protein engineering
- Column chromatography
- \textit{In vitro} reconstitution
Membrane proteins are present in an anisotropic and hydrophobic environment

i. Must remove the protein from lipid-rich membrane to separate it from other membrane proteins.

ii. Must be able to stabilize them as single “particles” in an aqueous environment.

(https://www.creative-proteomics.com/services/membrane-proteomics.htm)
Solubilization of membrane proteins using detergents:

Critical Micellar Concentration (CMC)

3.4. The critical micellar concentration. As detergent (or surfactant) is added to an aqueous solvent, the concentration of dissolved monomers increases until the critical micellar concentration (CMC) is reached. At that concentration, micelles form. Further addition of detergent increases the concentration of micelles without appreciably affecting the concentration of monomers. Redrawn with permission from Helenius, A., and K. Simons, Biochim Biophys Acta. 1975, 415:38.

(Mary Luckey, Membrane Structural Biology, 2008)
- Detergent solubilized proteins can be purified and crystallized, but too much detergent or types of detergents may denature the protein.

- Detergents are dynamic and can interfere with the formation of the protein-protein contacts in a crystal.

- You have to find the right detergent (size, shape, charge) and conditions (concentration) that solubilize well and generate homogeneous protein preparation for structural biology.

- Structural biology of membrane proteins is thus a distinct field!

(Mary Luckey, Membrane Structural Biology, 2008)
3. Ways to study membrane protein structures

- X-ray crystallography ✔
- Electron microscopy ✔
- Fluorescence ✔
- Magnetic resonance ✔
- Computer simulation, NMR, mass spec, cross-linking, ...
Membrane protein crystallography

Coarse vapor diffusion

i. Transfer purified proteins to a microcentrifuge tube
   Mix proteins with desired chemicals, other proteins, ligands, etc.

ii. Transfer the protein mixture to a robot-customized syringe

iii. Mix with the reservoir solution (by robot)
    Seal each crystallization well
    Monitor over time for crystal growth

Membrane protein crystallography

**Bicelle (stacked 2-D crystals)**

i. Transfer purified proteins to a microcentrifuge tube

Mix proteins with desired chemicals, other proteins, ligands, etc.

Add bicelle solution to the protein mixture

ii. Transfer the protein mixture to a robot-customized syringe

iii. Mix with the reservoir solution (by robot)

Set up hanging-drop crystal trays and seal each crystallization well

Monitor over time for crystal growth

(Ujwal & Bowie, Methods, 2011)
Bicelles (On Ice)   Purified detergent-solubilized membrane protein (On Ice)

Pipeette to mix and incubate on ice for 30'

Protein-Bicelle Mixture

Crystallization trials using standard set up including robotics

*(Ujwal & Bowie, Methods, 2011)*
Membrane protein crystallography

In *meso* lipid cubic phase (LCP)

i. Mix proteins with desired chemicals, other proteins, ligands, etc.

Transfer the protein mixture to a robot-customized syringe

Transfer pre-made LCP lipids (usually monoolein ± cholesterol) to another robot-customized syringe.

Mix proteins and the lipids until the mixture shows no cloudiness

ii. Mix with the reservoir solution on sandwich plates (by robot)

Seal the LCP sandwich plates

Monitor over time for crystal growth (usually at 20°C - room temperature)
(Caffrey, Acta Cryst F, 2015)
2.4.3. Cholesterol crystals. Cholesterol and MO at a 1 : 3 mole ratio were co-dissolved in methanol. Solvent was removed under a stream of argon initially and subsequently under vacuum (20 mTorr) for 24 h at room temperature (B20°C). The dry cholesterol/MO was then homogenized with water in a 3/2 weight ratio using the lipid mixer. The mixture formed a transparent and homogeneous cubic phase. Crystallization of cholesterol was initiated by the addition of 500 nL 0.3 M Na–K Fig. 2 Photographs of crystals grown by the in meso method in microcells at 20°C. (a) bR crystals growing in meso in the microcell. In this view, the entire window of the microcell is shown. (b) The same view as in (a) but taken between crossed polarizers to highlight crystal birefringence and the non-birefringent nature of the hosting cubic mesophase (dark background). At certain orientations the bR crystals appear as bluish birefringent objects. Clusters of such dots in the upper left hand corner likely originate from bR microcrystals that are not visible under normal light in (a). (c) A zoomed in view of several bR crystals growing in meso in a microcell. Crystals typically reach a size of 20–25 μm and are randomly oriented with respect to the microcell windows. (d) A crystal of lysozyme growing in meso in a microcell. (e) Plate-like crystals of cholesterol growing in meso in a microcell. The crystals were usually quite large and were aligned parallel to the microcell windows. Often crystals grew to fill the depth (25 μm) of the microcell. (f) X-Ray damage footprints left by the focused 400 nm beam after stepwise scans along orthogonal directions across a bR crystal. This picture illustrates the level of accuracy attained in positioning the sample in the X-ray beam. In this instance, scan lines were supposed to cross at the centre of the crystal. The mismatch of 6 μm represents the error in beam position determination. Such tracks of radiation damage were used during analysis to improve absolute positional accuracy.

