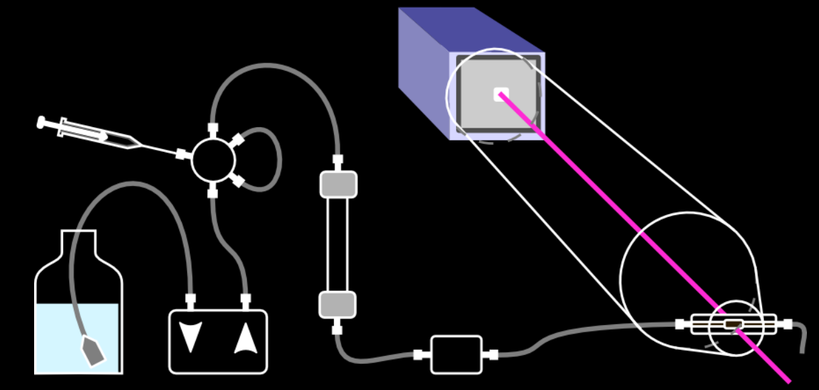
SEC-SAXS data collection and analysis

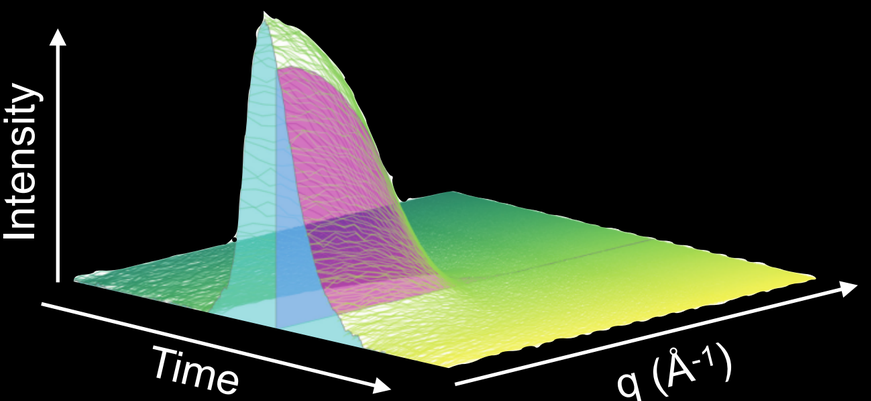
Steve Meisburger, Cornell University

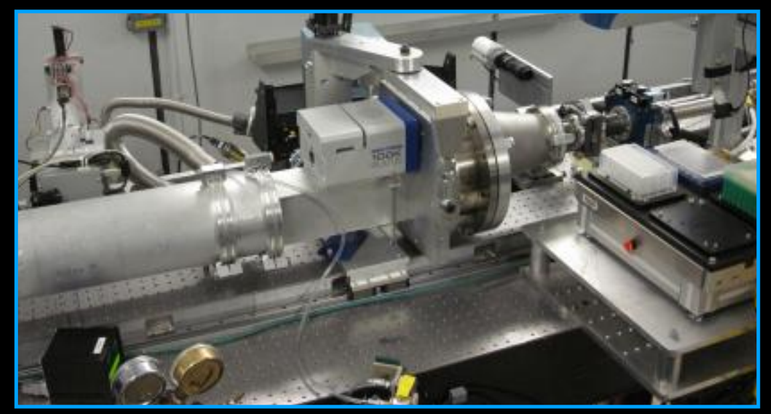
Everything BioSAXS 7 (BioCAT)

March 30, 2021

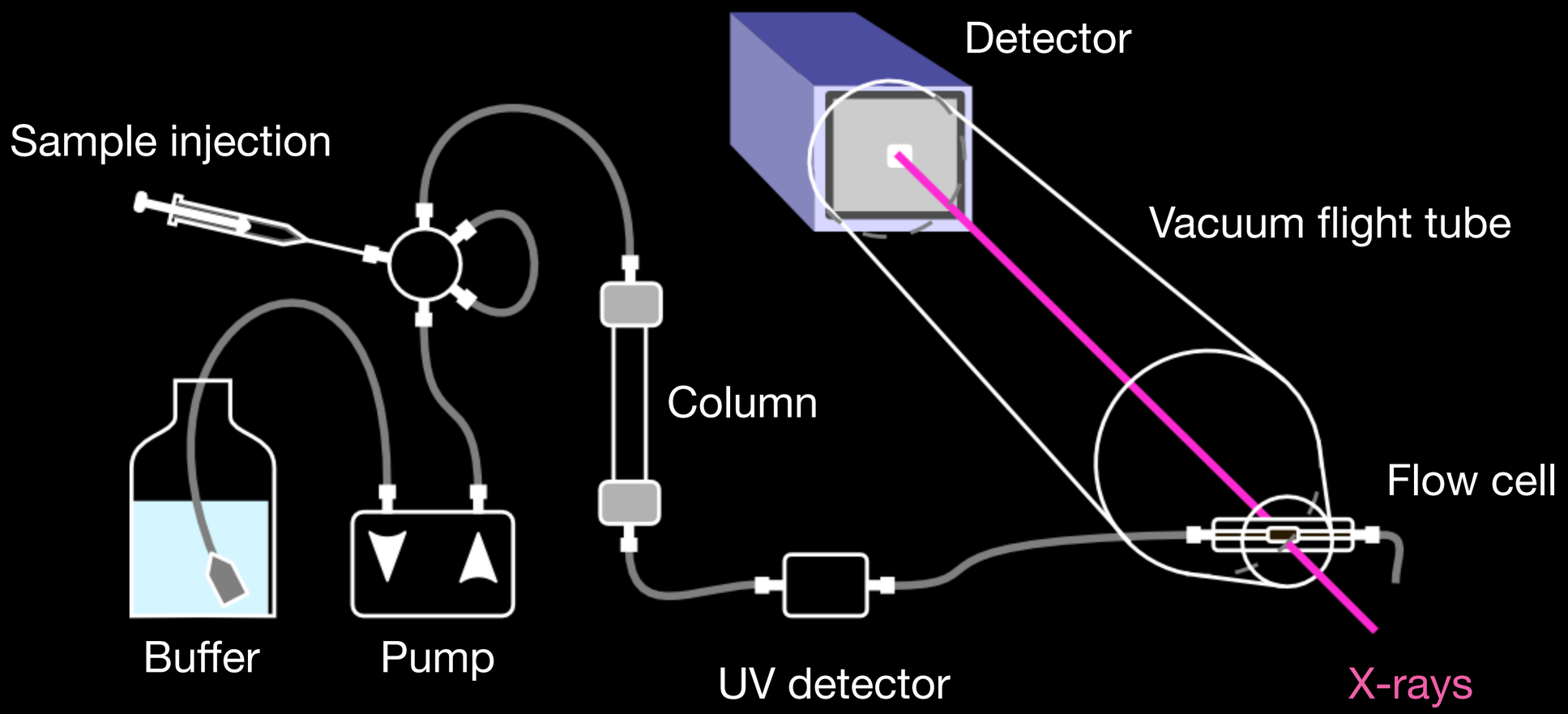
I: Introduction







SAXS with in-line chromatography



Why use SEC-SAXS?

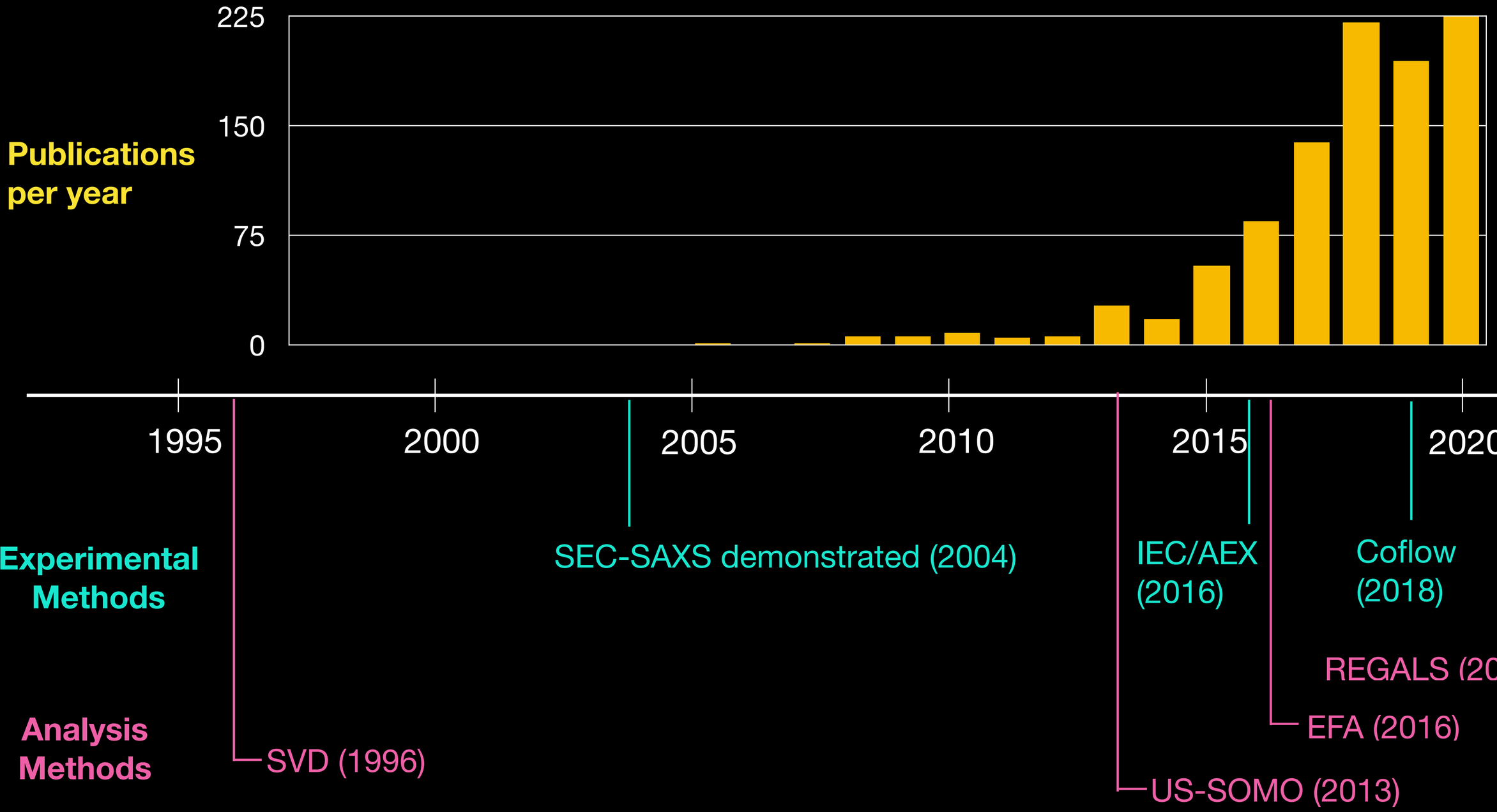
Pros

- Exact buffer match
- Remove aggregates
- Confirm monodispersity
- Separate mixtures
- Computationally deconvolve overlapping peaks

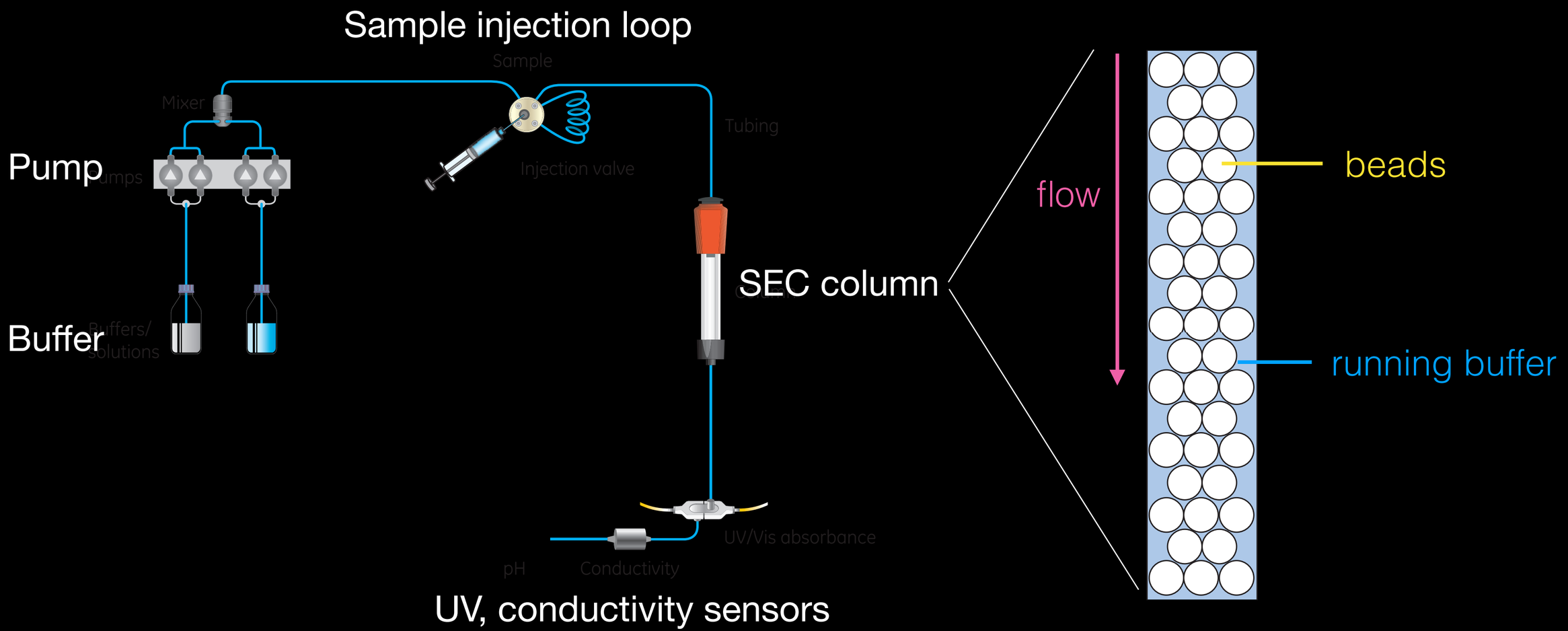
Cons

- Usually uses more material, takes longer, dilutes the sample
- Buffer is fixed (no titrations)
- Protein concentration varies (issue for weak complexes)
- Radiation damage can compromise experiment

