Purification of Membrane Proteins
History

1895 = W.C. Röntgen discovers X-rays (Nobel Prize 1901)
1910 = Max von Laue: Diffraction Theory (Nobel Prize: 1912)
1915 = W.L. Bragg & W.H. Bragg: NaCl, KCl (Nobel Prize Physics)
\[ 2 \cdot d \cdot \sin \Theta = n \cdot \lambda \]
1934 = D. Bernal & D. Crowfoot examine first Proteins
1950 = DNA double helix structure: Watson, Crick, Wilkins (Nobel Prize 1963)
1958 = Myoglobin Structure (Nobel Prize 1962 Kendrew, Perutz)
1971 = Insulin (Blundell)
1978 = First Virus Structure (S.C Harrison)
1988 = Nobel Prize: Photosynthetic reaction center (Huber, Michel, Deisenhofer)
1997 = Nobel Prize: ATP-synthase structure (Walker)
1997 = Nucleosome core particle (T. Richmond)
1998 = KcsA ion channel (MacKinnon) (first recombinant \( \alpha \)-helix structure)
1999 = Ribosome Structures (Steitz, ...)
2000 = Reovirus core structure (S.C. Harrison)
2000 = Rhodopsin structure, GPCR (Palczewski et al.)
2002 = ABC-Transporter (D. Rees et al.)
2012 = B. Kobilka: structures of GPCR (Nobel Prize Chemistry 2012)
Dimensions of Life

- Atoms
- Lipids
- T2 phage
- Protein
- Small molecules
- Chloroplast
- Most bacteria
- Plant and animal cells

Microscopic techniques:
- Light microscope
- Electron microscope

Scale:
- 0.1 nm
- 1 nm
- 10 nm
- 100 nm
- 1 μm
- 10 μm
- 100 μm
- 1 mm
Why do we use X-rays?

- Visible light: 400 – 700 nm
- X-rays: 0.01 nm – 100 nm (0.1 Å – 1000 Å)
- Atomic distances: ~0.15 nm (~1.5 Å)

In microscopy resolution is limited by the length of electro-magnetic radiation used.

Light Microscopy...
To observe a sample, a lens is used to refocus incident radiation that is diffracted in all directions by the sample.

X-ray’s can’t be refocused by a lens...so we record the diffraction pattern and mathematically back-calculate the sample..
Our Data

Our Calculation

Our Model

Our Blood, Sweat and Tears
Why do we need crystals?
Crystal acts like an amplifier.

How waves work

Constructive interference

Destructive interference
As of Sept 4, 2015

- 104,020 protein structures in the PDB
- 1005 (0.97%) are of Membrane Proteins
- 555 of which are of unique proteins

Challenges

- High level (mg) expression
- Membrane is heterogeneous, but homogeneity is often necessary for biochemical, biophysical and structural studies.
- Solubilization conditions that maintain the protein in a stable, functional and monodisperse form cannot yet be predicted.
- Crystallization conditions cannot be predicted yet.
- NMR more suited to smaller proteins (<200 kDa)
A roadmap to structures:
Expression Systems:

Natural tissue (eg. AQP from spinach leaf; ATP synthase from bovine heart mitochondria)

- Protein of interest must be highly expressed in that tissue.
- Affinity tags not usually available, therefore must use properties of the protein in order to purify (eg. Ligand affinity, pl, size, etc.)

E. coli (eg. )

- Cheap!
- Easy to culture and methods to handle large volumes readily available.
- Affinity tags can be used (metal affinity, anti-body affinity, strepatividin, GST, etc.)
- Easily ruptured (10 kPSI sufficient)
- Numerous vectors available with constitutively active promoters, inducible promoters (by IPTG) or autoinducible promoters.
- Common strains: ROSETTA, BL21*(DE3), C41(DE3), C43(DE3)
- **Cons:** Glycosylation, or other translational modifications not performed.
  - Often cannot handle formation of critical Cys bridges
  - Not all proteins properly folded (lacks correct chaperons, etc.)
  - Best yields from fresh transformants.
**Expression Systems:**

*S. cerevisiae* (eg.)

