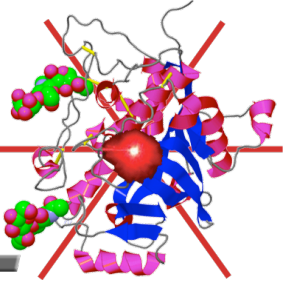

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Lipidic cubic phase injector facilitates membrane protein serial femtosecond crystallography

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15 April 2015

Membrane Proteins

- One third of the proteome[4]
- 60% of current drug targets in humans[2]
- Critical cellular and physiological functions
- Structure → Function

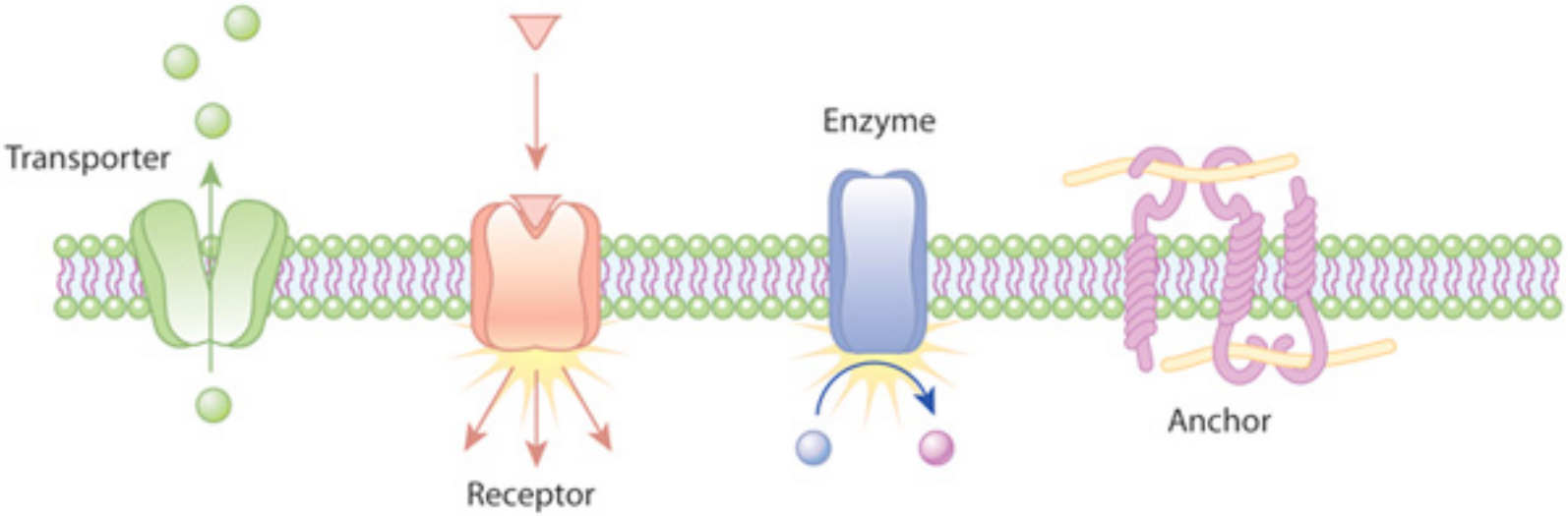


Figure 1. Membrane protein functions. (Nature)

Membrane Protein Crystallization

- Development of LCP almost 20 years ago (1996) Landau
- Detergent micelle solution
- Crystallization of membrane proteins, remove from native lipid bilayers
- Lack methods to crystallize membrane proteins for structure determination

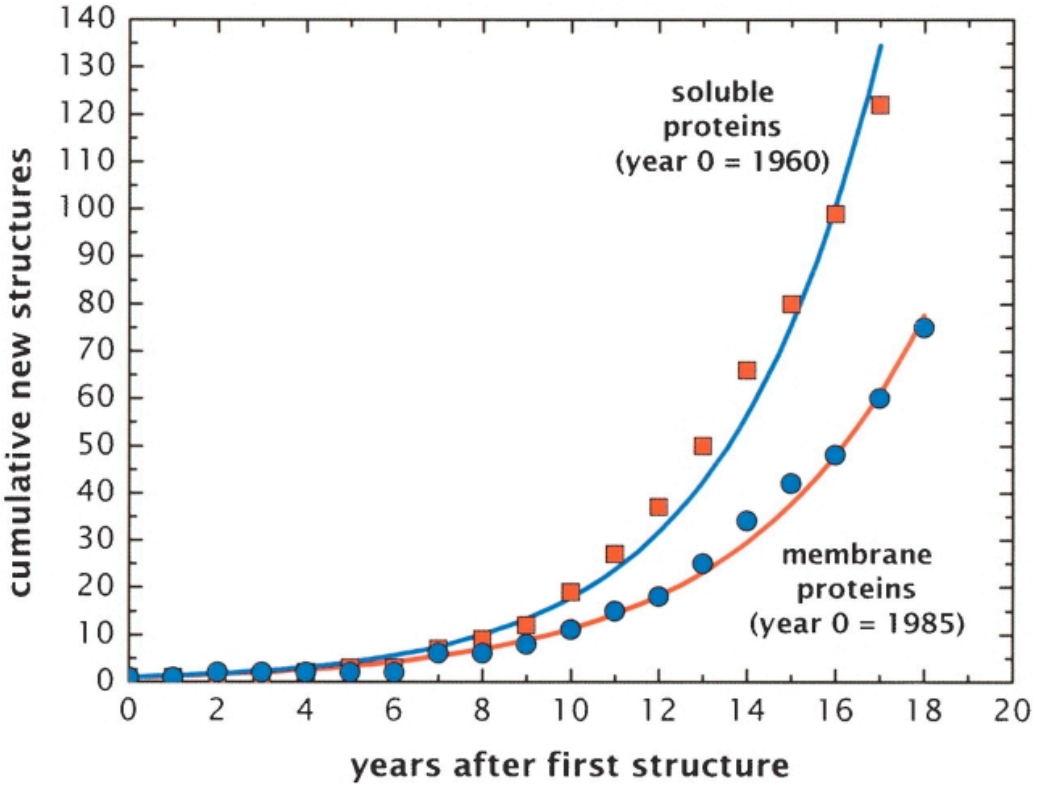


Figure 2. Membrane protein structures lag behind soluble membrane structures.[1]