Timeline of developments in SEC-SAXS

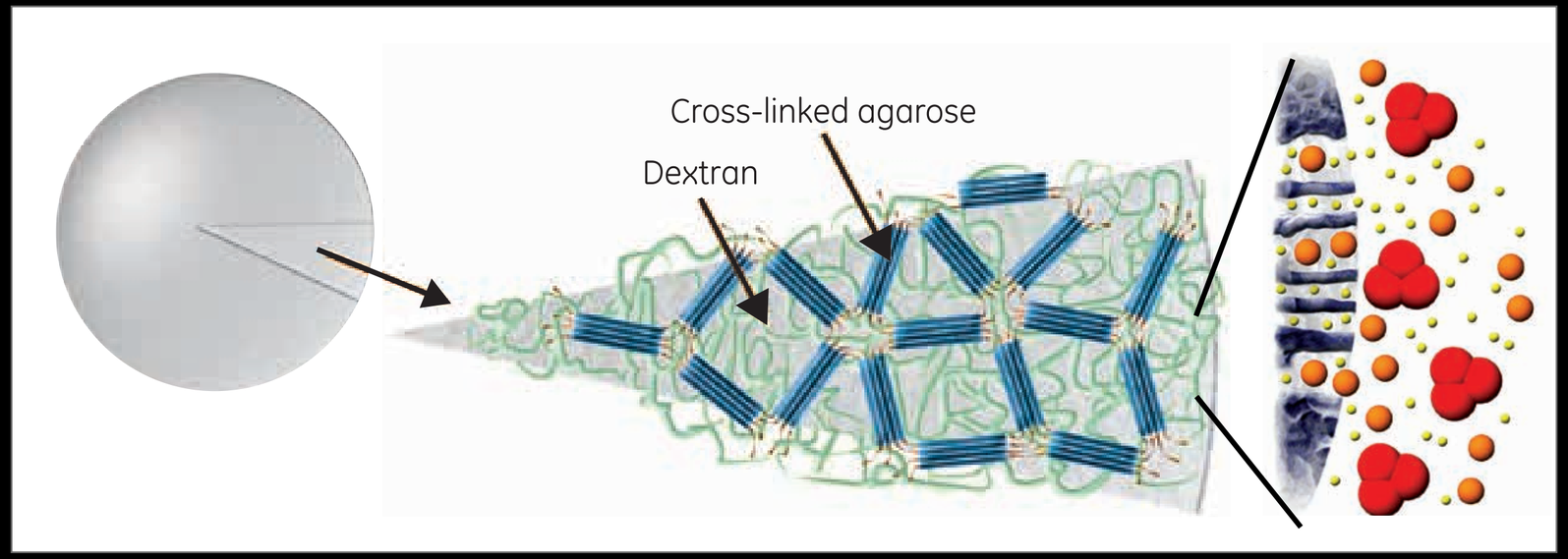


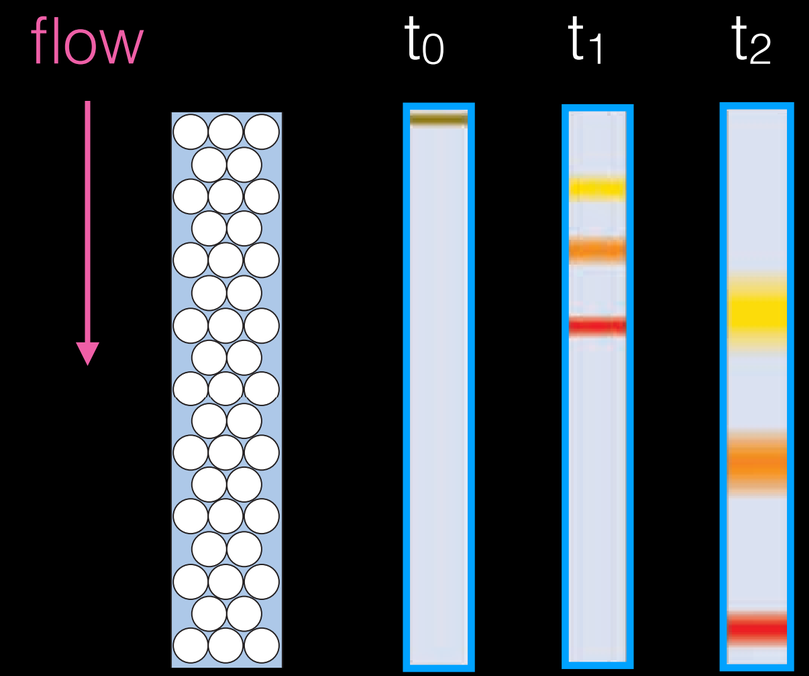
Size exclusion chromatography (SEC) setup



"Size exclusion chromatography: Principles and Methods", GE Healthcare Life Sciences.

Physical separation by SEC

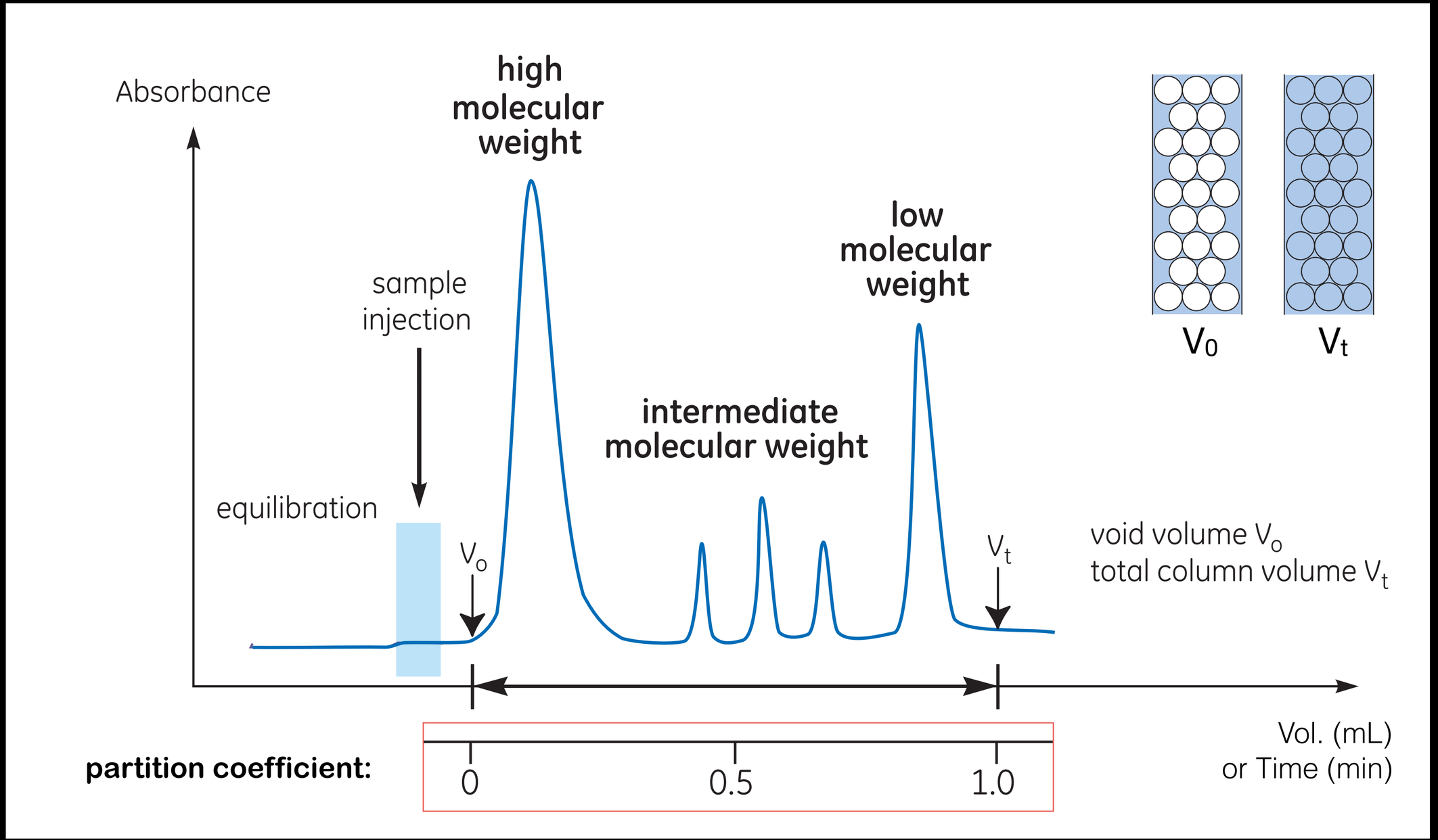




- Large objects are excluded → run quickly / elute first
- Small objects diffuse in the pores → run slowly / elute last

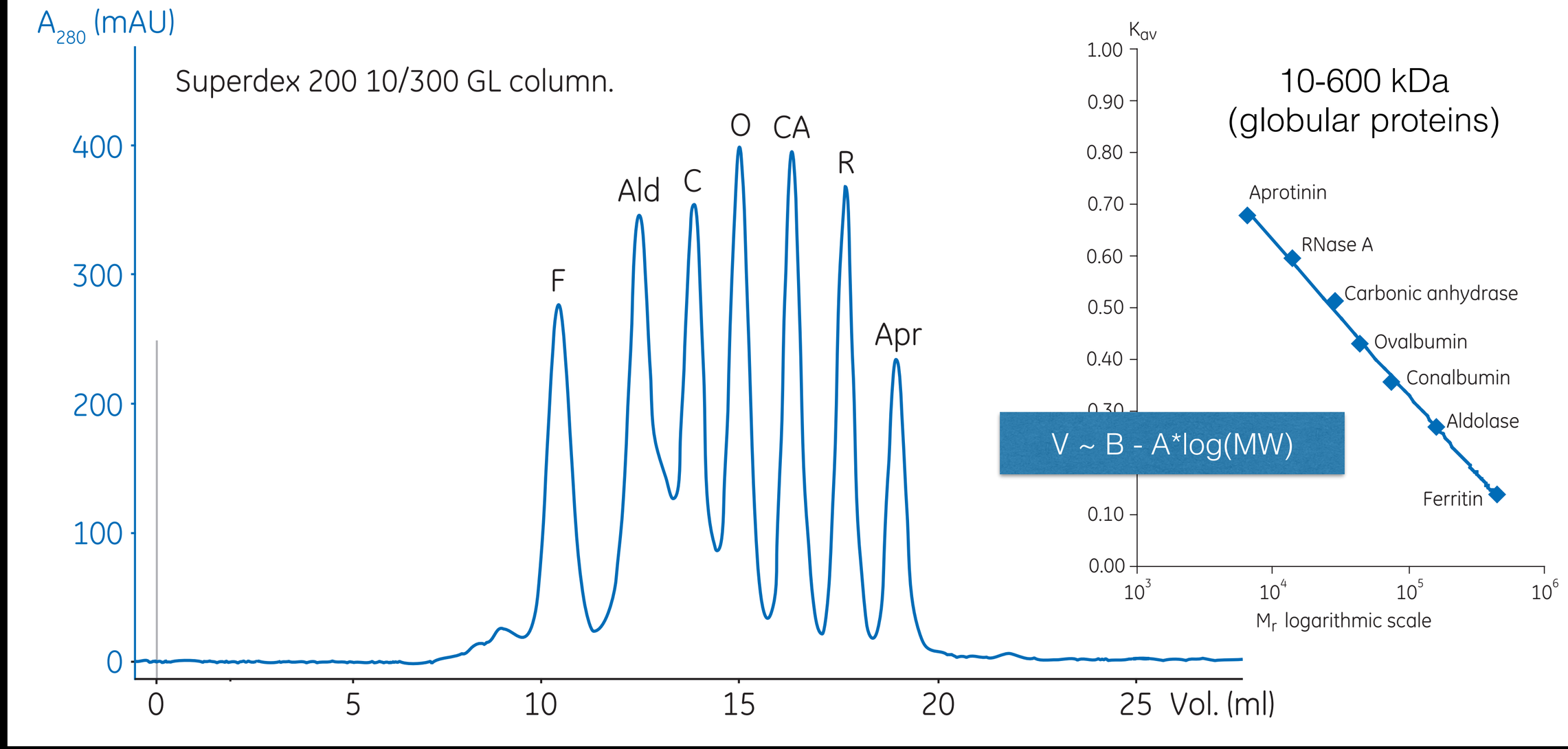
"Size exclusion chromatography: Principles and Methods", GE Healthcare Life Sciences.

Reading a chromatogram



"Size exclusion chromatography: Principles and Methods", GE Healthcare Life Sciences.