- Cheap!
- Easy to culture and methods to handle large volumes readily available.
- Can freeze stocks (so fresh transformations not needed every time).
- “The power of yeast genetics!” (A lot of strains available with knocked-genes that may help promote expression of your protein of interest)
- Plasmid based expression and so through homologous recombination can rapidly screen mutations or different constructs.
- Affinity tags can be used (metal affinity, anti-body affinity, strepatividin, GST, etc.)
- Glycosylation, or other translational modifications are performed.
- Great ERAD system that prevents misfolded protein from leaving the ER.
- Numerous vectors available with consitutively active promotors, inducible promotors (by GAL1, CuP) or autoinducible (ADH2) promotors.
- Selectivity through nutrient drop out (eg. –Uracil, -Leucine, -Histidine, etc.)
- **Cons:** More expensive than *E. coli* especially if GAL1 promoter used (since ultrapure Galactose must be used at $600 / kg).
  - High pressures (>30 kPSI) are needed to rupture cell membranes due to cell wall.
  - Cell densities are not high (~5-15 g wet pellet / L) so large volumes may be necessary.
  - Following transformations, cultures take 3 days to grow.
Expression Systems:

P. pastoris (eg. Kv1.2 channels, Kir2.2)
- Reasonable cost! Particularly since MeOH used for induction.
- Easy to culture and methods to handle large volumes readily available.
- Can freeze stocks (so fresh transformations not needed every time) or use stab cultures.
- Affinity tags can be used (metal affinity, anti-body affinity, strepavidin, GST, etc.)
- Glycosylation, or other translational modifications are performed.
- Cells can grow to ODs of 50 – 100 depending on strain, and growth conditions. That’s as thick as yogurt! Up to ~50-100 g wet pellet / L)
- **Cons:** Following transformations, cultures take 3 days to grow.
  - Genome integration necessary for construct, ie. constructs generated first prior to transforming cells.
  - Zeocin (antibiotic) is expensive for initial steps.
  - Up to 25% false positives (antibiotic resistant but no clone insterted)
  - High pressures (>30 kPSI) are needed to rupture cell membranes due to cell wall.

Insect cells (sf9, HiFive) (eg. )
- Higher eukaryotic system therefore complex translational modifications possible.
- Better machinery for folding mammalian proteins.
- Yields / L are generally high for proteins that express.
- Affinity tags can be used (metal affinity, anti-body affinity, strepavidin, GST, etc.)
- Easily ruptured (homogenization or sonication)
- **Cons:** Cost is significantly higher
  - Do not contain cholesterol or some other lipids that may be necessary for proper function of the protein.
  - Cell cultures must be constantly maintained.
  - Cells are much more fragile than yeast or bacteria to environmental stressors and variation.
  - Viral system for gene expression, so safety procedures must be in place
  - Cloning, virus generation, and titration take significantly longer yeast or bacterial systems.
Expression Systems:

Mammalian cells (eg.)
- Higher eukaryotic system therefore complex translational modifications possible.
- Best machinery for folding mammalian proteins, particularly challenging ones like C-class GPCRs which require specific Cys bridges.
- Yields / L are generally high for proteins that express.
- Affinity tags can be used (metal affinity, anti-body affinity, strepatividin, GST, etc.)
- Easily ruptured (homogenization or sonication)
- **Cons:** Cost is significantly higher
  - Cell cultures must be constantly maintained.
  - Cells are much more fragile than yeast or bacteria to environmental stressors and variation.
  - Viral system for gene expression, so safety procedures must be in place
  - Cloning, virus generation, and titration take significantly longer yeast or bacterial systems.