Lipidic Cubic Phase (LCP)

- Quasisolid membrane environment
- Viscoelastic properties, transparent, optically isotropic, viscous and sticky, gel like
- LCP one of many phases that form spontaneously
  - 2 nonintersecting networks of water channels
  - More native like environment rather than harsh detergent
  - Packing provides more hydrophobic contacts, lower solvent content

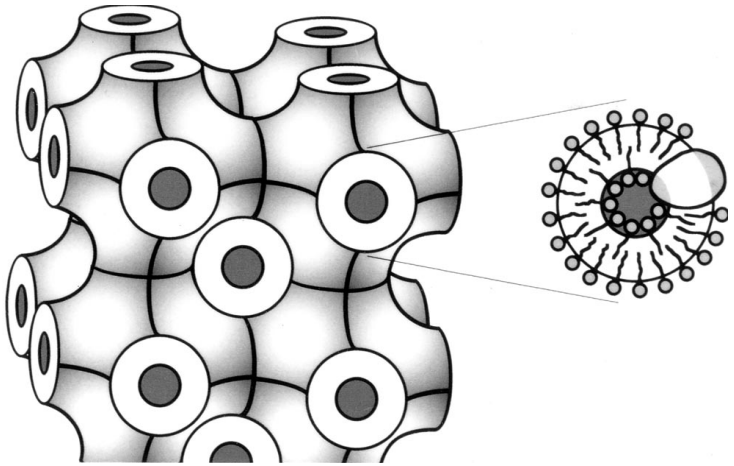


Figure 3. Representative LCP schematic. (Landau, PNAS, 1996)

Lipidic Cubic Phase (LCP)

- Produces highly ordered crystals, limited in size
- Micrometer-sized crystals in initial screening
- Not large enough for synchrotron, tedious to optimize larger crystals
- Resolution for crystals limited by radiation damage
- LCP grown microcrystals are suited for SFX serial femtosecond crystallography

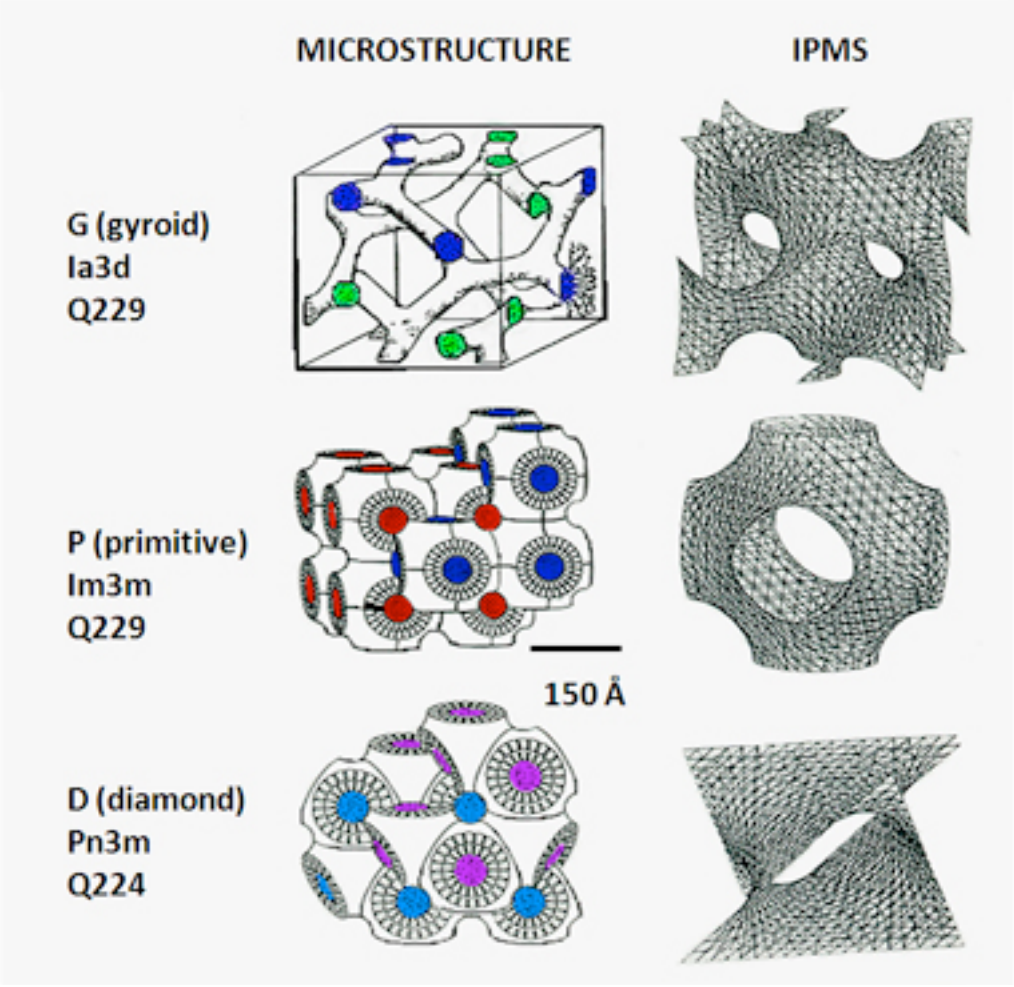


Figure 4. Pn3m is the microstructure in this proof of principle of Smoothened receptor. (Cherezov)

Serial Femtosecond Crystallography (SFX)

- "Diffract and destroy"
- Duration of X-ray pulses is such that diffracted photons exit sample before photoionization
- Data collection at more native temperatures
- Work with soluble proteins, aqueous delivery into beam
- Gas Dynamic Virtual Nozzle (GDVN)