Globular proteins elute according to log(MW)



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Column choice for in-line SAXS

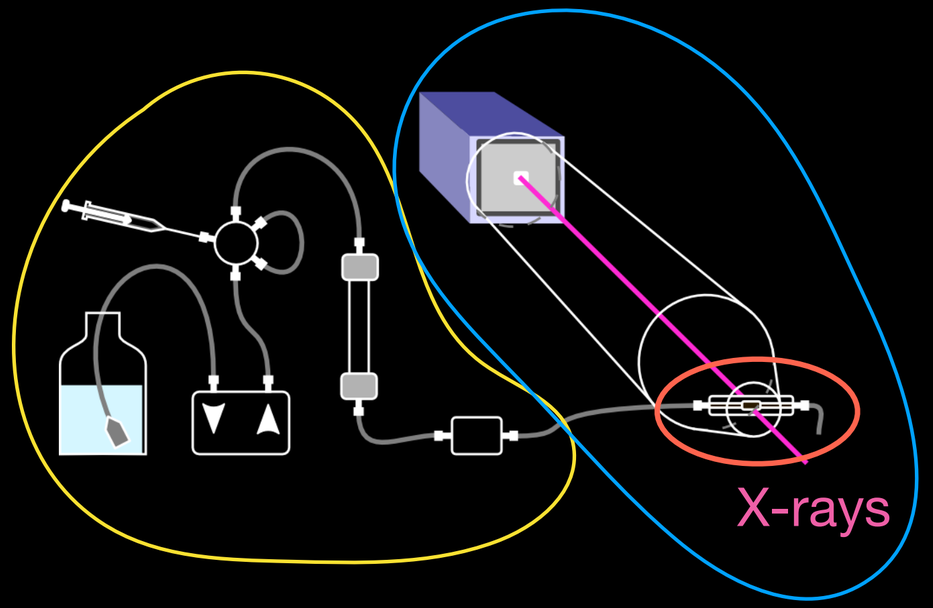
GE superdex 10/300, 3.2/300, 5/150

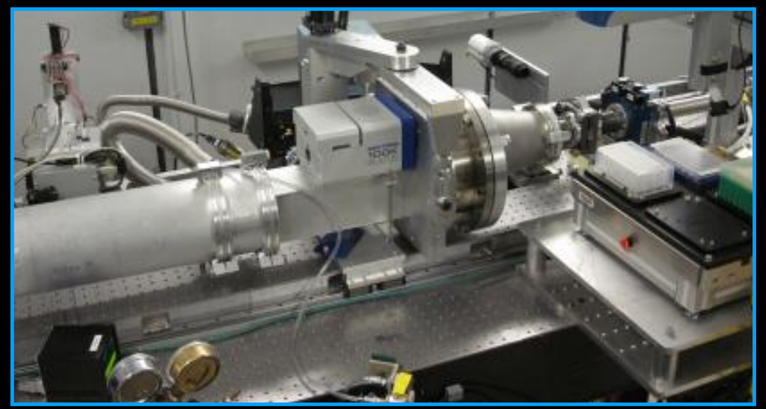
Shodex KW series

Property	Options	Considerations
Volume	analytical scale (2.4-24 mL)	• **smaller**: less dilution, less sample needed. • **larger**: faster flow, less peak broadening.
Length	typ. 150-300 mm	• **longer**: better resolution, TPN (“theoretical plate number”) • **shorter**: faster flow
Media	Polymer / sugar (GE superdex, superose)	• Common for protein purification. • Chemical compatibility
	Polymer-coated SiO_2 (Shodex KW)	• Small bead size → better resolution. • **Buffer pH < 7.5**
MW range	500-5000 kDa (superose 6)	• Choose range appropriate for sample of interest. • Given range is for globular proteins.
	10-600 kDa (superdex 200)	
	3-70 kDa (supderdex 75)	

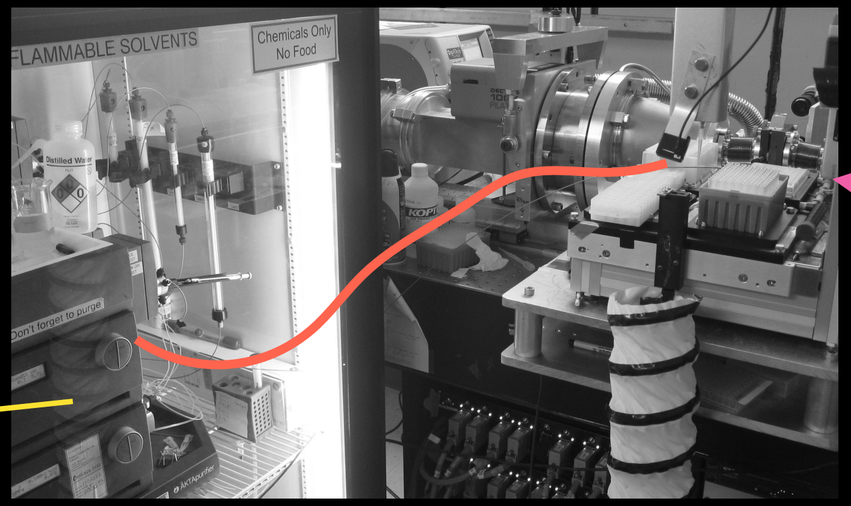
SEC-SAXS experimental setup

FPLC
(AKTA Purifier at CHESS G1)

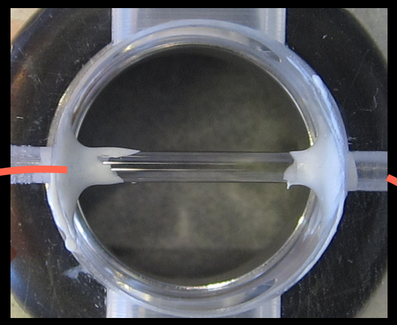




SAXS beam line
(CHESS G1)

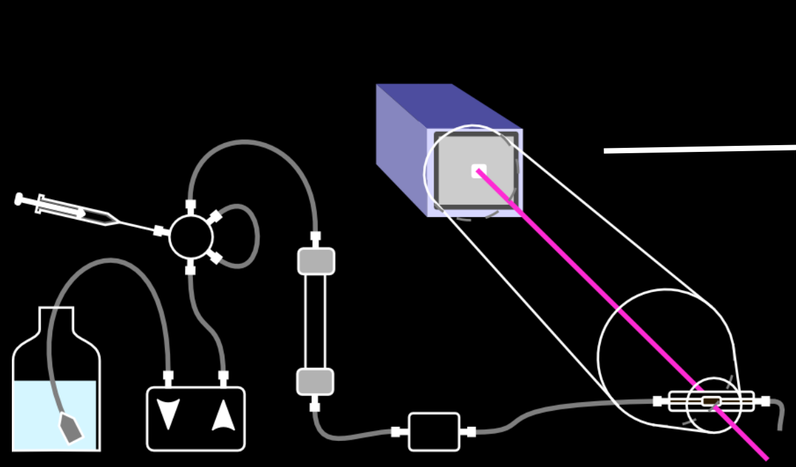


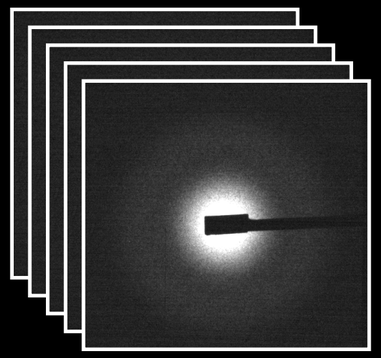
X-rays



Glass capillary flow cell

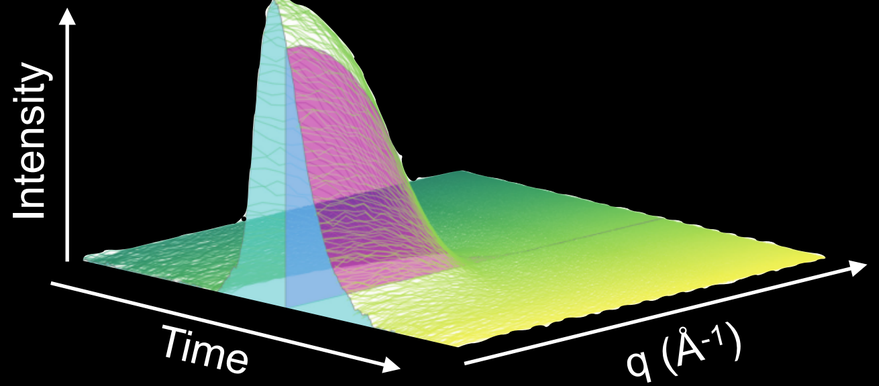
Procedure for data collection





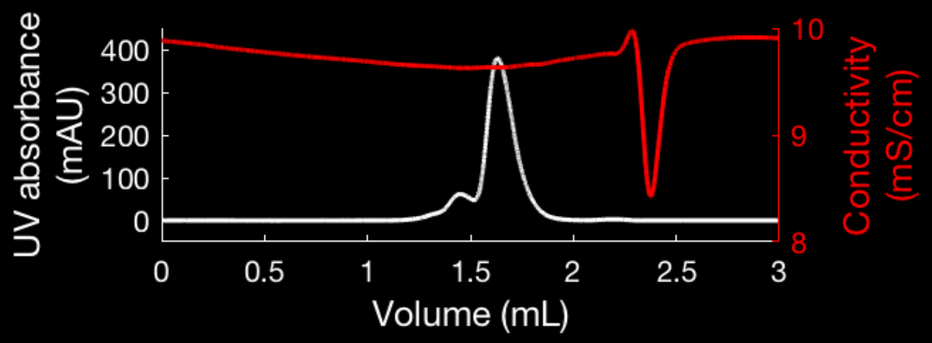
scattering images

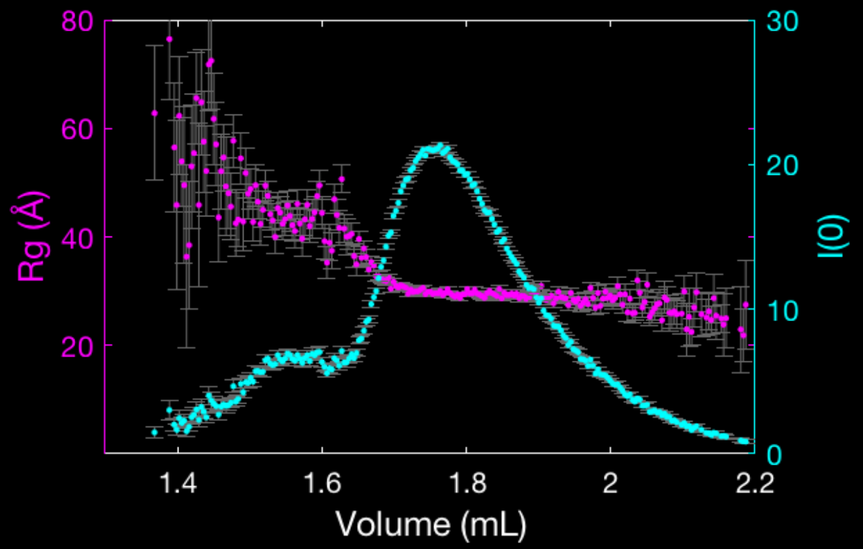
integration



Further analysis

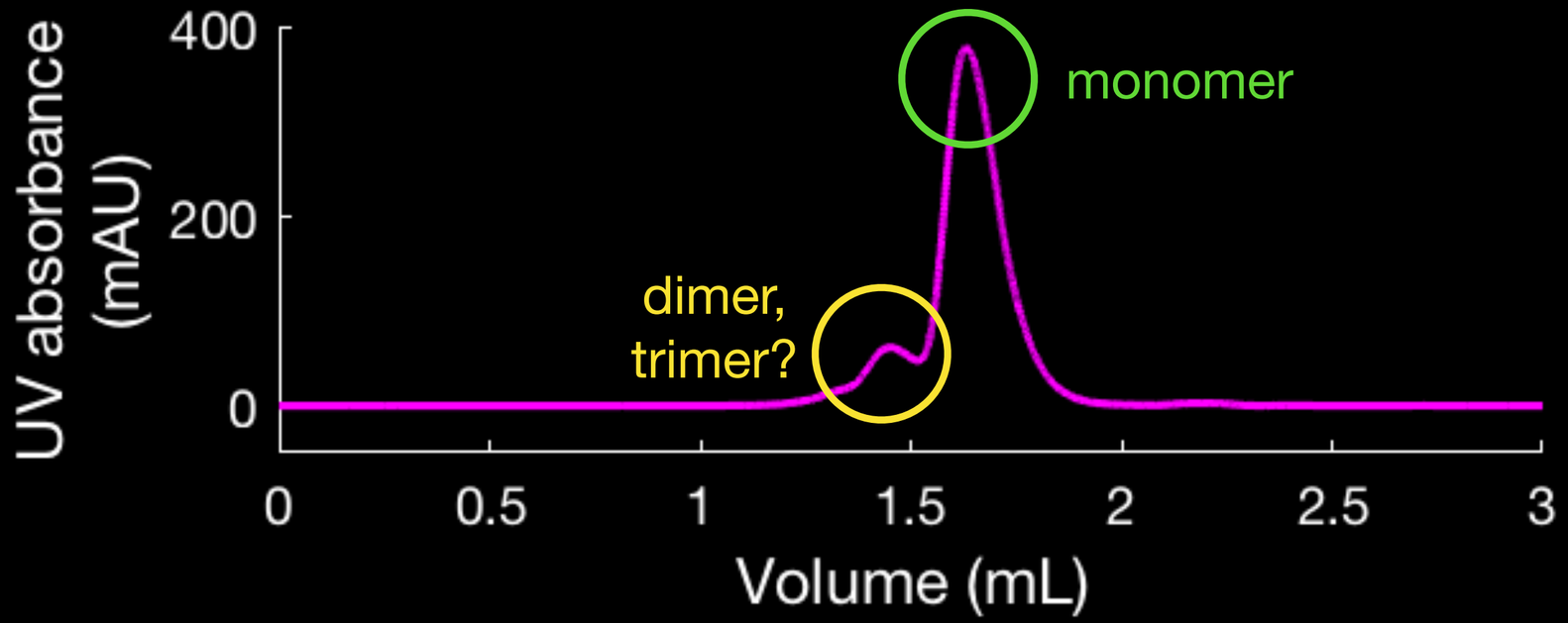
II: Basic Analysis





Example SEC-SAXS dataset

Bovine Serum Albumin (BSA)



Experimental details

Beamline	CHESS G1, Nov. 2015
Sample	BSA at 11 mg/mL in 50 mM HEPES, pH 7.5
Column	Superdex 200 Increase, 3.2/300
Running Buffer	50 mM HEPES pH 7.0, 100 mM NaCl, 5% glycerol
Injection Volume	50 uL
Flow Rate	0.1 mL/minute
Frames	1000 at 2s each