Cell-free systems (eg.)
- Purified RNA are mixed with ribosomal systems to directly transcribe proteins.
- Purification of toxic proteins
- Can easily insert isotopically labelled or unnatural amino acids
- Proteins can be directly inserted into detergent micelles or desired lipids during transcription.
- Easy to purify since system is relatively homogeneous
- **Cons:** Cost is significantly higher / mg of protein.
  - New system, so kinks for large quantities may not be fully worked out.
Establishing a high-expressing stable cell line:

Stable T-REx293 [rP2X4] cells

\[\xrightarrow{\text{Induction}}\] Cell sorting

\[\xrightarrow{\text{Culture}}\] High-expression stable line

Repeat 3 times

FACS profile

pre-sort

Cell number

GFP Fluorescence

T-REx293 [rP2X4]

Cell number

after 3rd sort

GFP Fluorescence
Determination of Structured Domains

[Diagram showing a graph with labeled axes and a molecule structure]

NH₃

[Image of a molecule structure with NH₃ and CO₂ indicated]
Determination of Structured Domains (GLOBPOT)

Disordered by Russell/Linding definition

Disordered by Russell/Linding definition

Potential globular domains (GlobDoms) by Russell/Linding definition
Construct Design:

Target Protein

Constructs Designed
The “co-translational tranlocation” process is initiated by an N-terminal ER signal sequence (red) that functions as a start-transfer signal. Following transcription of a stop-transfer sequence (orange), the stop-transfer sequence enters the translocator and interacts with a binding site, inducing a conformational change in the translocator that discharge the protein laterally into the lipid bilayer.
**Construct Design:**

A Signal Sequence that functions as a start-transfer signal binds to the translocator (+ side in the cytosol).

(single pass membrane protein: option 2)
Construct Design:

Signal Sequence?

(single pass membrane protein: option 2)

An internal ER signal sequence acts as a start-transfer signal and initiates the transfer of the C-terminal part of the protein. At some point after a stop-transfer sequence has entered the translocator, the translocator discharges the sequence laterally into the membrane.
Construct Design:

Affinity Tag?
- His6; His8, His10; His12 tags co-ordinate divalent cations (Ni$^{2+}$, Co$^{2+}$, Zn$^{2+}$, Cu$^{2+}$)

- glutathione S-transferase (GST)

- etc.

Detection Tag?