(Cherezov & Caffrey, Faraday Discuss, 2007)
(Warren et al., in “The Next Generation in Membrane Protein Structure Determination”, 2016)
Accordingly, we sought to exploit other characteristics of the assorted phases to assist in their identification. Under the prevailing experimental conditions the bulk cubic phase always gave rise to discrete Bragg reflections or spots (Fig. 3(a)). These arise because the domain size of the cubic phase exceeds the diameter of the beam ($r > 5 \text{ mm}$) and the thickness of the sample-holding microcell (25 $\text{mm}$). Accordingly, a spotty pattern plus the corresponding d-spacings of the discrete reflections were used to identify the cubic phase.

In contrast, the lamellar (L$_{a}$) phase tended to produce powder diffraction rings or arcs (Fig. 3(b)). Such patterns were never observed with the cubic phase in the microcells. Accordingly, powder-like diffraction along with a d-spacing range that is characteristic of the phase were used as hallmarks or signatures of the lamellar phase.

Our working hypothesis posits that a lamellar phase acts as a conduit between the bulk cubic phase and the crystal. Thus, diffraction characteristic of the lamellar phase was looked for in the vicinity of crystals growing in the cubic phase housed in Fig. 3.

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(Cherezov & Caffrey, Faraday Discuss, 2007)
X-ray crystallography: micro-diffraction

*Bicelle*

A: 50-500 µm, 25-30 Å

B: 100-300 µm, 7-10 Å

C: 50-150 µm, 3.5-4 Å
- Long exposure 2-5 sec @ APS 30 sec @ ALS
- Radiation damage 3-5 frames (< 5°)
- Signal ($I/I_0 = 1-1.5$ at 3.9-4Å)
Cryo-electron microscopy (cryo-EM)

- Electron crystallography ✔
  - 2-D crystals / MicroED
  - Helical crystals

- Single-particle cryo-EM ✔

- Cryo-electron tomography (cryo-ET)
Only 2-D projections are recorded \((x,y)!!\)
(Amos et al, Prog Biophys Mol Biol, 1983)
Microelectron Diffraction (MicroED) & Electron Crystallography

Cryo-EM applications in protein structural biology:

2) Microelectron diffraction:
(need to generate protein crystals, but much smaller than that for X-ray crystallography)

a. Prepare purified protein samples, crystallize and freeze them.

b. Take electron micrographs of protein crystals and diffraction images.

c. Use diffractions to establish the amplitude information and images to find the phase information, then calculate the structural factors to generate the electron clouds for the target proteins.

d. Model building like that in X-ray crystallography.

(Martynowycz & Gonen, Curr Opin Colloid Interf Sci, 2018)
Calculation of Amplitudes & Phases

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Correlation Averaging

Cross-Correlation Map

S/N-enhanced Image

FT
IFT

Fourier space
real space

25Å
20Å
15Å

k
h
Cryo-EM applications in protein structural biology:

1) Single particle analysis:
   (no need to generate protein crystals)
   a. Prepare purified protein samples and freeze them.
   b. Take electron micrographs of isolated protein particles.
   c. Collect several of “identical” images and add altogether to enhance the signal of the objects (darker area).
   d. Then place amino acid models in the electron density.

(Bai et al, eLife, 2013)
A. Raw image data typically seen from a cryo-EM experiments. Protein particles are usually shown as the dark objects. This image shows particles of a proteasome complex.

B. However, often time, each particle looks “blurred”, largely because of image drifting during the picture taking, as well as the microscopic movement of protein molecules in the ice. "Motion correction" is thus necessary to help enhance the image quality, i.e., making the images sharper.

C. We use power spectrum to evaluate the quality of an image. Power spectrum can be seen as a theoretical diffraction pattern of the image in A.

D. Corrected and sharpened images from A. As indicated in C, the corrected picture clearly reveals potential information that can be resolved as better as 3Å.
EM Samples: Membrane Proteins

- Use of detergents: starting with the one used for purification, e.g., DDM.
  - Mild non-ionic
  - Amphipols

- Nanoparticles: a membrane-mimetic scaffold that stabilizes proteins in the native lipid-bilayered environment.
  - MSP-nanodiscs (MSP: membrane scaffold protein)
  - SMA nanodiscs (SMA: styrene–maleic acid)
  - Bicelles
  - Peptidiscs
EM Samples: Membrane Proteins

Micelle  Amphipol  Bicelle

Nanodisc  SMA nanodisc  Liposome

Peptidisc

The past few years have been revolutionary for the field of single-particle electron cryo-microscopy (Cryo-EM), with over 50% of the total deposited structures being determined since 2014. Currently, there are over 1,300 unique (<95% sequence identity) Cryo-EM structures deposited in the PDB, over 150 of which are membrane proteins. Here, we have curated all of the commonly used tools from Anatrace and Molecular Dimensions in Cryo-EM experiments.

DETERGENTS FOR CRYO-EM

The following detergents have been successfully used in the Cryo-EM studies of membrane proteins. Want to learn more? Check out our compilations of membrane protein structures for 2016([Landing/2016/Cryo-EM-Update-Sept16]), 2017([Landing/2017/Cryo-EM-Update-Oct17]), and 2018([Landing/2018/Cryo-EM-Update-Oct18]).