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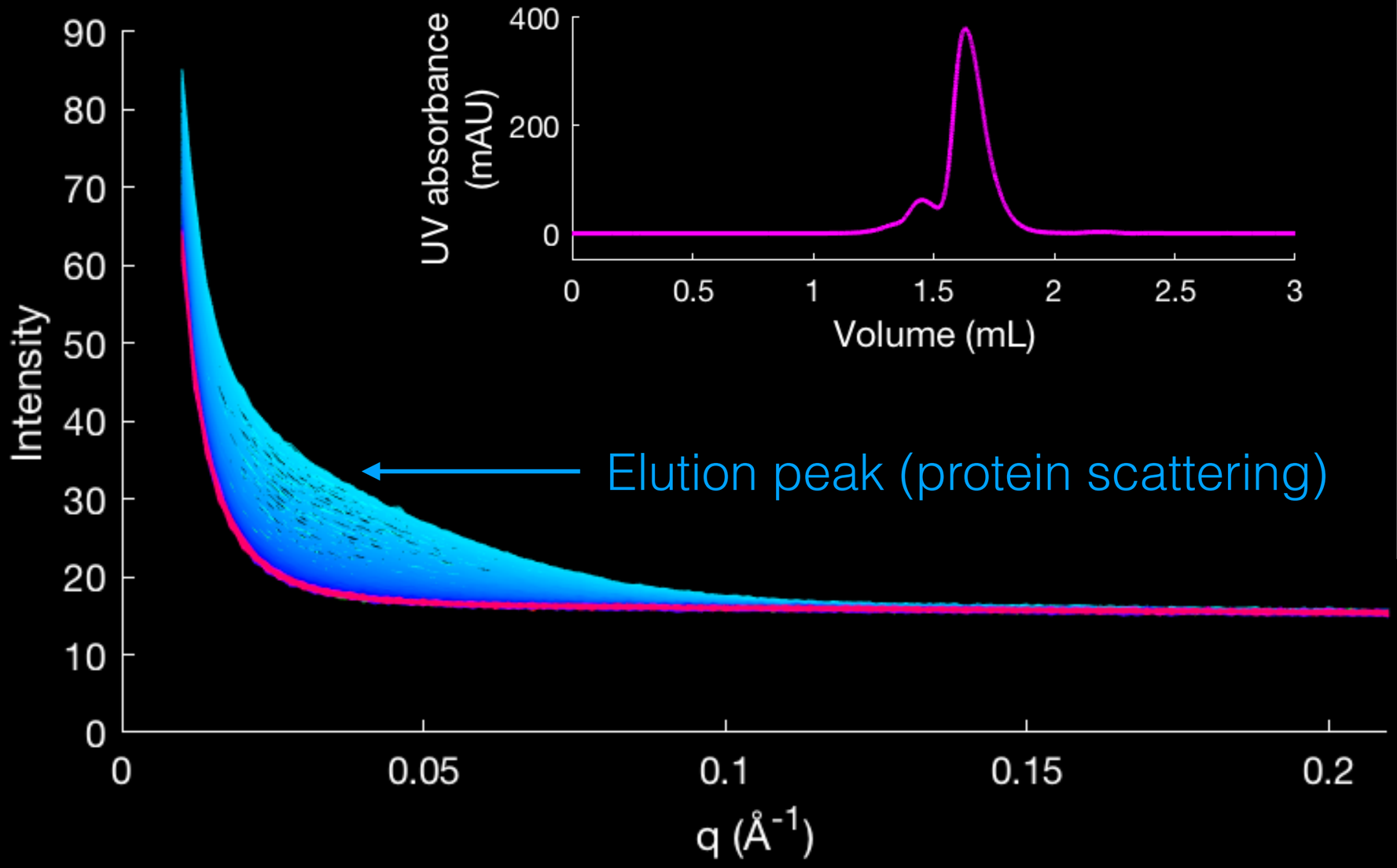

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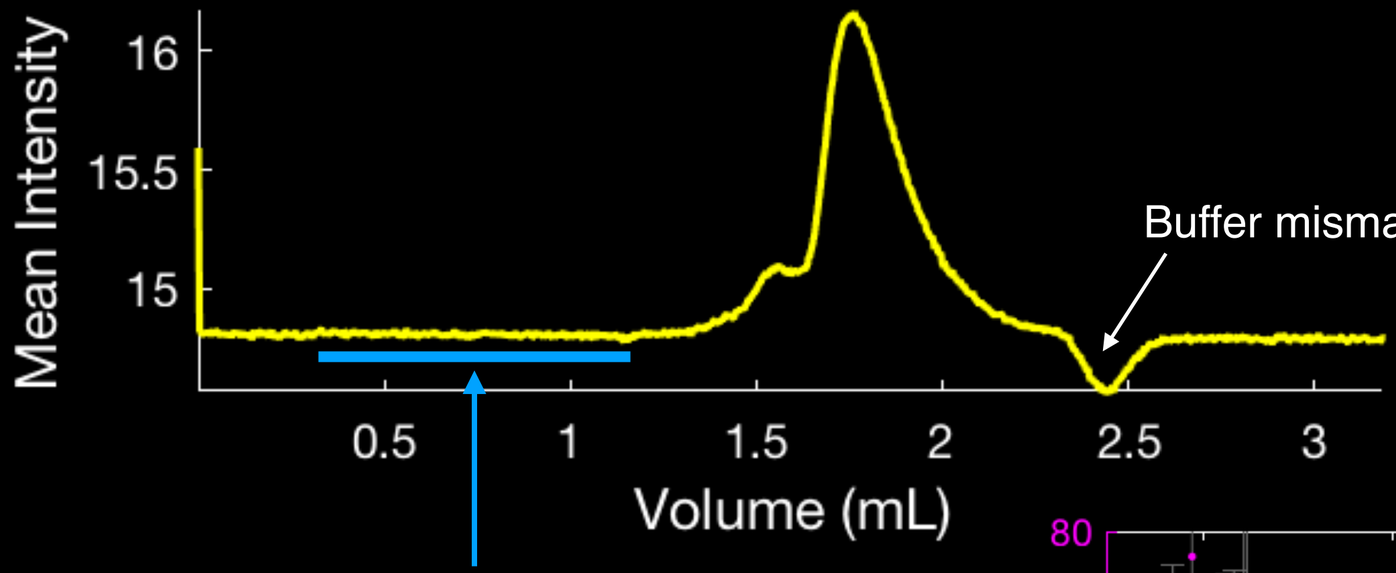
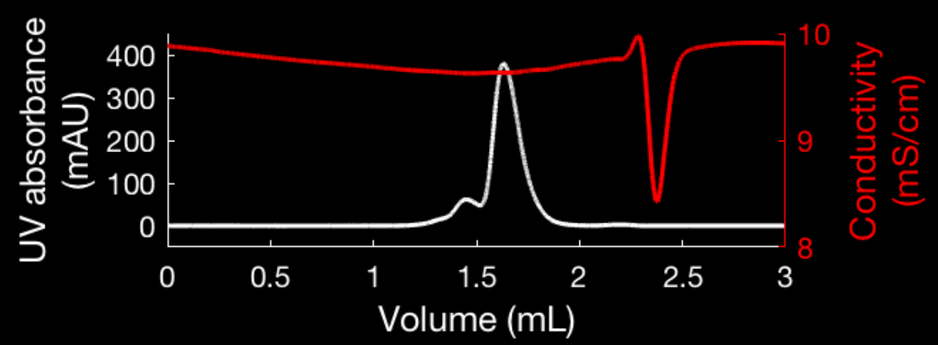
SAXS “chromatogram”

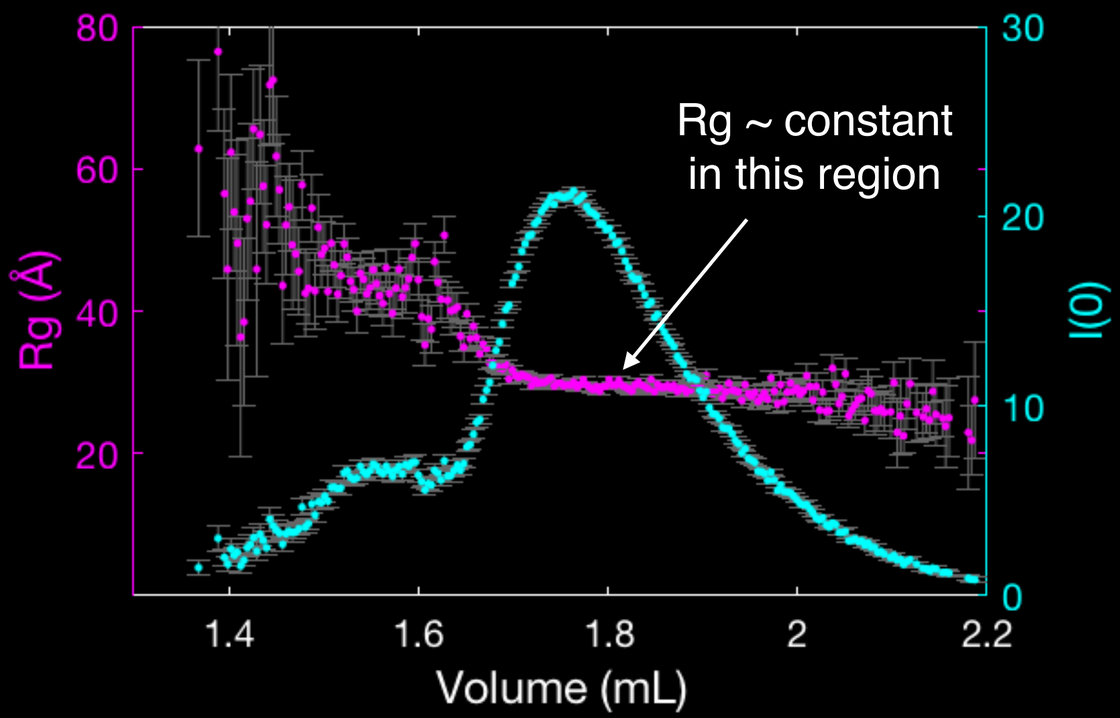
Mean intensity, radius of gyration (Rg), forward scattering (I(0))



Use a region before the main peak for background subtraction

Guinier analysis →



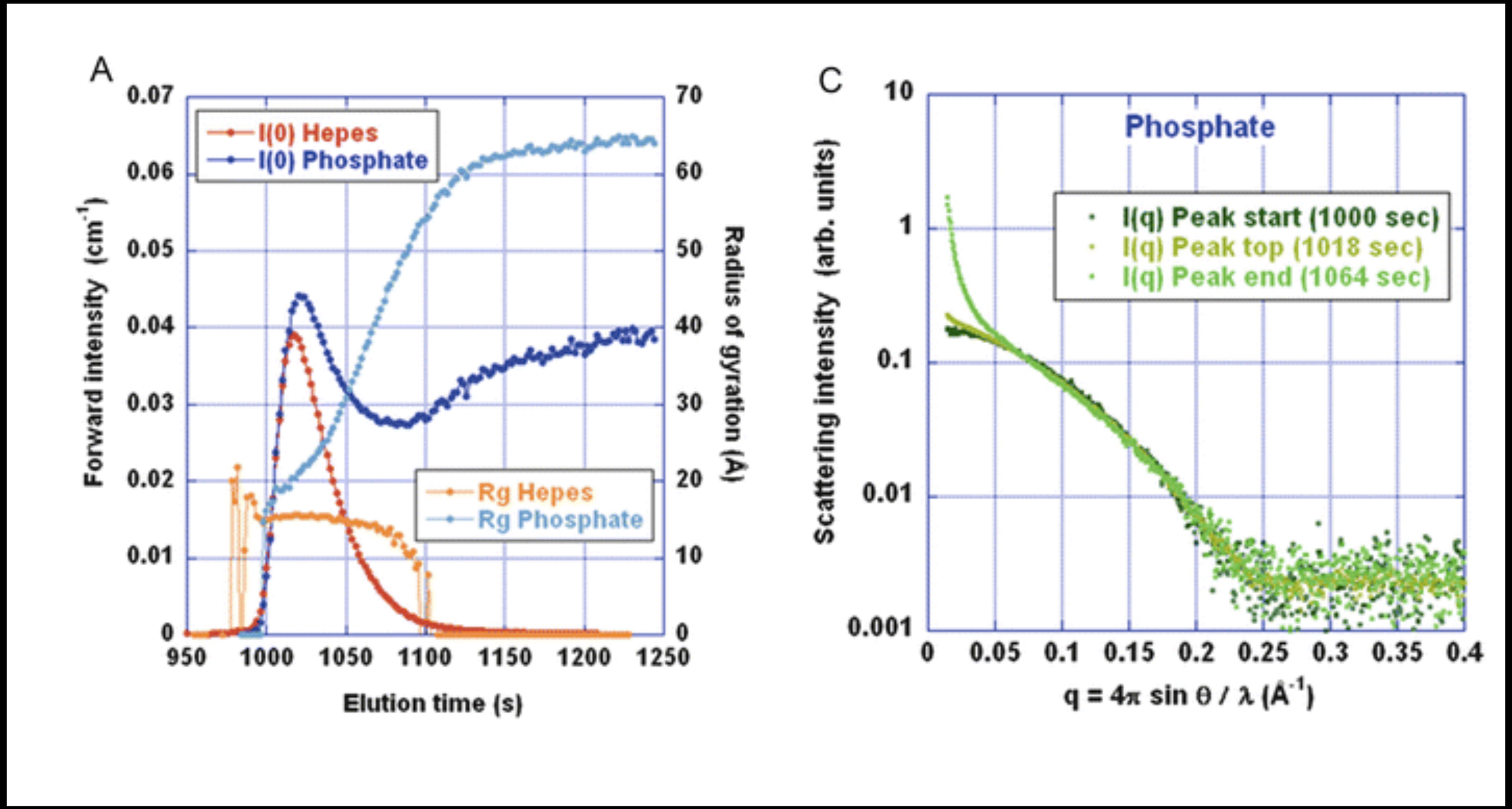


Troubleshooting

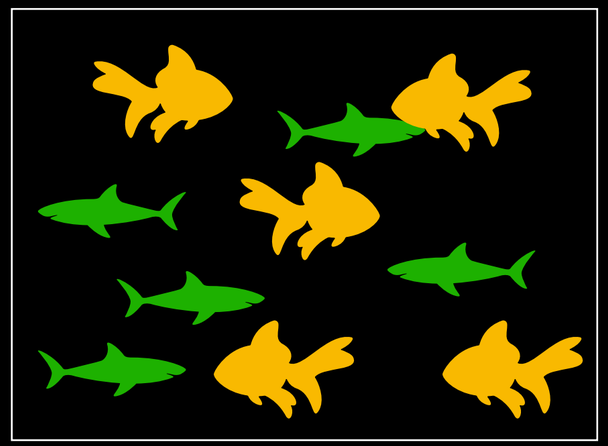
Issue	Possible Causes	Experimental solutions
SAXS profile does not return to baseline	Capillary fouling (X-ray damage)	• Attenuate beam or close shutter during aggregate peak • Buffer additives to reduce damage (3% glycerol, etc) • Increase flow rate (may require larger column)
	Sloping baseline	• Fully equilibrate column with running buffer (~2 c.v.)
Rg not constant across peak	Interparticle interference	• Reduce injection volume or use larger column
	Overlapping peaks	• (See below)
Overlapping peaks	Insufficient resolution	• Verify column health using calibration standard. • Choose a different column. • Reduce injection volume. • Optimize buffer components (pH, salt) to reduce non-specific association of protein with media.
	Peak broadening	• Use a larger column, bypass sensors (UV, cond. etc) between column and X-ray cell
	Re-equilibration of oligomers / aggregation	• Optimize conditions (pH, additives, temperature) for stability