Elution by free glutathione

Incompatible with reducing agents and EDTA/EGTA
<table>
<thead>
<tr>
<th>Tag</th>
<th>Length</th>
<th>Amino Acid Sequence</th>
<th>size (kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arg5-Tag</td>
<td>5-6</td>
<td>RRRRR</td>
<td>0.80</td>
</tr>
<tr>
<td>Hie6-Tag</td>
<td>2-10</td>
<td>HHHHH</td>
<td>0.84</td>
</tr>
<tr>
<td>Hat-Tag</td>
<td>19</td>
<td>KHDLIHNVHEKFHAHAN</td>
<td>2.31</td>
</tr>
<tr>
<td>V5-Peptide-Tag</td>
<td>14</td>
<td>GKP1INPLLGLDST</td>
<td>1.42</td>
</tr>
<tr>
<td>Flag-Tag</td>
<td>8</td>
<td>DYKDDE</td>
<td>1.01</td>
</tr>
<tr>
<td>3 x Faq-Tag</td>
<td>22</td>
<td>DYSKDIHYKDHYKDIDYKDDE</td>
<td>2.73</td>
</tr>
<tr>
<td>Strep-Tag I</td>
<td>9</td>
<td>AWREHPQFGG</td>
<td>1.00</td>
</tr>
<tr>
<td>Strep-Tag II</td>
<td>6</td>
<td>WSHPFQK</td>
<td>1.06</td>
</tr>
<tr>
<td>Nano-Tag9</td>
<td>9</td>
<td>MDVEAKLGR</td>
<td>1.15</td>
</tr>
<tr>
<td>Nano-Tag15</td>
<td>15</td>
<td>MDVEALKRARVPLVET</td>
<td>1.78</td>
</tr>
<tr>
<td>SBP-Tag</td>
<td>38</td>
<td>MDEKTTGWGGHVGGLAGELQELARLEHHPQGQEP</td>
<td>4.30</td>
</tr>
<tr>
<td>c-myc-Tag</td>
<td>11</td>
<td>EQKISIEDEL</td>
<td>1.20</td>
</tr>
<tr>
<td>S-Tag</td>
<td>15</td>
<td>KRRRKKNFIYAVSAAKFKK</td>
<td>1.75</td>
</tr>
<tr>
<td>Camodulin-Binding-peptid</td>
<td>26</td>
<td>KRRKVINFIYAVSAAKFKK</td>
<td>2.96</td>
</tr>
<tr>
<td>Cellulase-Binding-Domain (different)</td>
<td>27-189</td>
<td>MTWVGQCGGGWSGPTNCAPG6SACSTLNFYYAQC</td>
<td>3.00-20.00</td>
</tr>
<tr>
<td>Chitin-Binding-Domain</td>
<td>51</td>
<td>TNPVGVSAWQNTAYTASQLVTVNGKTYKLCQPHICIALGWEPNSV PALWQLQ</td>
<td>5.59</td>
</tr>
<tr>
<td>GST-Tag</td>
<td>211</td>
<td>LGYWKIKKLGVQFTRLLELYELKKE YEEILYERDEGDKWKNKPKELGELEFPLFYYIDIGYDKLTQSWAIIYIAD</td>
<td>26.00</td>
</tr>
<tr>
<td>Maltose Binding Protein MalE-Gene, MEP-Tag</td>
<td>396</td>
<td>MKITKAGTIALSALTMMFSAASALAKTEEGKLVITWNGKGYNGVLUAGKVGKKFDITGKLTVHEHPKLEKFT PQVAACTCGGPIIIFWAADHPFGYQSGGLLEAVTEPKAFOQKLVPYFTWDARVNYGKLIAPIAFLKLKLYK DPLPENPKWRFTIPPALDKELKKGKSAIMPFLNQEPYFTWPLIAADGGAFKYNKGKDYKTIVGVINAGASKAGT FLVDLKNKHNMADNTSYSMASEAAFNKGETAMTINGPNWAYNISITDSKVNFRGTVLPFRKGQSKPFVGVSLAGI NAASPNSKUELAKLEFELYLLTTDEGELAVKNDKIPGLAVALKSYEELKADPKLIAITAMIENKQGEIMPNI PQMSAF WYAVRATIVNASGRQGTVDEALKDADIGTRK</td>
<td>40.00</td>
</tr>
<tr>
<td>Bietin Carboxy Carrier Protein</td>
<td>122</td>
<td>MDIRIKKIKLILVESSESISELEISEGEEVESRISAAAPASFPMQQAAYAAPMNMQOPFAQSNAAPATVPSMEEP AAATFEGHSTVSPVGTFFIYRTPSYPDRAKAFIEVEQGKVNVGDLTCLSVEAMHMONQIEADSGTSDKVAILVESGQPV EDFEPVVE</td>
<td>16.70</td>
</tr>
<tr>
<td>Thioredoxin-Tag</td>
<td>109</td>
<td>MDSDKIIIHLTEDeDFDIDVLKADGAILDVBPWAENCGFPCKMIAPLDEIADEYQGKLVTAKLNDQNPSTAPKYGI RMIFILLFNMGEVAAKVGALKSGKQIKFLDANLIA</td>
<td>11.80</td>
</tr>
</tbody>
</table>
Does N-terminal or C-terminal tag make a difference?

- [x] YES
- [ ] NO
- [ ] MAYBE
- [ ] WTF?

For some proteins: expression, folding, localization, function can be affected.

For others: expression, folding, localization, function are unaffected.

For some proteins: expression, folding, localization, function are unaffected, but tag is buried so unavailable for binding resins.
Expression testing from a practical perspective:

Cells are grown at small scale (eg. 1 – 50 mL) and screened for expression.

3 useful techniques:

**Coomassie staining an SDS/PAGE gel**

**Western Blotting**

**Fluorescence Detection**


Detergents:

- Detergents are monomerically distributed until they reach a threshold concentration (CMC) where they spontaneously form micelles.
- At 1-3 x CMC detergents are effective at solubilising.
- CMC is inversely related to the size of the acyl chain.
- CMC is sensitive to both temperature and salt conc.
Lipopeptide Detergents:

polymeric surfactants, such as the amphipols (amphiphilic polymers):

Structure of A8-35 and of PC-amphipols.

Artist's view of a protein (pink) complexed by an amphipol (grey). The polymer adsorbs onto the hydrophobic transmembrane surface of the protein, keeping it soluble while stabilizing it biochemically.