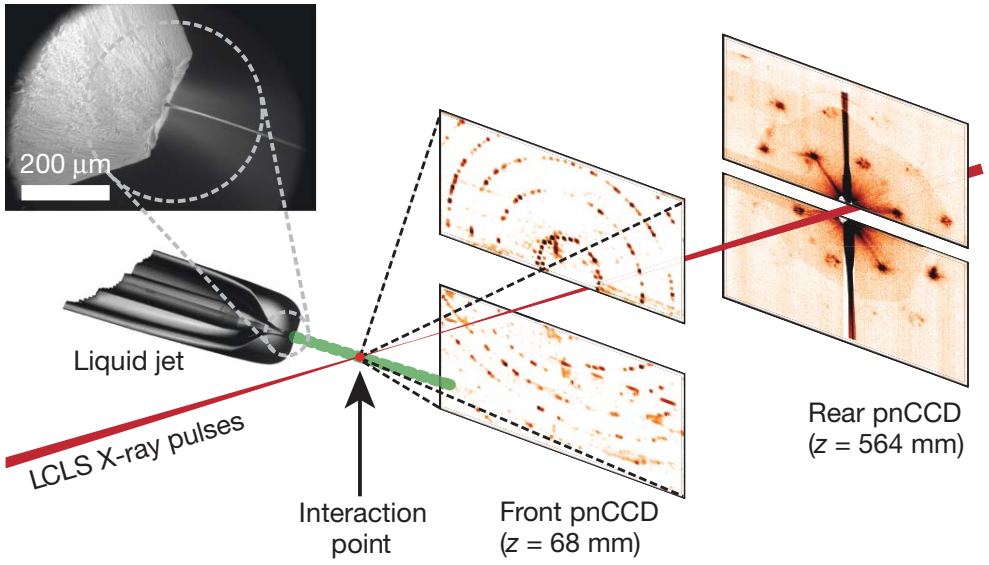


Figure 5. Experimental set-up for SFX. (Nature 470, 73 – 78 (2011)

X-ray Free Electron Laser (XFEL)

- Stanford 2009 – DOE
- Peak brightness of an XFEL is a billion times higher than that of third generation synchrotrons
- 50fs XFEL energy = 1s synchrotron energy
- 120Hz pulses
- 10^{12} photons per pulse
- Ability to diffract and destroy

Figure 6. Aerial view of SLAC National Laboratory, including XFEL

Gas Dynamic Virtual Nozzle (GDVN)

- Gas focusing sheath
  - supplies hydrated nanocrystals to the pulsed X-ray beam

- Not feasible for LCP (highly viscous) – Need for LCP injector

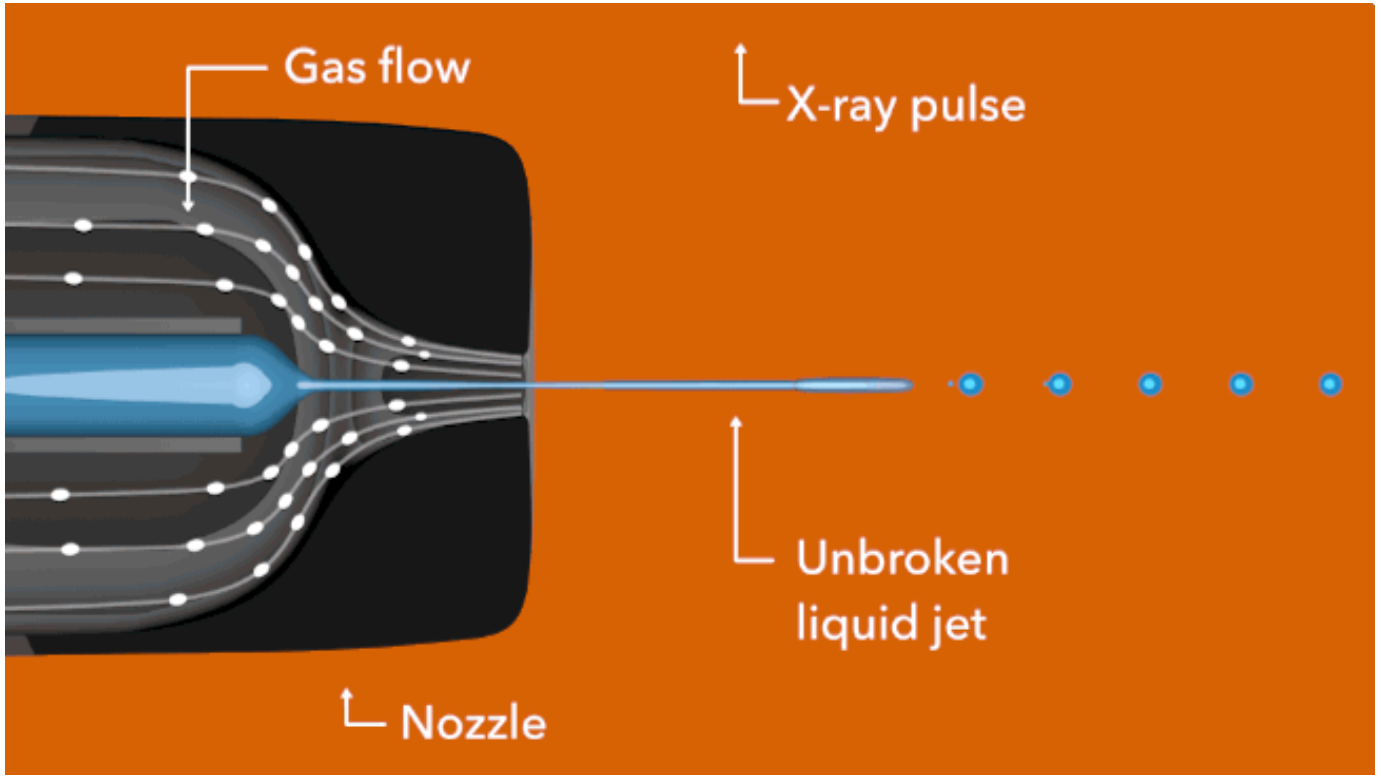


Figure 7. Gas Dynamic Virtual Nozzle. (SLAC National Lab)