Issues can also be addressed computationally, using more advanced analysis

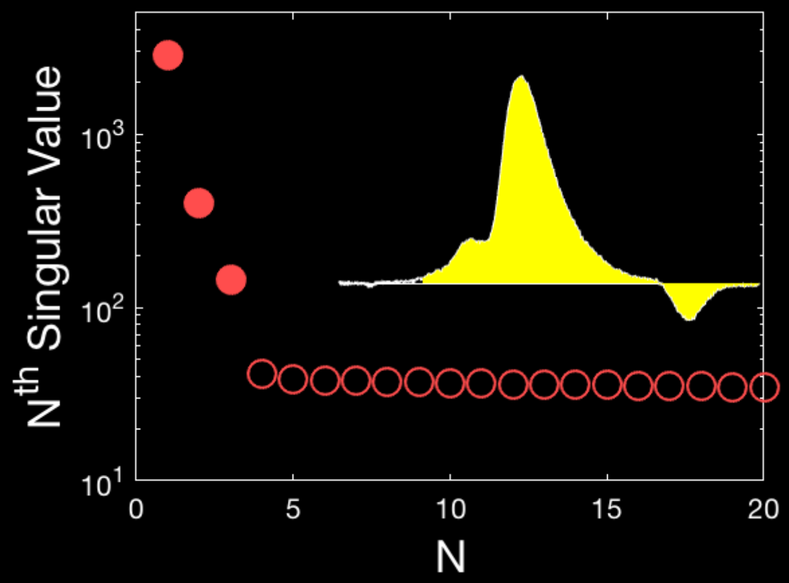
Example of capillary fouling (severe)



Pérez & Vachette in Biological Small Angle Scattering: Techniques, Strategies and Tips.183–199 (Springer, 2017)

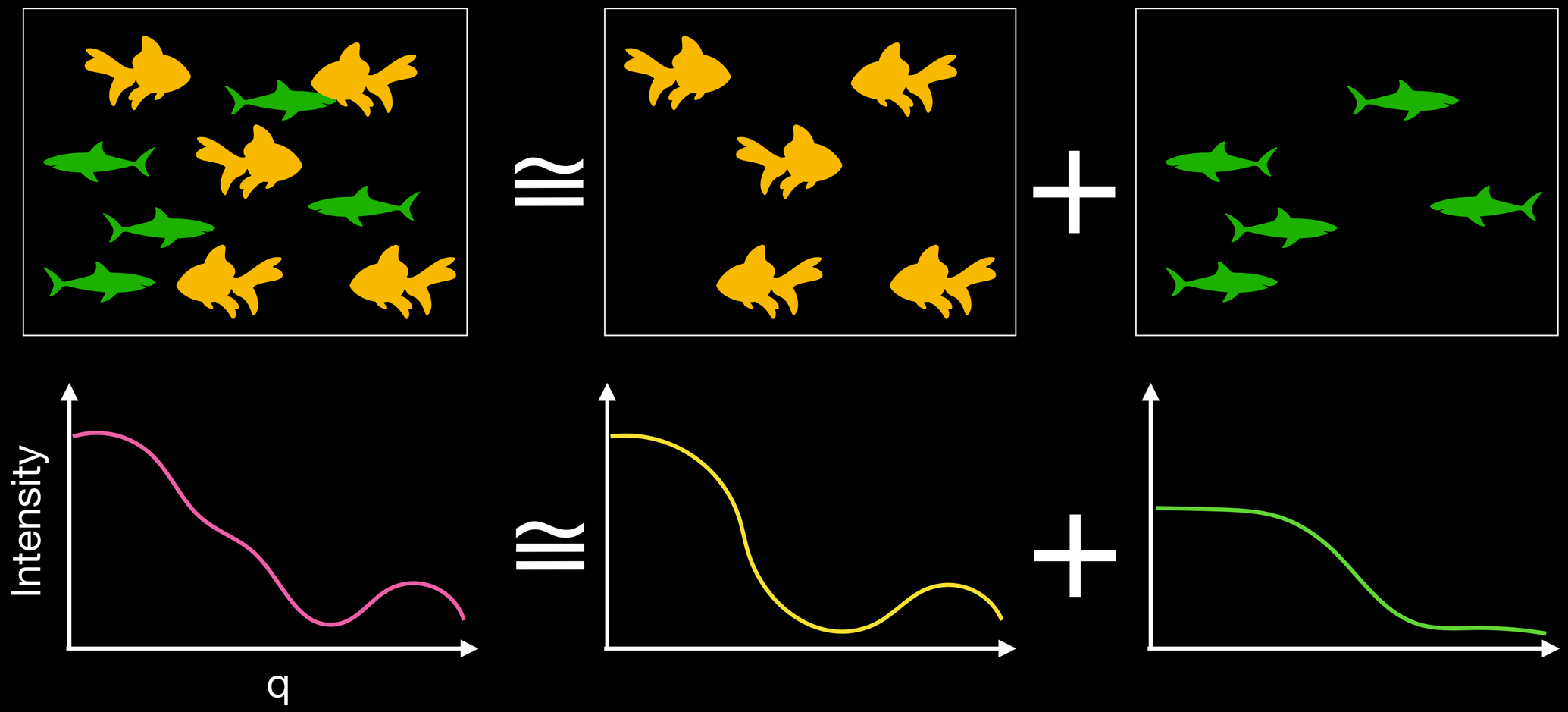


III: Advanced Analysis



Theory of SAXS from mixtures

Intensities add in dilute solution



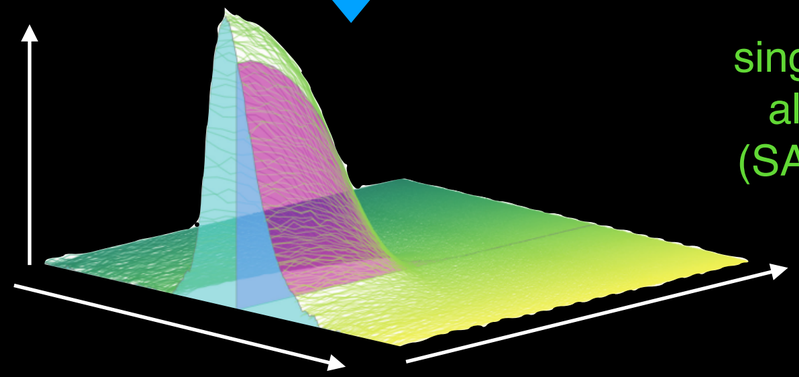
SAXS profiles combine linearly → use methods from linear algebra to deconvolve

Singular value decomposition (SVD)

Method to factor a matrix into components

First used in SAXS by Chen, Hodgson, & Doniach. J. Mol. Biol. 261, 658–671 (1996).

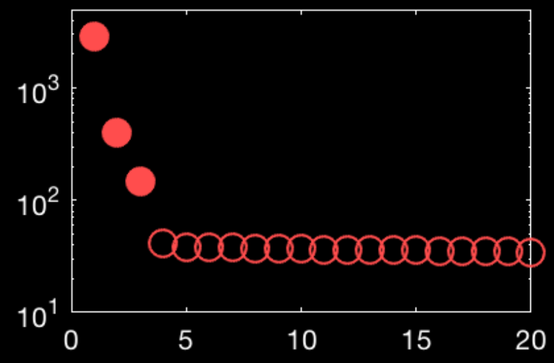
$A = U \times S \times V^T$



SEC-SAXS dataset

singular vectors along *I* vs. *q* (SAXS profiles)

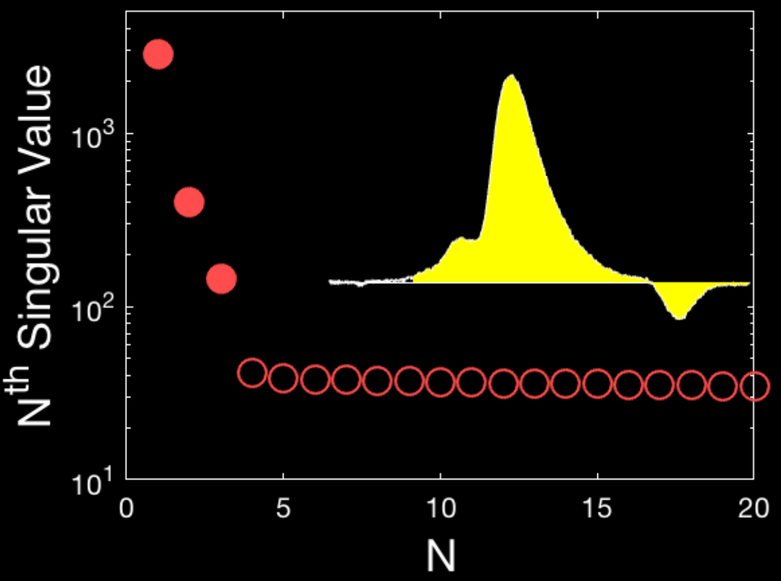
singular values



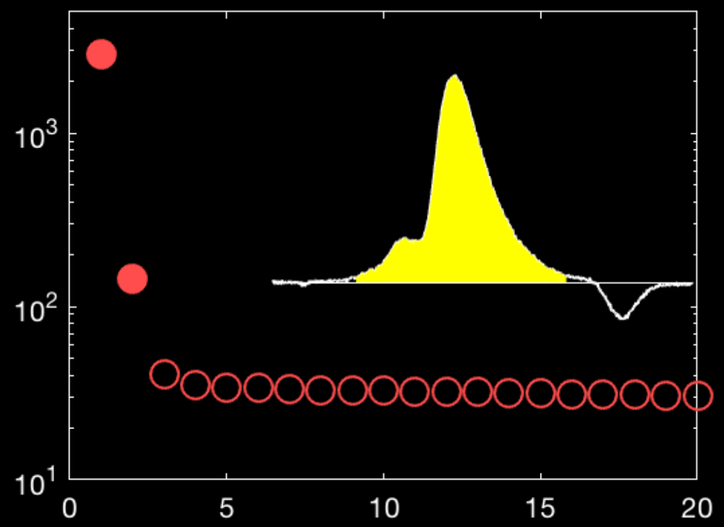
singular vectors along *I* vs. frame (elution profiles)

Using SVD to select a single component

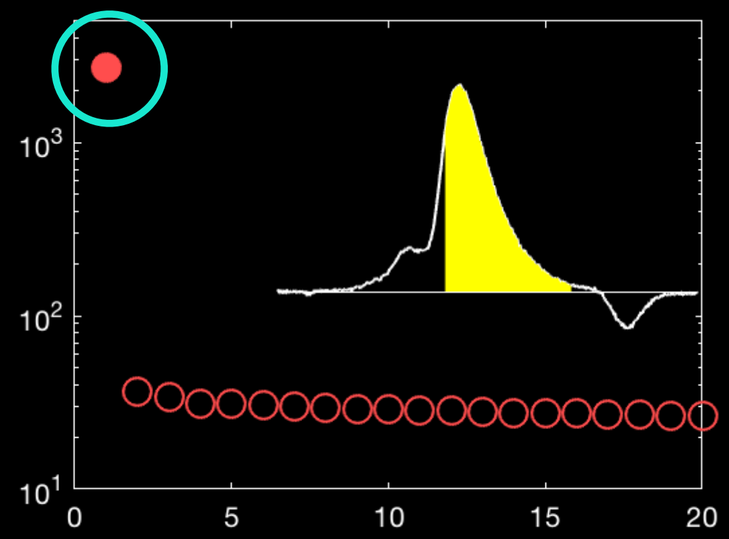
whole peak: 3 s.v.

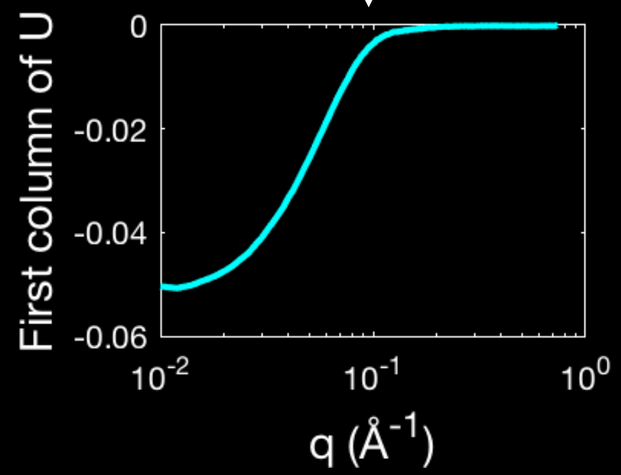


first part: 2 s.v.

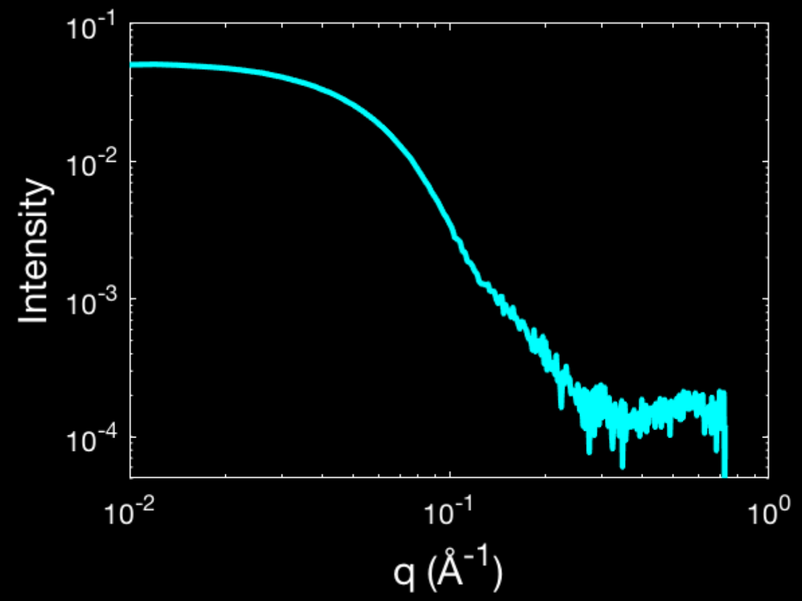


middle part: 1 s.v.

