Methods Development for Membrane Proteins

- **Pre-crystallization screening**
  - measurement of detergent by RI
  - characterization of PDC by SLS/RI/UV
  - rapid exchange into 90+ detergents
  - in silico analysis
  - other assays

- **Crystallization screening**
  - detergent-independent and -specific, & additive screens
  - parameterization of crystallization buffers
  - software for optimization screens by hand or machine

- **Crystallization chaperones**
  (target-dependent or target-independent)
  (generation of binding protein vs. insertion of a 'knob')

Validate with several test proteins, publish/disseminate, apply to human membrane protein target set

Disorder and/or flexibility precludes good crystals (characterize/reduce?)
**Methods Development for Membrane Proteins**

Tools to make it faster, cheaper, easier (% of time, $, effort spent on sample preparation)

- Pre-crystallization screening
  - explore a vast experimental space
  - eliminate possibilities based upon experimental observation
  - try to avoid *ad hoc* exclusions at the outset
  - assays are imprecise but accurate

- Explore 'membrane-mimetic space' quickly & easily

- Crystallization screening
  - for a specific protein construct in a specific membrane-mimetic, how much screening is enough?
Pre-crystallization screening (examples)

- Proteins that are polydisperse crystallize less well (DLS)
- Proteins w/ disordered regions can crystallize less well (limited proteolysis/MS, HDX, *in silico* analysis)
- Solubility characterization to get good [protein] for set-ups
- $B_{22}$ measurements
- The protein is a very important parameter to vary (Kwong et al. [1999], variational crystallization)
Membrane-Mimetic Space (MMS)

Native membrane – purple membrane

Modified native membrane - AchR

Liposomes/vesicles/bilayers - 2D xtals, 3D LHC

Lipidic cubic phase, bicelles
(nanodiscs? other detergent phases?)

‘Classical’ PDCs

Organic solvents - peptides, fragments

• Preserve structure/function and obtain adequate samples
• MMS has been inadequately screened/explored
• Successes in ‘uncommon’ detergents reported
Method described by Strop, Brunger (2005)
SLS for Molecular Weight Determination (P&D of PDC)

- Obtain oligomeric state with amount of bound detergent
- For RI, apply peak fitting for more accurate [det]
- 5-10 μl injection, small Superdex-200 column
- A280 alone looks perfectly ok, but micelles (may be) too high
Detergent type is a significant factor in crystallization

- How to examine protein stability in different detergents?
  - ‘traditional gold standard’ - gel filtration/SEC w/ UV-VIS (but false positives occur - good SEC but not good crystals)
  - Kawate & Gouaux (2006) fluorescence-detection SEC w/GFP
  - Wiener (2004) $A_{280}/A_{320}$ to look for aggregation, use μplate (Dilute ~10x into a new detergent, so false positives due to low concentration and/or incomplete exchange.)
  
  Example of false-positive: $A_{280}/A_{320}$ looks good, so scale up. Solubilize membranes with original good detergent, bind to IMAC, wash & elute with new ‘good’ detergent, nothing or nearly nothing elutes.

  Therefore, getting stuck on the column is a robust assay for eliminating bad detergents.

  Miniaturize and parallelize this approach, and obtain ...
Detergent Footprint Assay

- Bind purified protein in known good detergent to resin
- Aliquot to a filter plate
- Wash with good detergent buffer
- Exchange (20CV) with test detergent buffer
- Elute (30 μl)
- Run gel and blot to look for presence of protein (3 μl)
- Use remaining 27 μl elution volume for SEC, other expts
- Note: detergent solutions made as 2x stocks in single-use heat-sealed SBS plates

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Detergent Footprint Assay

- Use 96-well E-PAGE gel (Invitrogen)
- Can stain the gel, but takes several hours or overnight
- Instead, use iBLOT (Invitrogen) and stain blot (minutes)
- Resin to Readout in less than an hour
- How much protein is required for the entire 96-sample set?

For E-PAGE/iBLOT, 0.5-1.0 mg (conservative estimate)
For E-PAGE/iBLOT + SEC, 5 mg (due to UV detection)
A fluorescence detector is 100-1000x more sensitive than UV, so fluorescence-SEC of intrinsic Trp fluorescence brings it back down to 0.5-1.0 mg
(actually, if E-PAGE/iBLOT skipped, then 50 μg sufficient)
**E. coli**
water channel
AqpZ

**E-PAGE Gel**
Coomassie Stain

Stable in many detergents

Tetramer to monomer ratio varies
Human MP

E-PAGE Gel
Nitrocellulose Blot
MemCode Stain

Stable in few detergents

Pattern of detergents has information
SEC with a Single Mobile Phase

Fast, information in size/presence of peaks but lose retention time information (no SLS/RI PDC)
Slow, information in size/presence of peaks + peak position, line-shape, SLS/RI PDC
Crystallization Screening

A. Commercially-available membrane protein screens

- MembFac (Hampton)
- MemStart, MembSys, MemGold (Molecular Dimensions)
- JBScreen Membrane (Jena Biosciences)
- MbClass, MbClassII (Qiagen)
- Optimix5 (Fluidigm)

+ soluble protein screens, esp. PEG/ion conditions
Crystallization Screening

B. Laboratory-developed membrane protein screens

1. Detergent-independent
   Matched solubility pre-screen + biased random screen

2. Additives
   Heavy atoms, detergents (small amphiphiles), others

3. Detergent-dependent
   Based on proximity to phase boundaries
35 two-component buffers, fit to smooth curves, for simple formulation and dispensing
ScreenGem: design & formulation of optimization grids

- Automated or manual options
- Generation of recipes for manual dispensing, or machine-readable for fluid-handling robotics
Questions
What do ‘complex’ membrane-mimetics ‘do’ to help?
Can ‘simpler’ membrane-mimetics be tuned to do the same?
What are the ‘bad’ length-scales and time-scales of motions?
What assays can give read-out on this ‘bad-ness’?
Can pre-crystallization assays yield useful correlative (or even predictive) data to improve throughput?
[apply to proteins, ligands, membrane-mimetics, all of it]

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