LCP Injector Set-up

- Injector attached to nozzle rod for insertion to experimental chamber
- Water line (blue) provides pressure 300psi, drives hydraulic plunger
- Teflon balls provide tight seal against LCP leakage from 20uL LCP reservoir
- Gas line (green) for reliable extrusion of LCP and to maintain coaxial flow

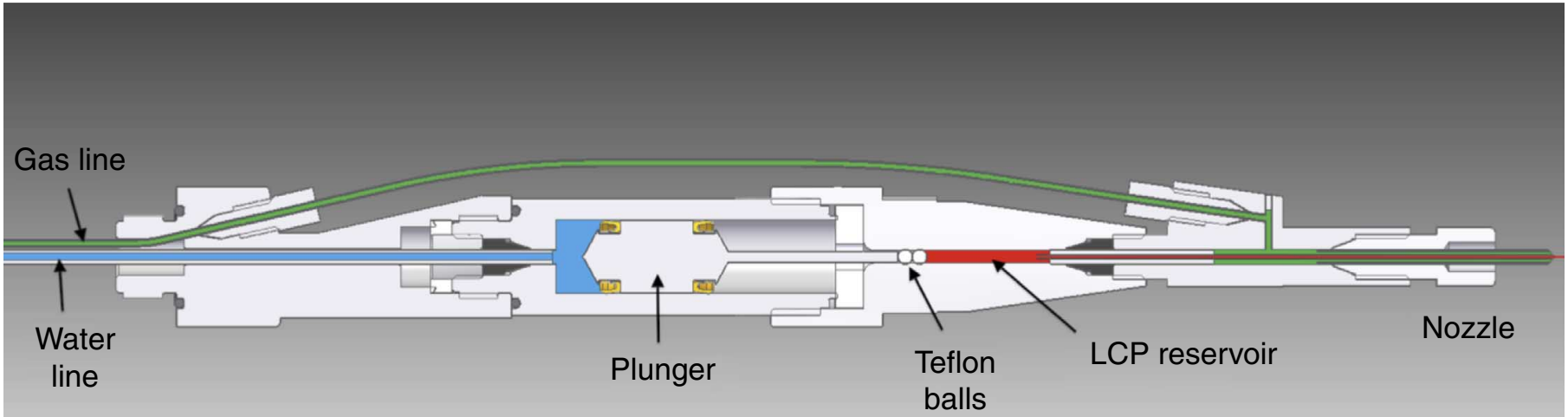


Figure 8. LCP Injector schematic. (Weierstall, U. *et al. Nat. Commun.* 5:3309)

LCP extrusion

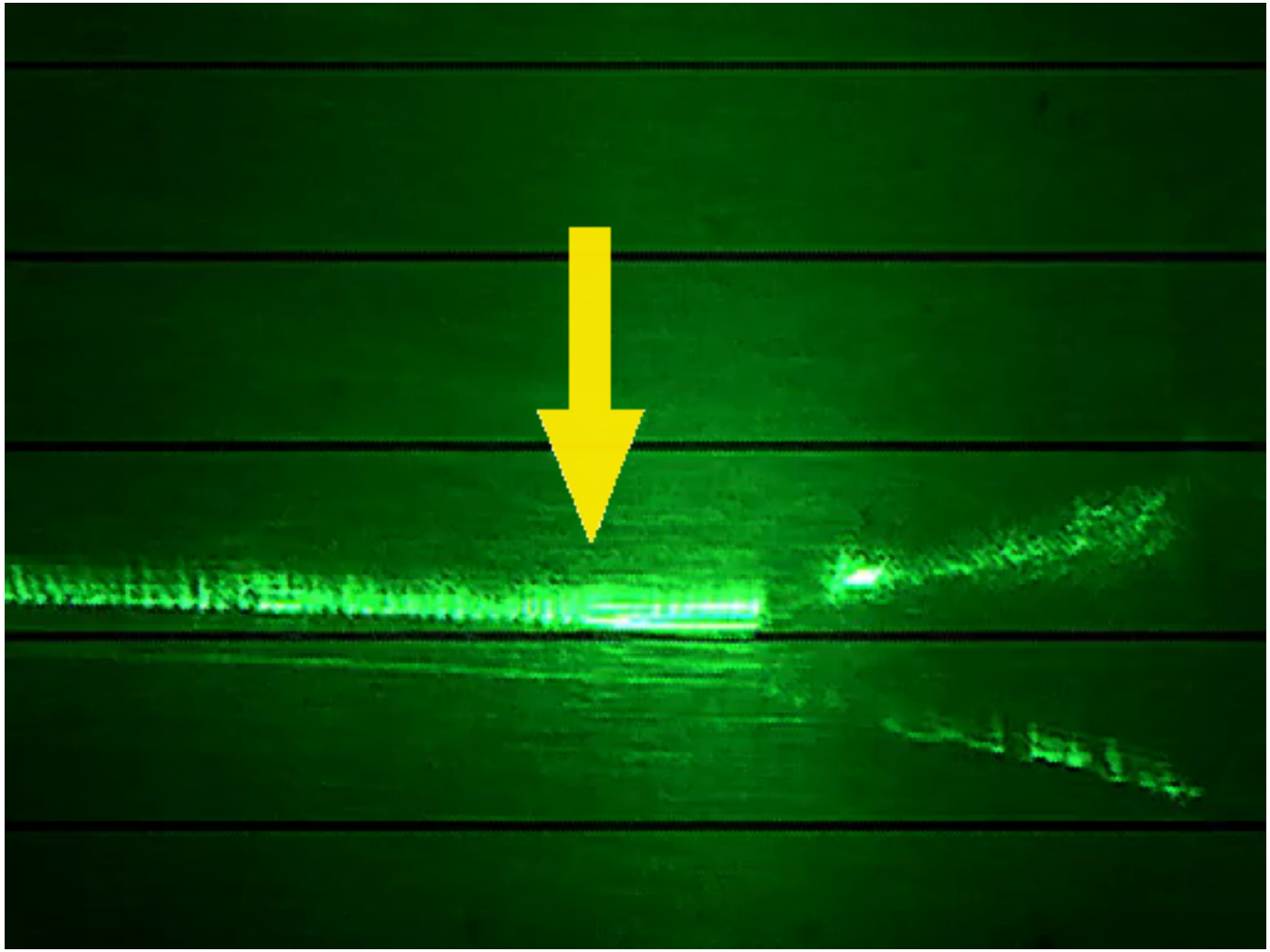


Figure 9. LCP Injector movie. (Weierstall, U. *et al. Nat. Commun.* 5:3309)