Choosing Detergents:

Coomassie staining or WB

Fluorescence size-exclusion chromatography


(Structure. 2006 Apr;14(4):673-81)
Purification methods:

- Disrupt the harvested cells by mechanical force (high pressure) using cell disrupter.

- Remove unbroken cells, debris that is not membrane, and organelles such as inclusion bodies with a “low speed” centrifugation (4000xg).

- Collect the membrane fraction by centrifuge the supernatant from the last step with a “high speed” ultra-centrifugation (120,000xg). This step removes all the soluble proteins.

- Resuspend membranes in solubilization buffer & add detergent (typically >10x CMC).

- Spin solubilized membranes down to remove unsoluble material.

- Add affinity resin to sample for several hours, and follow protocols for elution.

- Sometimes protein not stable in elution buffer (eg. due to high imidazole or salt)...if so, desalting or dialysis might be required...otherwise...
Purification methods:

- Size-exclusion chromatography.

**Column Superose 6 10/300 GL**

- **Column**: Superose 6 10/300 GL (Tricorn)
- **Sample**
  1. Thyroglobulin (M, 669,000) 5 mg/ml
  2. Ferritin (M, 440,000) 0.4 mg/ml
  3. BSA (M, 67,000) 8.0 mg/ml
  4. Ribonuclease A (M, 13,700) 1.0 mg/ml
- **Sample volume/load**: 500 μl
- **Eluent**: 0.05 M Phosphate buffer, 0.15 M NaCl, pH 7.0
- **Flow rate**: 0.5 ml/min
- **System**: AKTA PLC
- **Detection**: 280 nm

**Column Superdex 200 10/300 GL**

- **Sample**
  1. Thyroglobulin (M, 669,000) 5 mg/ml
  2. Ferritin (M, 440,000) 0.4 mg/ml
  3. BSA (M, 67,000) 8.0 mg/ml
  4. Ribonuclease A (M, 13,700) 1.0 mg/ml
  5. Bovine Serum Albumin (M, 66,000) 2 mg/ml
  6. Cytosol A (M, 13,600) 1.5 mg/ml
  7. Aprotinin (M, 6,512) 2 mg/ml
  8. Vitamin B12 (M, 1,355) 0.1 mg/ml
- **Sample volume**: 500 μl
- **Eluent**: 0.05 M Phosphate buffer, 0.15 M NaCl, pH 7.0
- **Flow rate**: 0.4 ml/min, room temperature
- **Detection**: 280 nm

**Column Superdex 75 10/300 GL**

- **Sample**
  1. BSA (M, 67,000) 8 mg/ml
  2. Ovalbumin (M, 43,000) 2.5 mg/ml
  3. Ribonuclease A (M, 13,700) 5 mg/ml
  4. Aprotinin (M, 6,512) 2 mg/ml
  5. Vitamin B12 (M, 1,355) 0.1 mg/ml
- **Sample volume**: 500 μl
- **Eluent**: 0.05 M Phosphate buffer, 0.15 M NaCl, pH 7.0
- **Flow rate**: 0.4 ml/min, room temperature
- **Detection**: 280 nm
Sources of heterogeneity (other than contaminating proteins and nucleic acids):

- Partial proteolysis products
- Oxidation of cysteines
- Deamination of Asn and Gln to Asp and Glu
- Post-translational modifications
- Oligomerization
- Isoforms
- Misfolded population
- Structural flexibility
What if protein still not pure?:

- Ion exchange:
  
  **Cation exchange (vely charged stationary phase):**

  A mixture of proteins in Mes buffer is loaded into the column, displacing sodium cations. A pulse of Mes is formed. To avoid drastic ionic strength increase and pH decrease in column micro-environments, applied concentration of adsorbing proteins should be <5 mg/ml. The max total protein concentration is 15 mg/ml.