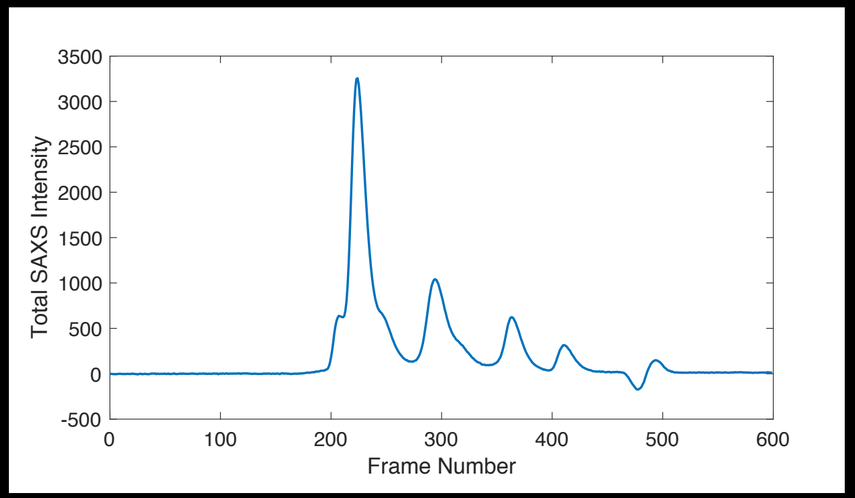




flip sign



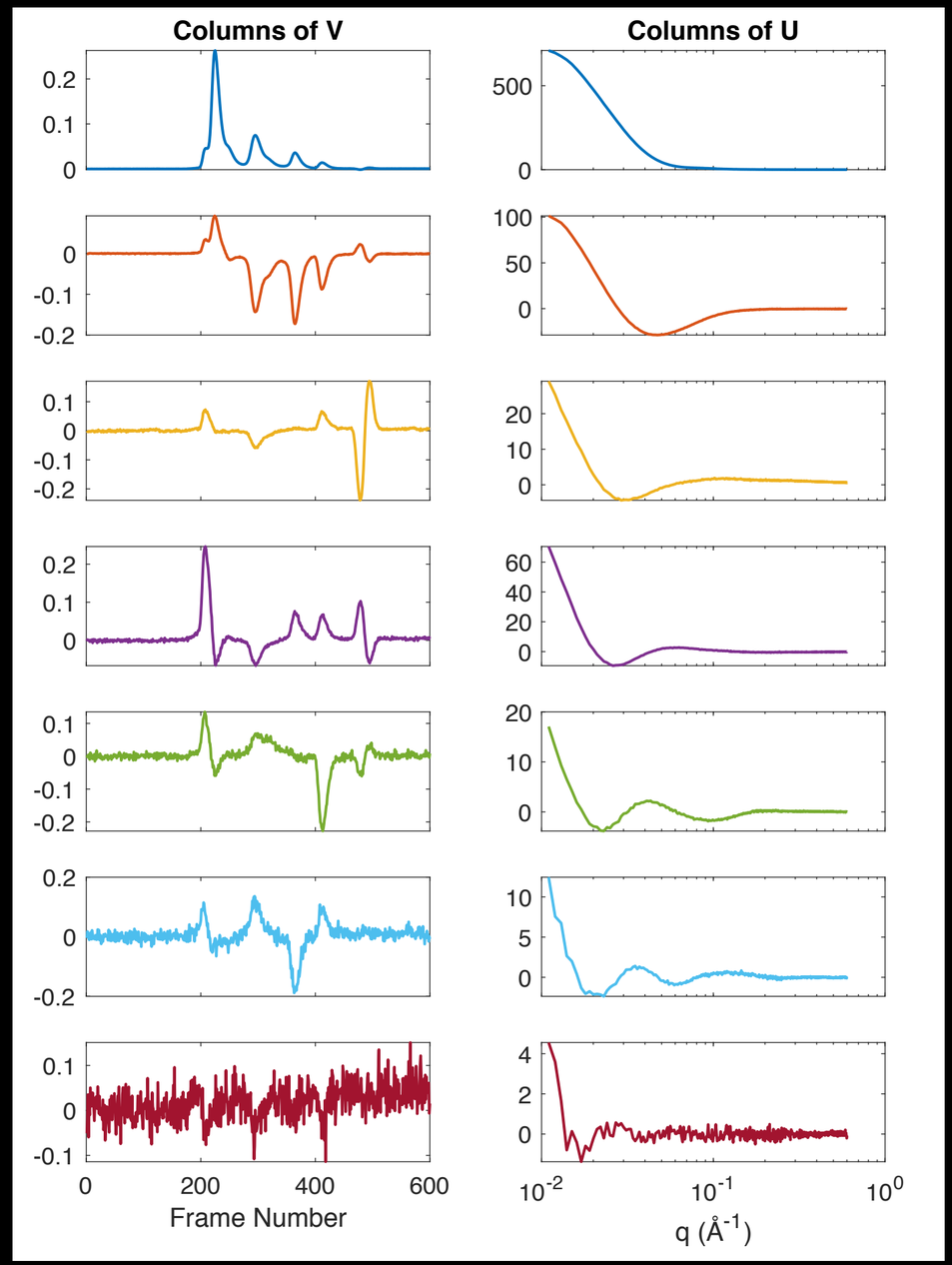
Basis vectors from SVD are usually non-physical



Sample: Bio-rad chromatography standard (thyroglobulin, γ-globulin, ovalbumin, myoglobin, vitamin B12)

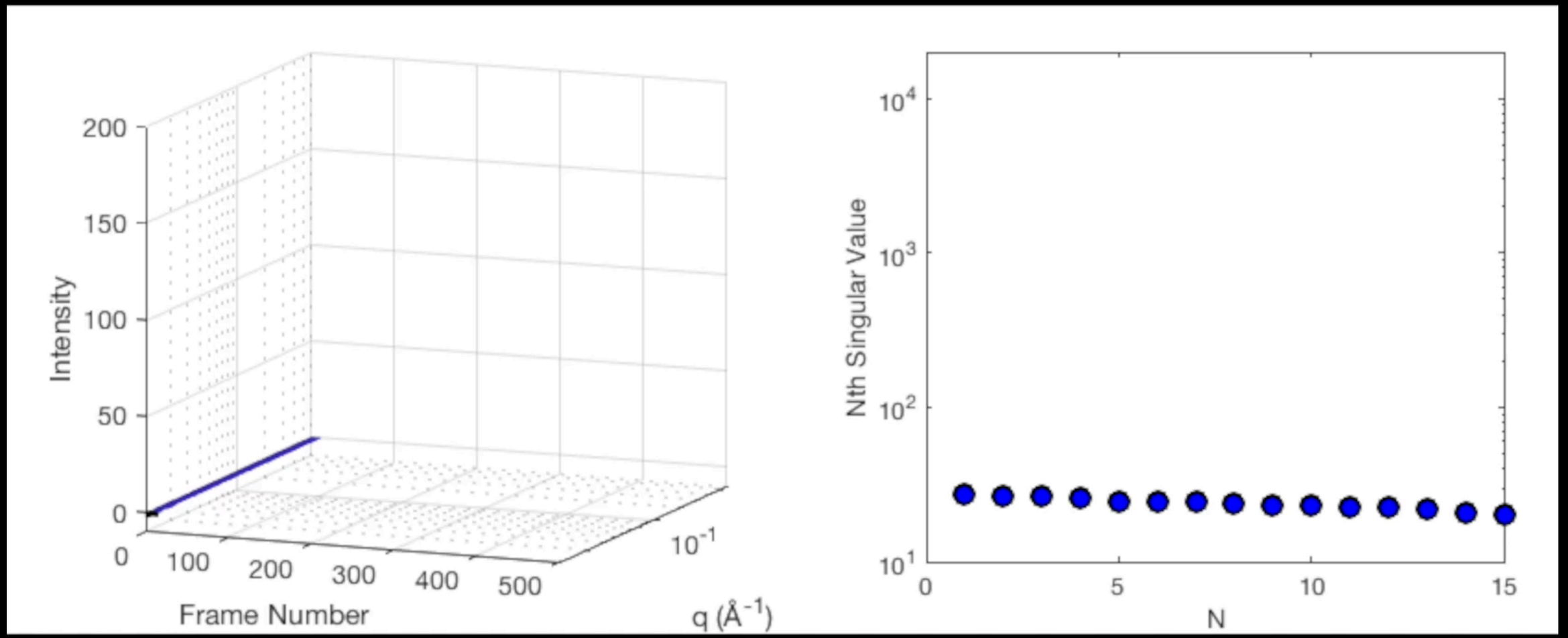
SEC-SAXS, SVD

First 7 basis vectors



- Cols. of V (concentration) go negative
- Cols. of V have multiple peaks
- Cols. of U (SAXS profiles) have negative intensity

SVD vs. time shows when new components elute

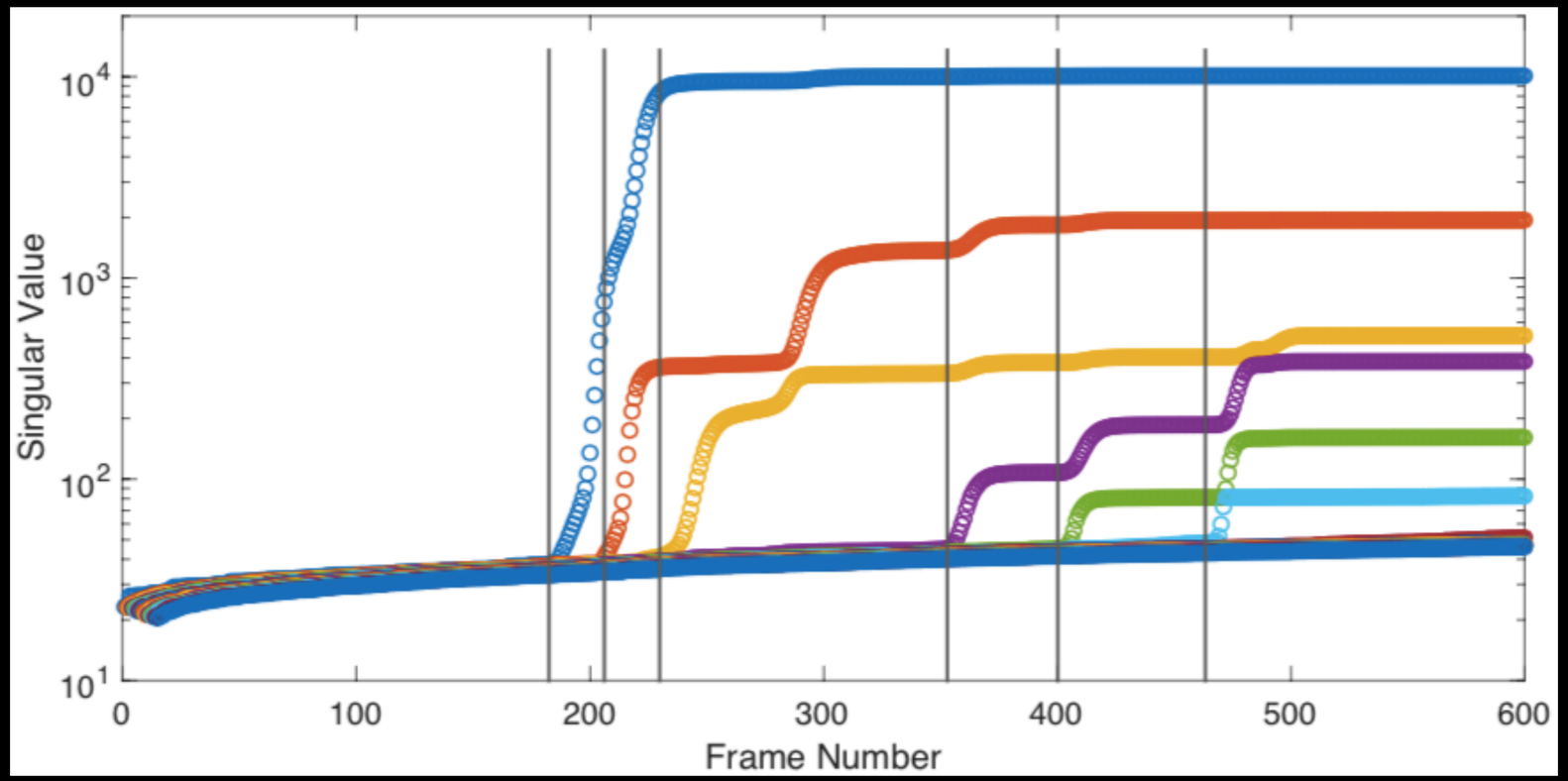


Evolving factor analysis (EFA)

A powerful method for analyzing the time-dependent SVD

First described in: Maeder, M. (1987) *Analytical chemistry*, *59*(3), 527-530.

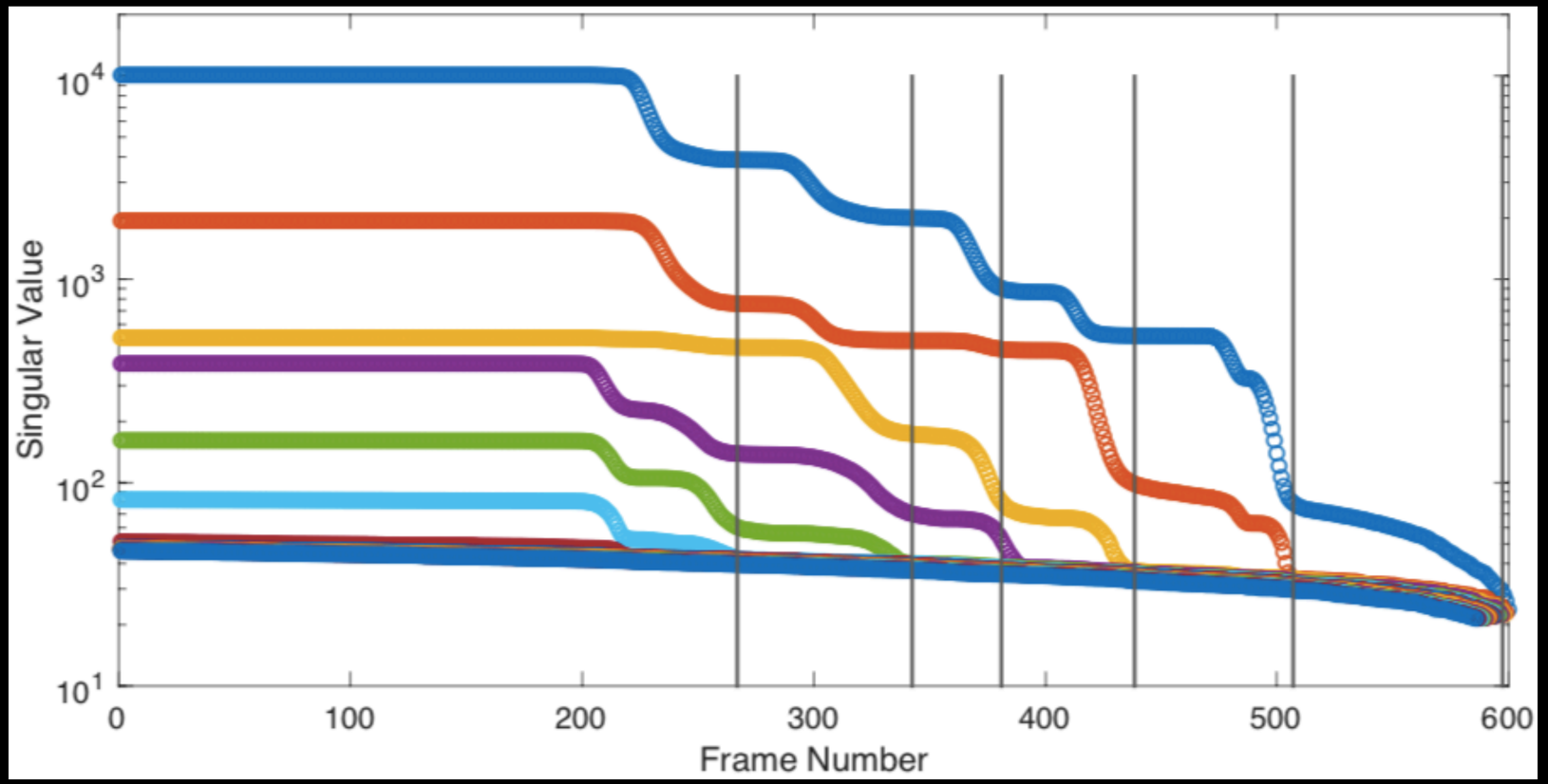
“Forward evolving factors” = singular value spectrum as components are added



Inflection points occur whenever a new component elutes from the column.