Model Protein: Human Smoothened Receptor

- Human Smoothened Receptor
  - GPCR-like, frizzled class receptor
  - Hedgehog signaling pathway, development, tissue maintenance
  - cyclopamine ligand (antagonist) derivatives for cancer treatment
  - Corn lily and one-eyed sheep

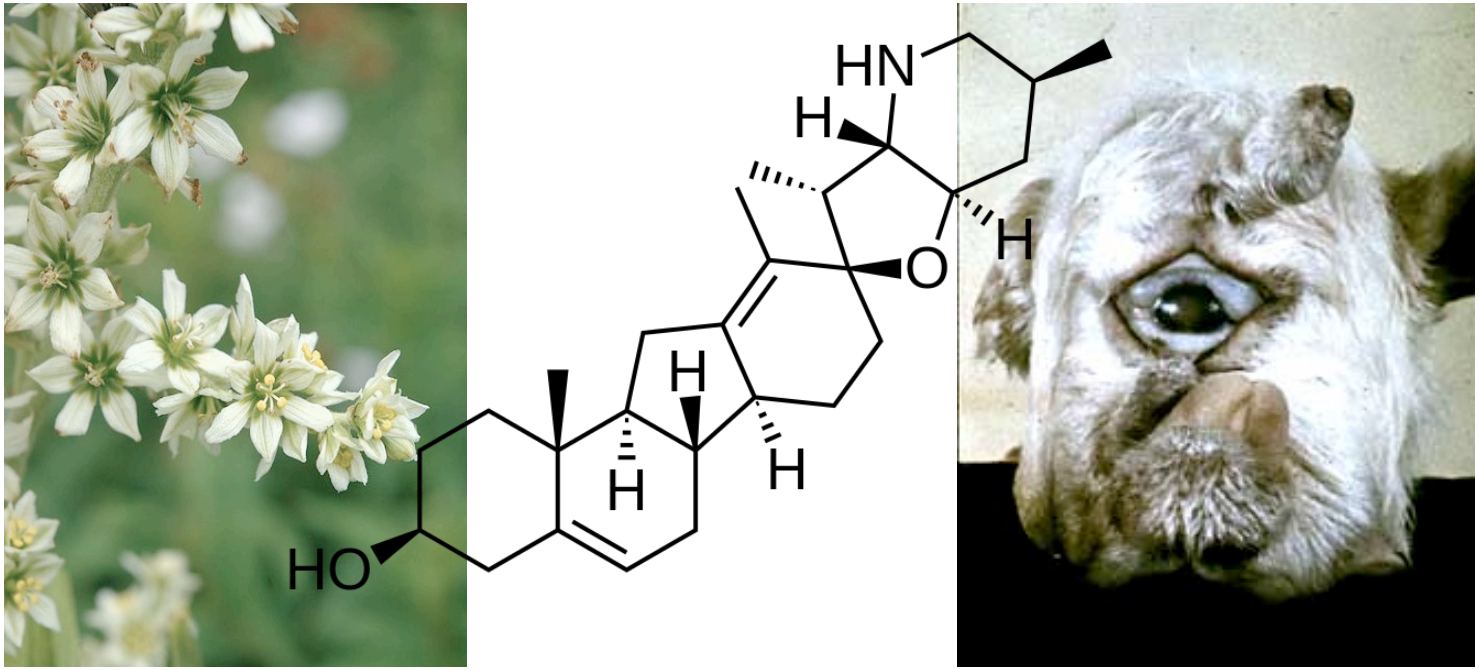


Figure 10a, b, and c.) Corny lil, cyclopamine, and one-eyed sheep. (Wikipedia)

Crystal Growth

- Human Smoothened Receptor crystals
- SFX (above) Synchrotron (below)
- 9.0 MAG / 10% (w/w) cholesterol
- 2:3 (protein:lipid)
- 100mM Hepes pH 7.0, 30% (v/v) PEG 400, 100mM NaCl