  Proteins are eluted with increasing salt (NaCl) gradient

  ![Diagram of ion exchange process](image)

  **Legend:**
  - Sodium cation, Na⁺
  - Mes anion, Mes⁻
  - Chloride anion, Cl⁻
  - Carboxymethyl anion (CM), R-CO⁻
  - Protein bearing a number of positive charges (as marked)

  **Elution profile:**
  
  ![Elution profile graph](image)

  Increasing salt concentration → Eluted volume

  1. quaternary ammonium (Q) - strong anion exchanger

  ![Quaternary ammonium](image)

  2. diethylaminoethyl (DEAE) - weak anion exchanger

  ![Diethylaminoethyl](image)

  1. methyl sulfonate (S) - strong cation exchanger

  ![Methyl sulfonate](image)

  2. carboxymethyl (CM) - weak cation exchanger

  ![Carboxymethyl](image)

- Note: While **hydrophobic interaction chromatography** is a useful technique to separate proteins based on hydrophobic character, this will be dominated by detergent in membrane proteins and will be non-specific at best, and induce aggregation at worst. This technique is best used on soluble proteins only.
What if protein still not pure?:

- Ligand affinity chromatography (nucleotides; Lectin(ConA); etc).

If ligand for your protein is known, you can consider conjugating it to matrix to generate a specific ligand-affinity resin.

Table 1. Experimental conditions for the binding and elution of brain enzymes to Con A-Sepharose.

<table>
<thead>
<tr>
<th>Brain enzyme</th>
<th>Binding characteristics to Con A-Sepharose</th>
<th>Elution with α-methyl glucoside</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-D-Galactosidase</td>
<td>Binds at pH 6.0. Both galactosidases A and B bind</td>
<td>Elutable at 25°C. No significant elution at 4°C.</td>
</tr>
<tr>
<td>α-L-Fucosidase</td>
<td>Binds at pH 6.0. All the three forms of fucosidase bind</td>
<td>Elutable at 25°C</td>
</tr>
<tr>
<td>α-D-Mannosidase</td>
<td>The cytosolic neutral mannosidase does not bind but lysosomal acid mannosidase binds at pH 6.0</td>
<td>Elutable at 25°C</td>
</tr>
<tr>
<td>β-D-Glucuronidase</td>
<td>Both lysosomal and microsomal glucuronidases bind at pH 6.0</td>
<td>Both enzymes are eluted at 28°C. The lysosomal enzyme is eluted at low and the microsomal enzyme at high concentration of α-methyl glucoside</td>
</tr>
</tbody>
</table>

What if protein still not pure?:

- Dye-affinity chromatography:

Dye chromatography uses mimics of natural protein ligands to act as pseudo-affinity ligands for protein separations. Reactive dyes are immobilized to a solid support matrix and act as a competitive inhibitor for a protein’s normal ligand.

Example: Blue Sepharose® 6B-CL

useful in the isolation of enzymes requiring NAD\(^+\) and NADP, albumin, interferon, steroid receptors, and, 25-Dihydroxyvitamin D3-receptor.
What if protein still not pure?:

- Negative purification

Protein-tag & contaminating proteins  +  Affinity resin → Affinity resin bound to tag & contaminating proteins  +  Purified protein
**Protein solubility:** will usually increase as you add salt to your aqueous solution (salting in), then begin to decrease when the salt concentration gets high enough to compete with the protein for hydration (interaction with water molecules) (salting out).
Protein solubility (A real life example):

HbCO (carboxyhemoglobin) solubility as a function of ionic strength of various precipitating salts

Typical Precipitating agents:

Salts
- Ammonium sulfate
- Sodium chloride
- Potassium phosphate

Organic reagents
- MPD (2-methyl-2,4-pentandiol)
- Isopropanol

Polyethylene glycol
- PEG 3000
- PEG 6000
- PEG 20000

http://www2.vuw.ac.nz/staff alan_clark/teaching/index.htm
**Nucleation:**
A phenomenon whereby a “nucleus”, such as a dust particle, a tiny seed crystal, or commonly in protein crystallography, a small protein aggregate, starts a crystallization process. Nucleation poses a large energy barrier, which is easier to overcome at a higher level of supersaturation.