Evolving factor analysis (EFA)

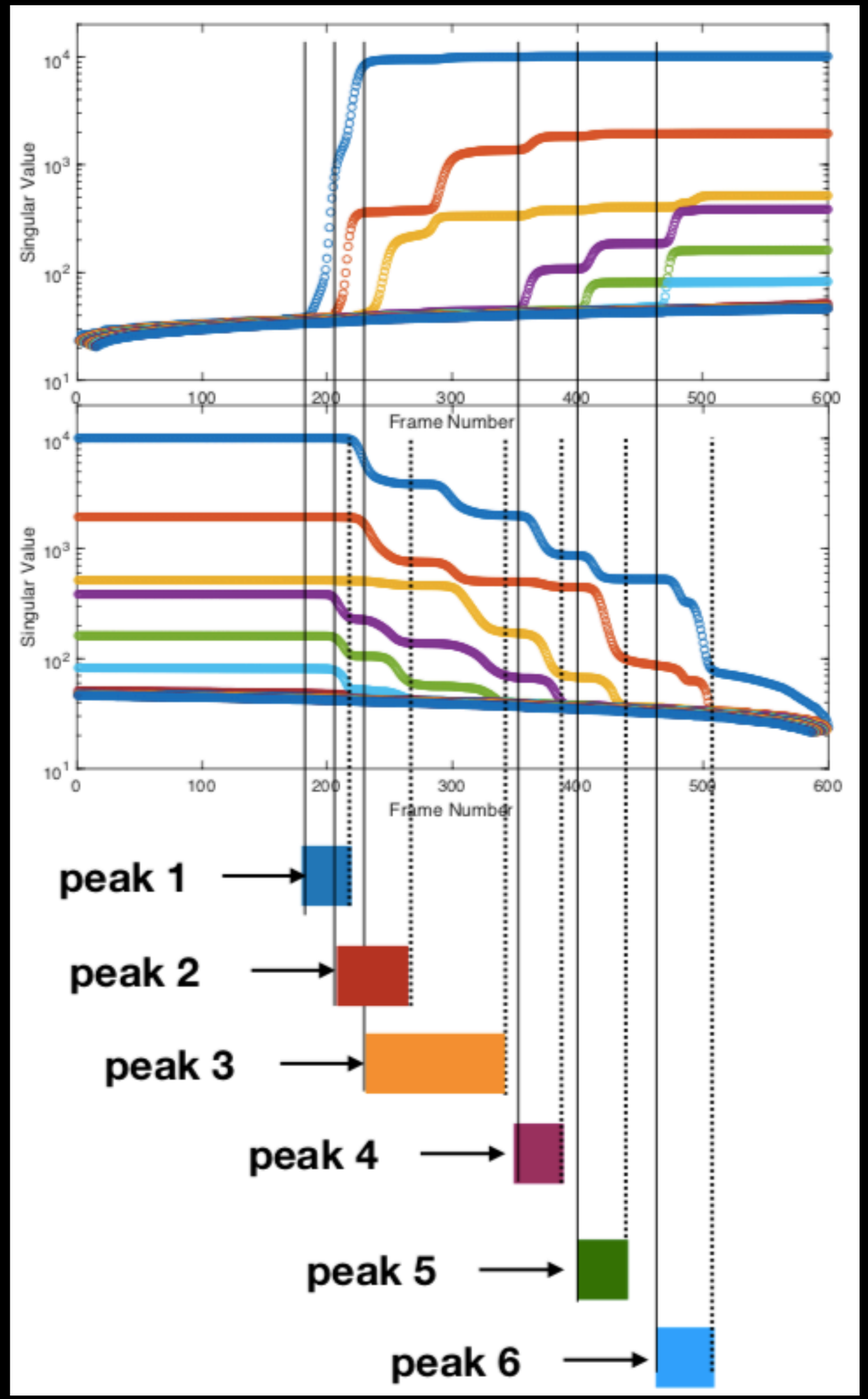
“Reverse evolving factors” = singular value spectrum as components are removed



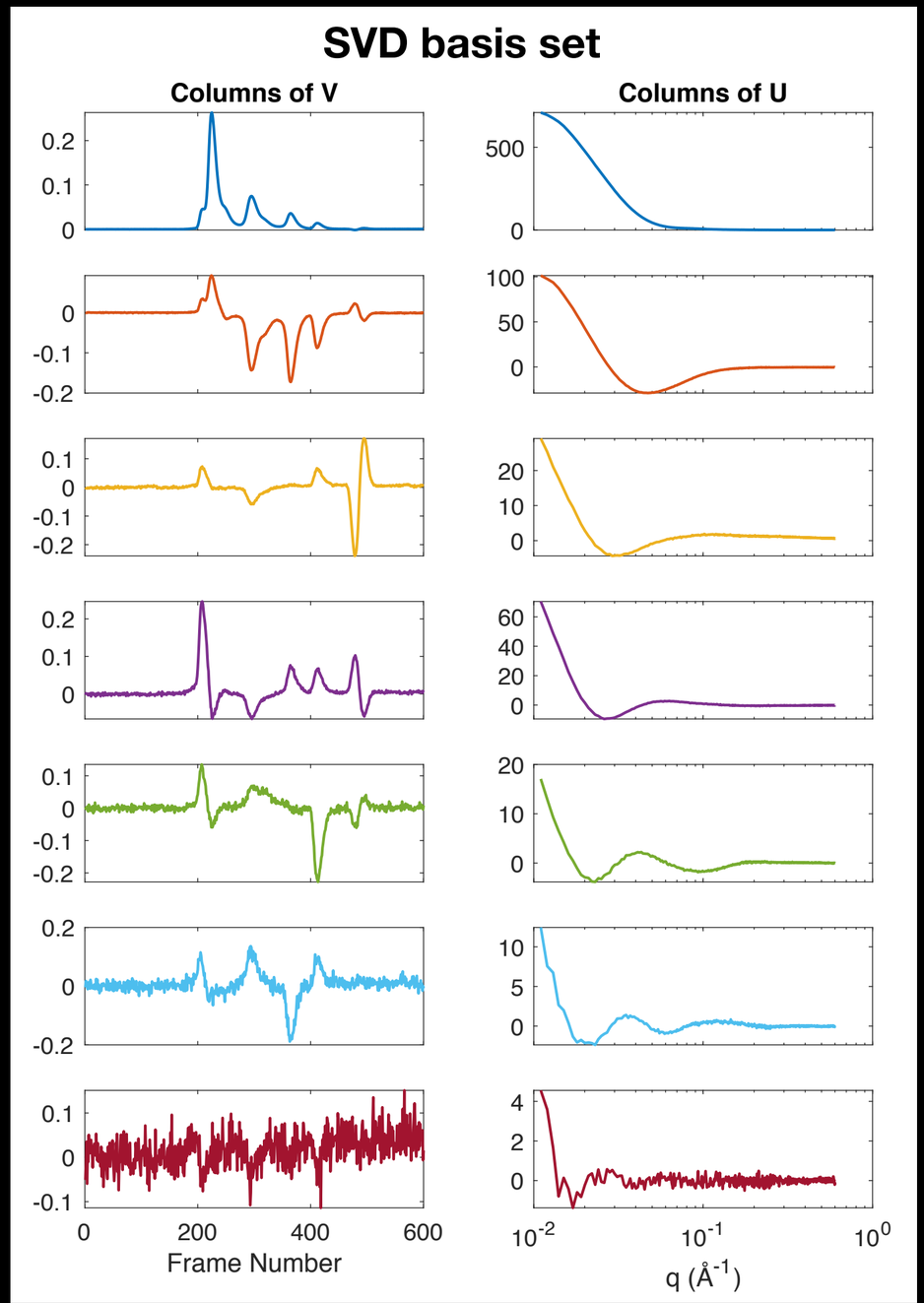
Inflection points occur whenever a component “leaves” the scattering volume.

Evolving factor analysis (EFA)