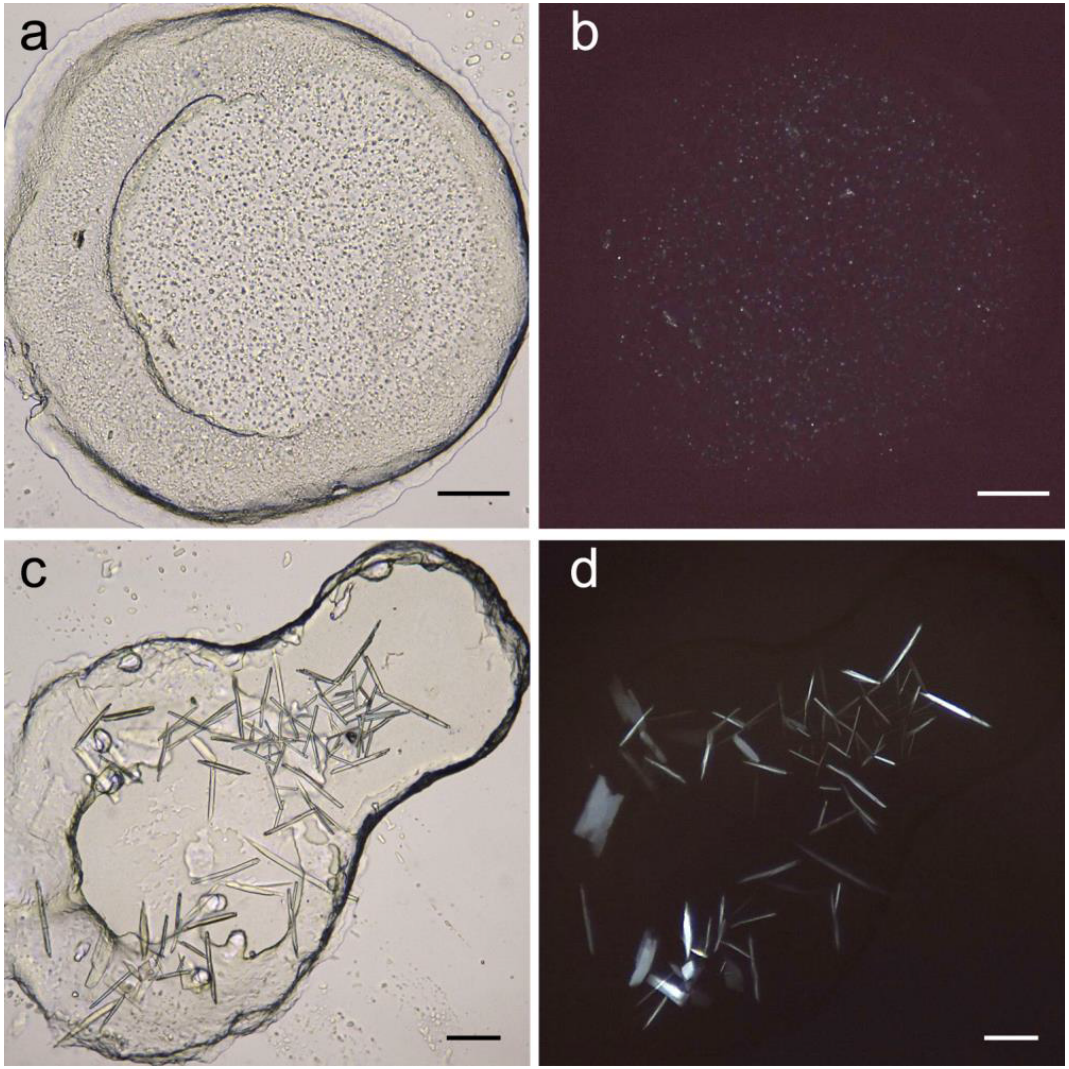


Figure 9. LCP grown human smoothened protein crystals. (Weierstall, U. *et al. Nat. Commun.* 5:3309)

Structure Recovery

- Most commonly used lipid in LCP is monoolein, 9.9 MAG
- Phase transition to lamellar crystalline (L_c) phase at 18°C
- LCP injected into evacuated sample chamber at ~10^{-3} Torr and 20°C, so evaporative cooling can transform it into L_c phase
  - Causing strong, sharp diffraction rings from $L_{c\ phsae}$
  - Increases background and poses danger to detector

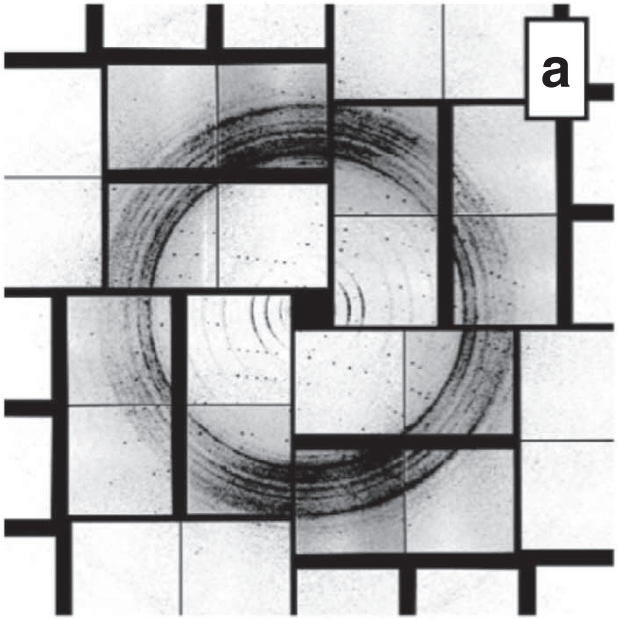
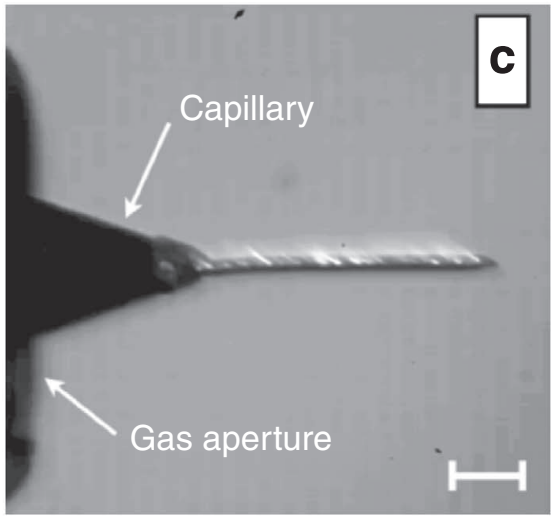


Figure 11a. Diffraction patterns and LCP extrusion. (Weierstall, U. *et al. Nat. Commun.* 5:3309)

Structure Recovery

- Changing co-flowing gas from He (figure c) to N_2 (figure d) the formation of the L_c phase is suppressed but not competely eliminated with LCP prepared with 9.9 MAG