**Common difficulties:**
If supersaturation is too high, too many nuclei form, hence an overabundance of tiny crystals. In supersaturated solutions where spontaneous nucleation is difficult, crystal growth often only occurs in the presence of added nuclei or “seeds”
Sparse matrix screens:

**Crystallization conditions** chosen based on limited number of solution and precipitant conditions (screens) that are empirically derived and based on known or published macromolecular crystallization conditions (ie. intentional bias towards combinations of conditions that have worked previously.)

Screens sample a large range of buffer, pH, additive and precipitant variables as possible, while using small amounts of proteins.
Crystallization Methods (Hanging Drop Vapour Diffusion):

Reservoir contains the crystallization condition to be screened

Protein in the drop (typically between [5-20mg/mL] but can be more or less) is diluted by 1/3 (2:1) or ½ (1:1) by mixing with the reservoir solution and placed onto a glass cover slide.

Usually wells are large enough to enable 2 or 3 drops to be placed on the coverslip so different dilutions can be tried.

The cover slip is flipped over to cover the reservoir and sealed with grease.

The precipitant concentration in the drop will equilibrate with the precipitant concentration in the reservoir solution by vapour diffusion. Hopefully, the protein will concentrate, nucleate and crystallize in this process as well.
Crystallization Methods (Sitting Drop Vapour Diffusion):

Again, the precipitant concentration in the drop will equilibrate with the precipitant concentration in the reservoir solution by vapour diffusion. Hopefully, the protein will concentrate, nucleate and crystallize in this process as well.

Same general principle as hanging drop.

Previously the advantage was that this technique enabled larger drop sizes due to surface tension issues in hanging drop.

Today, sitting drop actually enables smaller drops (100 – 500 nL) than hanging drop due to the robotics used in performing crystallization experiments.

Again, the precipitant concentration in the drop will equilibrate with the precipitant concentration in the reservoir solution by vapour diffusion. Hopefully, the protein will concentrate, nucleate and crystallize in this process as well.
Crystallization Methods (Oil Immersion Micro Batch):

Sample size 1-6µL

Paraffin oil does not allow for diffusion of water and other reagents through the oil. All reagents are present at a specific concentration.

Al’s oil is a 1:1 mix of paraffin oil and silicon that allows for slow evaporation. Protein and other reagents slowly concentrate in the drop.
Crystallization Methods (Lipidic-cubic phase - LCP):

**Lipidic cubic phase (LCP)** is one of many liquid crystalline phases that form spontaneously upon mixing lipids with water at proper conditions.

The protein is mixed with Monoolein and other lipid additives in tightly coupled syringes.

Drops are laid down on a glass slide and precipitation solutions are added. This requires different robotics from vapour diffusion methods, or can be done manually. Note that not all sparse matrix conditions are compatible with LCP due to their ability to induce non-cubic phases.

Crystals are typically small and therefore it is best to generate them sandwiched between 2 glass plates rather than in sitting drop form, due to optical constraints when trying to identify crystals.
Crystallization Methods (Bicelles):

Protein in Membrane → Solubilized Protein → Purified Protein in Detergent → Crystallization of membrane protein in bicelles

DMPC-DHPC bicelles
Metastable Zone – The solution may not nucleate for a long time, but will sustain growth. Seeding may be necessary.

Labile Zone – Protein crystals nucleate and grow

Precipitation Zone – Proteins do not nucleate but precipitate out of solution
Types of Membrane Protein Crystals

Type 1 crystals

Type 2 crystals

“Type 3” crystals

“Type 4” crystals
Atomic Force Microscopy

Protein Crystal Growth

Molecules attach to the edges of these layers (called steps) at specific sites. These attachment sites are called \textit{kinks}. Layers can be generated at \textit{screw dislocations}, by \textit{two-dimensional nucleation}, or by \textit{three-dimensional nucleation}.
Protein Crystal Growth

Screw dislocations

2D nuclei

3D nuclei

Multilayer stacks – droplets
Crystal imperfections

Vacancies

Grain boundaries & Stacking faults
Interpreting results of crystallization experiments:

- **Hit**

- **Phase separation**
  - amorphous precipitate
  - Crystals growing at phase boundary

- **Decreasing nucleation**
Interpreting results of crystallization experiments:

<table>
<thead>
<tr>
<th>Clear Drop</th>
<th>Precipitate/Phase</th>
<th>Needle Cluster</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skin/Precipitate</td>
<td>Quasi Crystals</td>
<td>Plates</td>
</tr>
<tr>
<td>Precipitate</td>
<td>Microcrystals</td>
<td>Rod Cluster</td>
</tr>
</tbody>
</table>
Why we need pure protein

Images of crystallization experiments with RC samples of increasing purity (as reflected by the $A_{280}/A_{800}$ ratio) using LCP, microfluidics or sitting-drop vapor-diffusion techniques. (a) Holistic view of crystallization trials and (b) enhanced magnification, to a uniform scale, for comparison of crystal size and quality.

Crystallization trials in Lipidic-cubic phase more tolerant of contaminants than other methods.

Overall: The purer the protein, the more likely to form crystals.

Fluorescence screening of Crystals:

**UV:** Takes advantage of intrinsic fluorescence of Trp residues. Most proteins have at least 1 Trp residue that can absorb UV light, and therefore protein crystals can be distinguished from non-protein crystals (salt, detergent, etc.). However, some proteins may weakly fluoresce yielding false negatives.

**Green screens:** A non-covalent fluorescent dye (emission at 490 nm) conveys fluorescence to most proteins. This is helpful particularly for small crystals and those without intrinsic Trp fluorescence. Need to use in conjunction with UV transparent plates for optimum performance.
## a

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**Crystal hit condition**

## b

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**Crystal hit condition**

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**Fine screening**
pH:

- Protein surface charges affect “crystal packing” or the relationship between one protein and another in the crystal.

- Electrostatic interactions and hydrogen bonds are pH sensitive due to the pKa’s of the amino acid residues which participate in these interactions.

- They are also more directional than hydrophobic interactions and therefore contribute to the protein orientation during protein interaction during lattice incorporation.
Temperature:

Temperature can affect the protein stability and how a protein solution reaches supersaturation states.

• Ideally crystallization experiments should be kept at constant temperature.

• Each set of conditions should be tested at different temperatures.

• Typically 4 °C and room temperature are tested but also 12 °C and 15 °C may be worth trying.
Additives:

Sometimes the stability of a protein can be increased and/or conformational heterogeneity by including additives in the crystallization screens.

- Detergents
- Reducing agents (DTT)
- Substrates / Ligands
- Co-factors
- Detergents
- Cryo-protectants (glycerol, ethylene glycol, etc.)
What if still no crystals or only those of low quality diffraction:

Different Detergents!

The best detergent for solubilisation is not necessarily the best detergent for crystallization. It is important to assess many detergents in crystallization experiments.

Detergents can also be used as an additive in crystallization trials which alter the PDC complex dimensions and the nature of the hydration shell.

Data from the Kawate Lab
What if still no crystals or only those of low quality diffraction:

- High speed spin to remove aggregates
- Deglycosylate/Dephosphorylate/etc.
- Add a ligand to reduce conformational variation.
- New construct (with additional domains?)
- Homologous Proteins
- Reduce the flexibility of loops
- New construct with additional protein partners
- Clever reworking of loops (eg. Lysozyme)
- Antibody fragments / nanobodies
Highspeed spin to remove aggregates:

Aggregates can poison the crystal lattice, disrupting crystal growth.
Deglycosylation can improve crystal quality:

Note that there are several different enzymatic and chemical methods for removing glycosylation. Also, if glycosylation sites are known, these residues can be mutated to Gln (Q) to prevent glycosylation in the expressed protein. However, glycosylation may be necessary for proper folding and/or expression. Often, yields decline with the mutant protein.
Lysozyme was added into the flexible loop of a GPCR:
Fab fragments and nano-bodies:

A

B

C

D

E

Antigen Binding site

Fab fragment

Hinge

Fc fragment

Antigen Binding site

Hinge

Fab fragment

scFv fragment

nanobody
Fab fragments and nano-bodies:

Antibody fragments can help stabilize a protein conformation, and provide additional crystal contacts. As an additional bonus, it can also assist in phasing by molecular replacement.