- **GDN101 - GDN** ([PRODUCTS/SPECIALTY-DETERGENTS-PRODUCTS/COMPLEX/GDN101](/PRODUCTS/SPECIALTY-DETERGENTS-PRODUCTS/COMPLEX/GDN101))
- **A835 - AMPHIPOL A8-35** ([PRODUCTS/SPECIALTY-DETERGENTS-PRODUCTS/AMPHIPOL/A835](/PRODUCTS/SPECIALTY-DETERGENTS-PRODUCTS/AMPHIPOL/A835))
- **P5008 - AMPHIPOL PMAL-C8** ([PRODUCTS/SPECIALTY-DETERGENTS-PRODUCTS/AMPHIPOL/P5008](/PRODUCTS/SPECIALTY-DETERGENTS-PRODUCTS/AMPHIPOL/P5008))
- **D310 - DDM** ([PRODUCTS/DETERGENTS/MALTOSIDES/D310](/PRODUCTS/DETERGENTS/MALTOSIDES/D310))
- **NG310 - LMNG** ([PRODUCTS/DETERGENTS/NG-CLASS/NG310](/PRODUCTS/DETERGENTS/NG-CLASS/NG310))
- **LIPID** ([PRODUCTS/LIPIDS/LIPIDS](/PRODUCTS/LIPIDS/LIPIDS))

GDN has been shown to be an effective drop-in substitute for Digitonin which is being used in a number of recent structures. First described in 1996 by Jean-Luc Popot, amphipols are a class of polymers that can stabilize membrane proteins in a detergent-free, aqueous solution. To date, there have been over 20 Cryo-EM structures of membrane proteins determined using Amphipol A8-35.

In recent years, PMAL-C8 has been gaining traction for use in Cryo-EM ([Landing/2018/PMAL-July18]) with a number of unique structures published. PMAL amphipols are zwitterionic, and contain repeating units of a carboxyl, ammoniumamide, and alkyl chain.

The most commonly used detergent in membrane protein crystallization, Dodecyl Maltoside (DDM), has also been used in the Cryo-EM structures of a number of proteins. DDM is also often used as a mixture with Cholesteryl Hemisuccinate (CHS) ([Products/Detergents/MALTOSIDES/10-1-DDM-CHS-Pre-Made-Solution](/Products/Detergents/MALTOSIDES/10-1-DDM-CHS-Pre-Made-Solution)).

Due to its very low CMC, the concentration of LMNG in the buffer can often be reduced to low concentrations, reducing the amount of free detergent micelles, and reducing background. Like DDM, LMNG is often used as a mixture with Cholesteryl Hemisuccinate (CHS) ([Products/Detergents/NG-CLASS/LMNG-CHS-Pre-Made-Solution](/Products/Detergents/NG-CLASS/LMNG-CHS-Pre-Made-Solution)).

Lipid nanodiscs allow for the reconstitution of a detergent solubilized membrane protein into a lipid environment, and are being increasingly used in Cryo-EM. Anatrace offers a full selection of the lipids commonly used in nanodisc reconstitution.

(Anatrace, Inc.)
Fluorescence as a mean to study membrane protein structures:

Fluorescence Resonance Energy Transfer (FRET)

The shift from excitation wavelength to emission wavelength is called the stokes shift. The ability of a fluorophore to absorb encountered light is known as the extinction coefficient. Once energy is absorbed, the fluorophore has some probability less than one of releasing this absorbed energy as light. This characteristic is called the quantum yield. Together, these properties dictate the basic fluorescent properties (brightness and spectra) of an individual fluorescent dye.

The fluorescence of many dyes can be modulated by environmental factors. These factors include soluble quenchers, pH, and the local chemical environment. Thus, when a fluorophore moves from one position to another, moving from one local environment to another, a change in the emission strength or peak emission wavelength of a fluorophore can result. In this regard, tracking the brightness (quantum yield) or spectrum (stokes shift) of a fluorophore are key methods for mapping a protein’s structure with fluorescence (Figure 1).

Some of the earliest structural measurements that use fluorescence took advantage of intrinsic changes in a dye’s fluorescence. Similarly, intrinsic changes in the fluorescence of the native amino acid tryptophan have been used to map structures.
Magnetic resonance as a mean to study membrane protein structures:

Nuclear magnetic resonance (NMR): function and dynamics

(Liang & Tamm, Nat Struct Mol Biol, 2016)
Magnetic resonance as a mean to study membrane protein structures:

Nuclear magnetic resonance (NMR): function and dynamics

Asymmetric water accessibility in *E. coli* multidrug transporter EmrE

Magnetic resonance as a mean to study membrane protein structures:

Spin-labeled Electron paramagnetic resonance (EPR) spectroscopy

(Mchaourab et al, Structure, 2011)
4. Other strategies in studying membrane protein structures

- Fusion proteins ✓
- Antibody
- Ligands
- Library of small molecules
- Protein re-engineering
- ...


Fusion protein strategy:

(Kobe et al, Acta Cryst F, 2015)