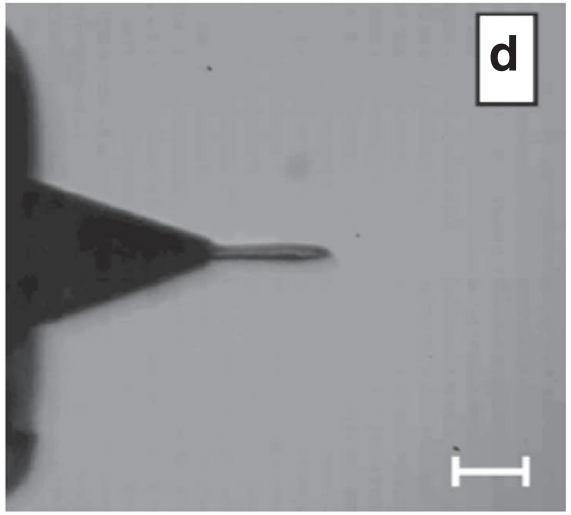


Figure 11c, d. Diffraction patterns and LCP extrusion, scale bar 100um. (Weierstall, U. *et al. Nat. Commun.* 5:3309)

Structure Recovery

- Replacing the 9.9 MAG with shorter chain MAGs (7.9 MAG[22] or 9.7 MAG (monopalmitolein) caused the preventation of the L_c phase altogether.
- Diffraction patterns confirmed the expected cubic-Pn3m phase
- Discovered that 7.9 MAG can be added post crystal growth and no damage observed upon addition

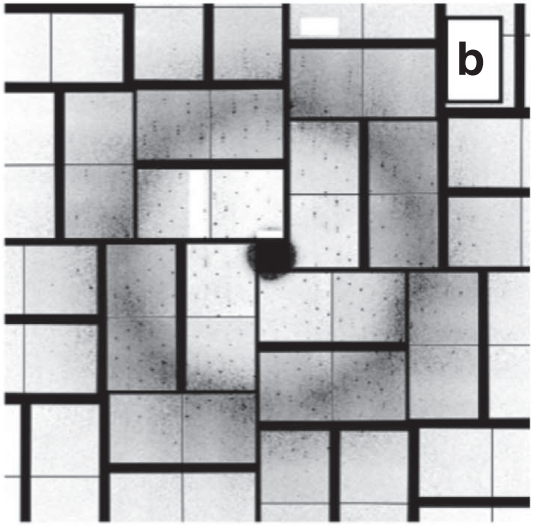


Figure 12. Diffraction patterns and LCP extrusion.
(Weierstall, U. *et al. Nat. Commun.* 5:3309)

Diffraction Data and Sample Consumption

- LCP injector flow rate of 170nl min^{-1}
- 5-10h data collection using less than 100uL of sample (<0.5mg of protein)
- Vast improvement over GDVN nozzle which requires 10mL (10mg protein) for complete data set
- Crystals < 5um at room temperature

Statistics

- 61,964 microcrystals to recover 3.2/4.0Å resolution structure.
- Solved by molecular replacement
- Resolution is not very high, but confidently able to locate small molecule
- 3,510,525 diffraction patterns → 61,964 indexed images with monoclinic lattice

Table 1 | Data collection and refinement statistics.

	SMO/cyclopamine
Data collection	
Space group	$P2_1$
Cell dimensions,	
a, b, c (Å)	40.5, 157.3, 52.4
α β γ (°)	90.0, 97.0, 90.0
Resolution (Å)	40–3.20 (3.26–3.20)*
R_{split}, (%)	9.8 (63.2)
$I/\sigma(I)$	7.4 (1.8)
*CC**	0.9991 (0.28)
Completeness (%)	100 (100)
Multiplicity	2,515 (1,820)
Refinement	
Resolution (Å)	40–3.20
Anisotropic truncation (Å)	3.4, 3.2, 4.0
No. reflections/test set	8,082/399
R_{work}/R_{free}	0.232/0.278
No. atoms	
Protein	3,389
Cyclopamine	30
B-factors (Å²)	
Wilson B/overall B	101.6/78.4
Receptor/BRIL	76.8/83.7
Cyclopamine	76.3
R.m.s. deviations	
Bonds lengths(Å)	0.003
Bond angles (°)	0.61

Abbreviation: SMO, smoothened.
This data set includes diffraction from 61,964 crystals.
*High-resolution shell is shown in parentheses.

Table 1. Data collection and refinement statistics.. (Weierstall, U. *et al. Nat. Commun.* 5:3309)

Structure Recovery: PDB ID 4O9R

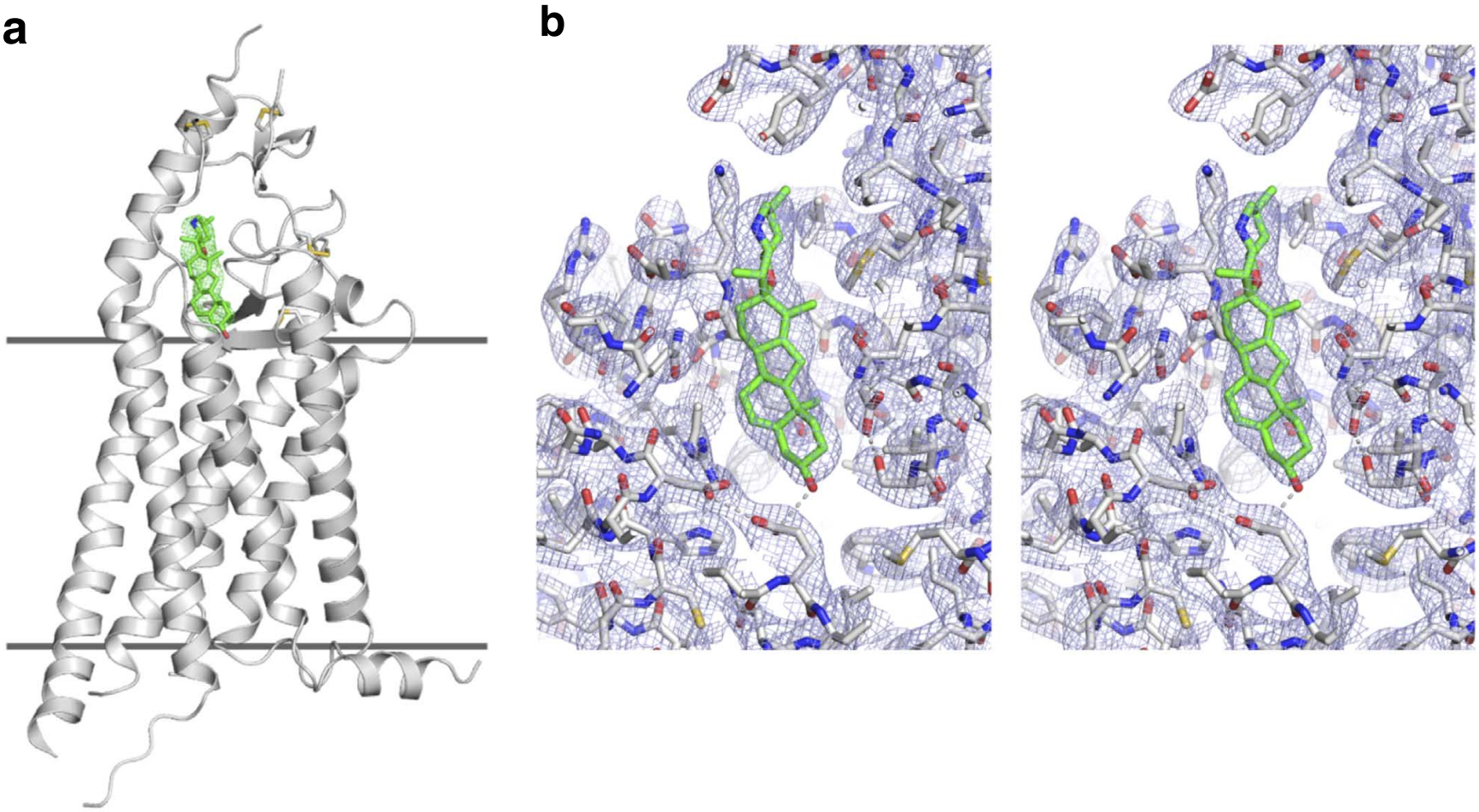


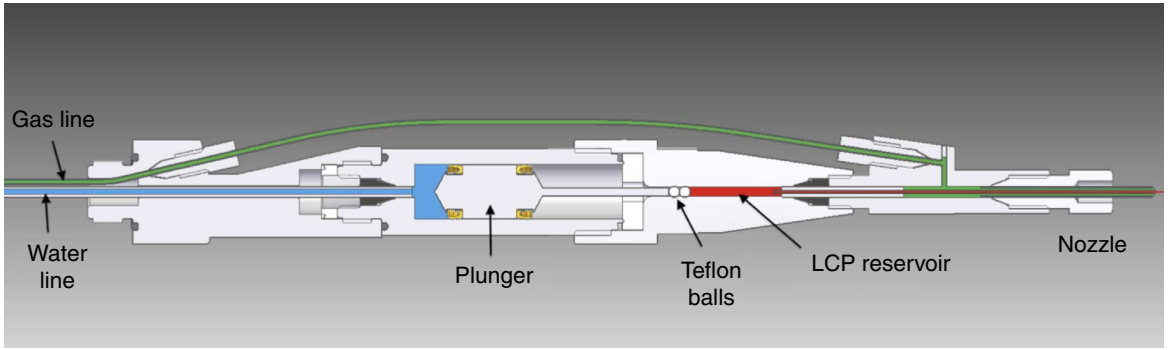
Figure 13. Cyclopamine binding to smoothened receptor. (Weierstall, U. *et al. Nat. Commun.* 5:3309)

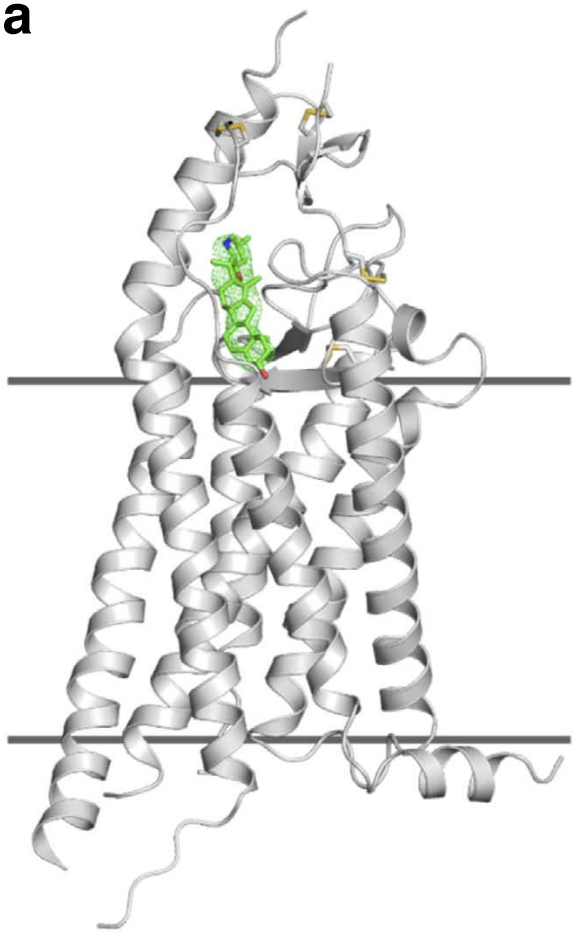
Implications of LCP Injector development

- Previously, aqueous SFX possible with GDVN
- Now, LCP—SFX possible with LCP injector
- Dramatic reduction of protein sample required
  - large amount of pure protein can be difficult to obtain
    - Doesn't purify well (i.e. – toxic to overexpress in cell)
    - Protein requires PTM (euk proteins) lack robust cell lines with capability
- Expect an increase in number of structures
  - Especially elusive, relevant eukaryotic ones that have characteristically been poorly diffracting microcrystals

In Summary

- Development of LCP micro-extrusion injector [a]
- SFX studies of membrane proteins





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