“Peak Windows” are determined on the “first in, first out” principle

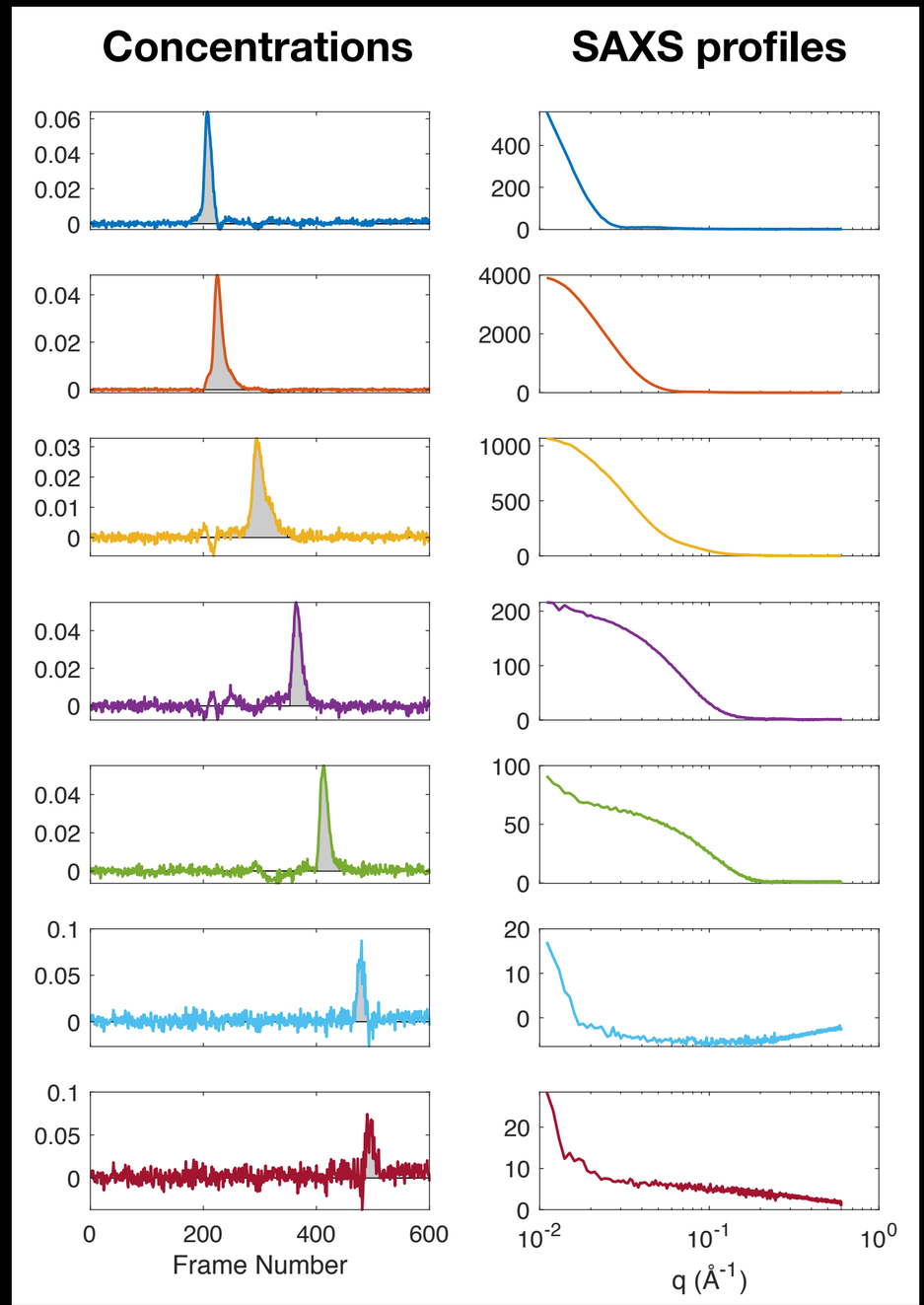


Final step: basis rotation



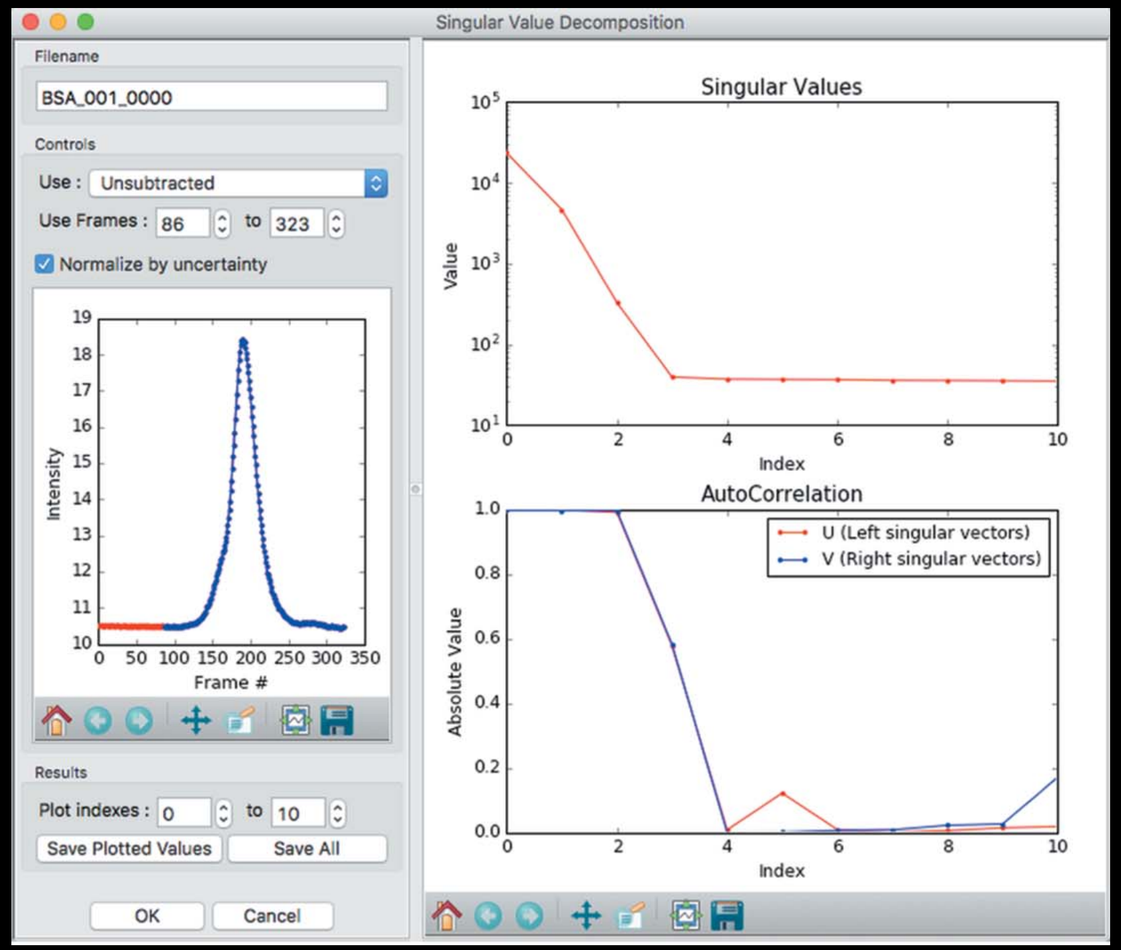
Rotate Basis

(Least-squares minimize residual outside peak windows)

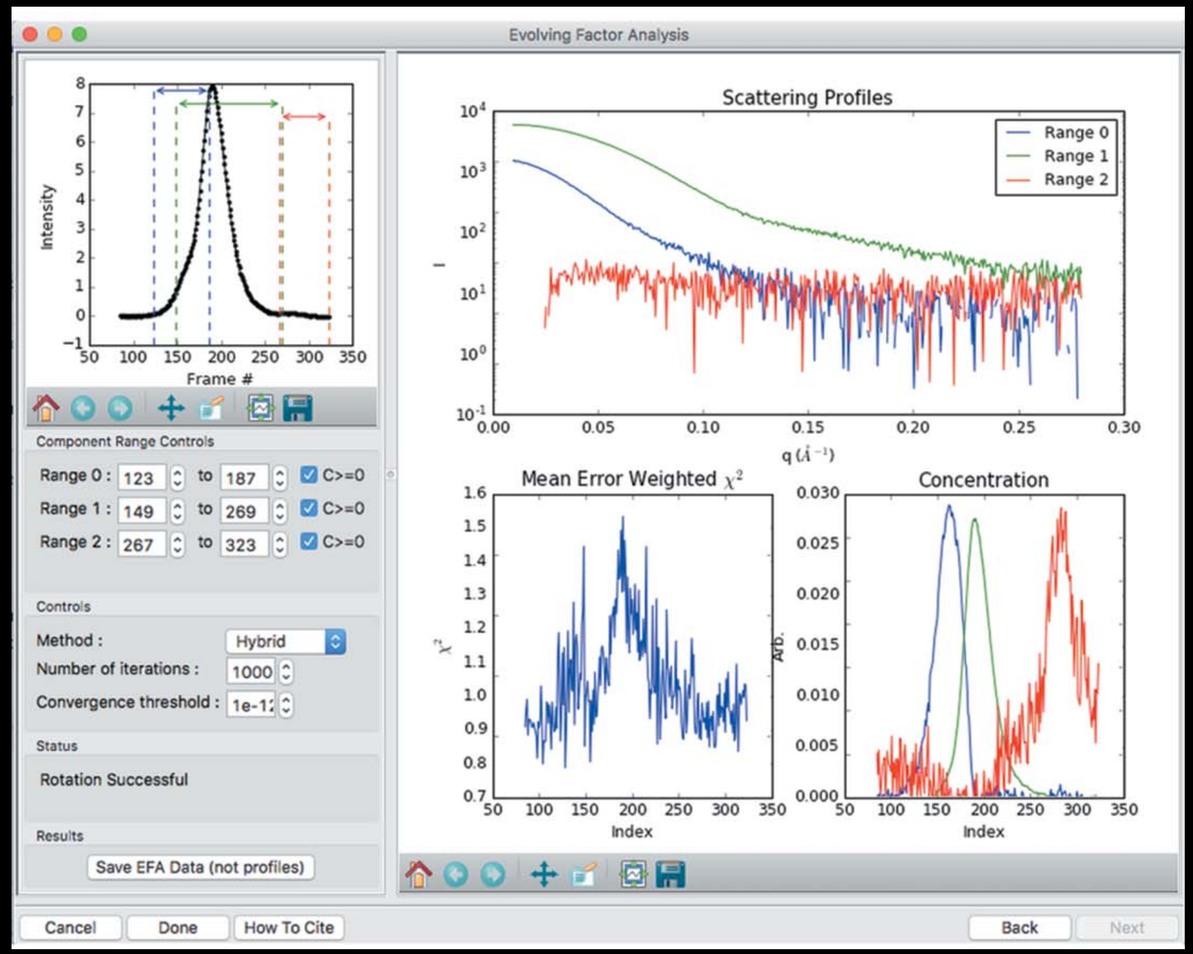


Analysis in BioXTAS RAW

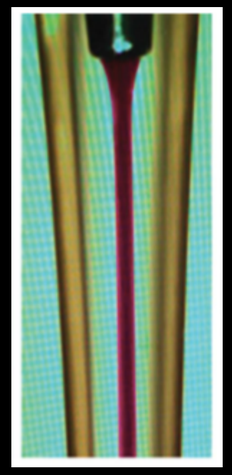
SVD

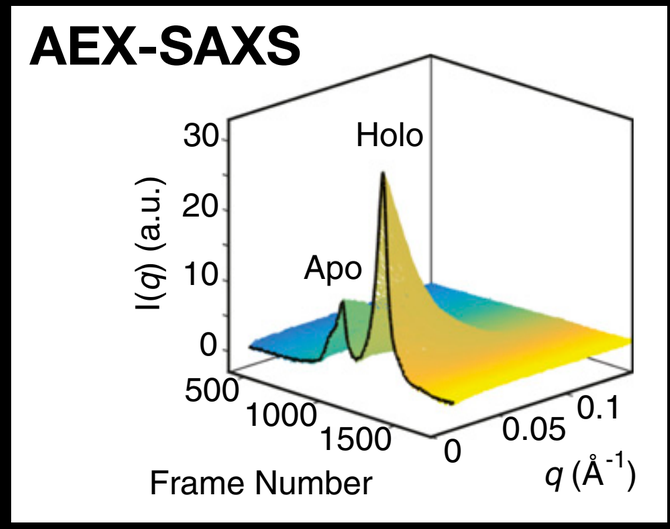


EFA

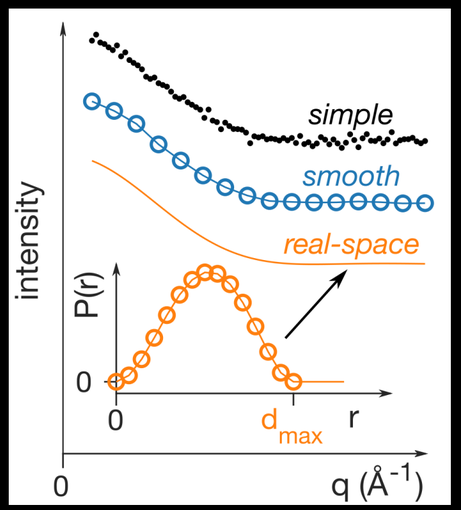


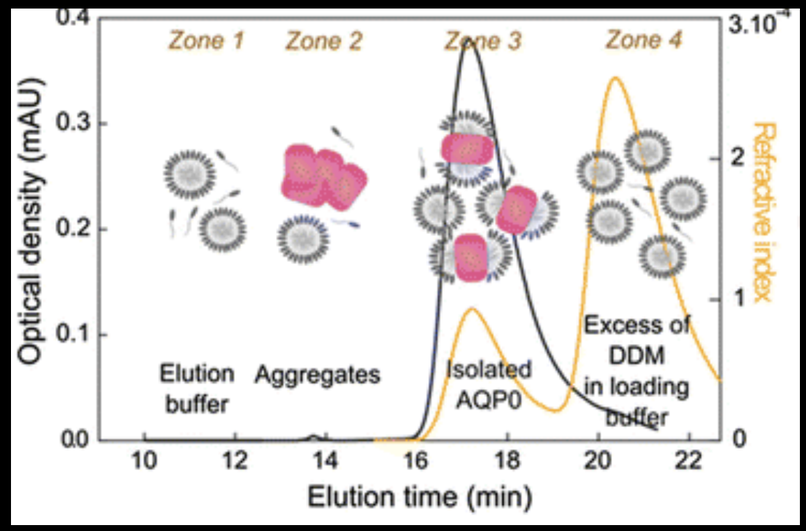
Hopkins, J. B., Gillilan, R. E. & Skou, S. *J Appl Crystallogr* **50**, 1545–1553 (2017).





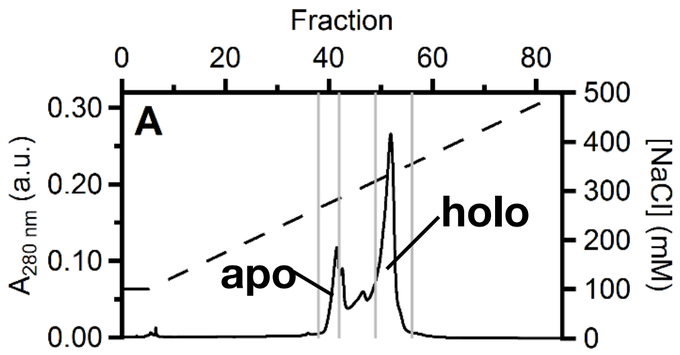
Advanced Applications and Methods

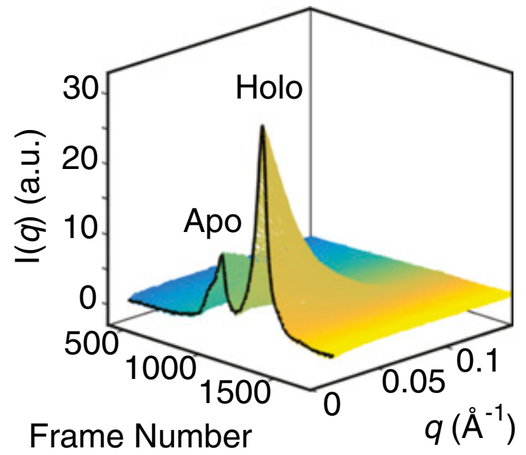


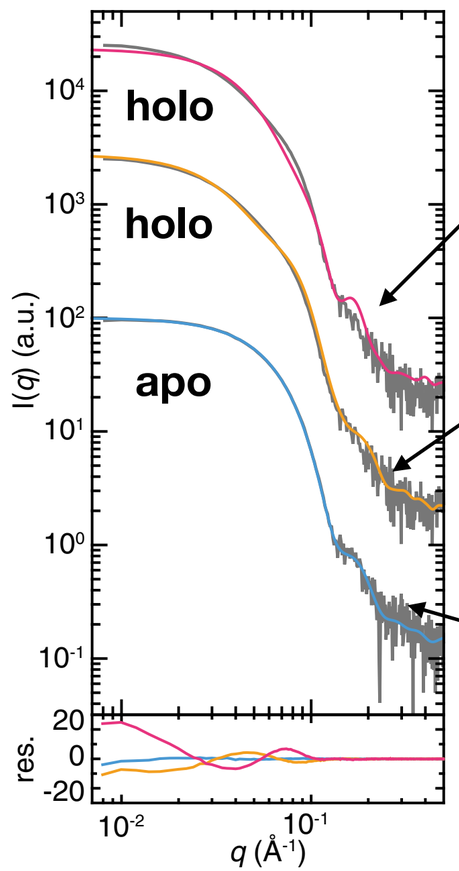


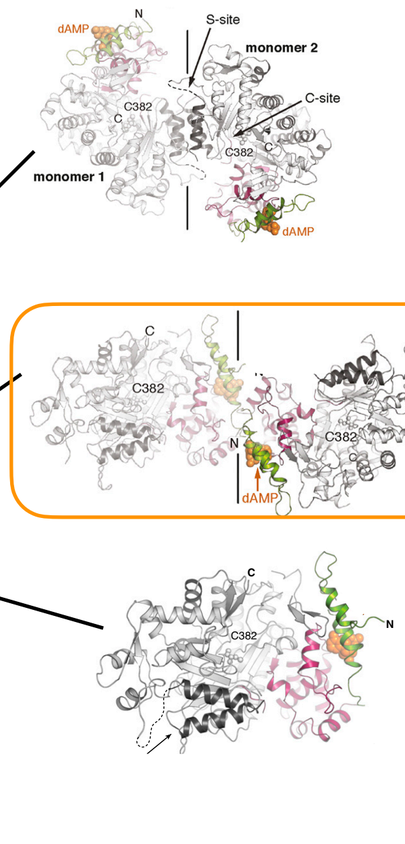
SAXS with Anion-Exchange Chromatography (AEX)

- Ribonucleotide reductase (RNR) from B. subtilis
- Co-purified with endogenous ligand, dAMP, and separated into two peaks by AEX (holo / apo)
- Performed AEX-SAXS
- Introduced new deconvolution method (REGALS)
- SAXS data + modeling —> holo peak corresponds to new structure (non-canonical dimer).









Canonical dimer (poor fit)

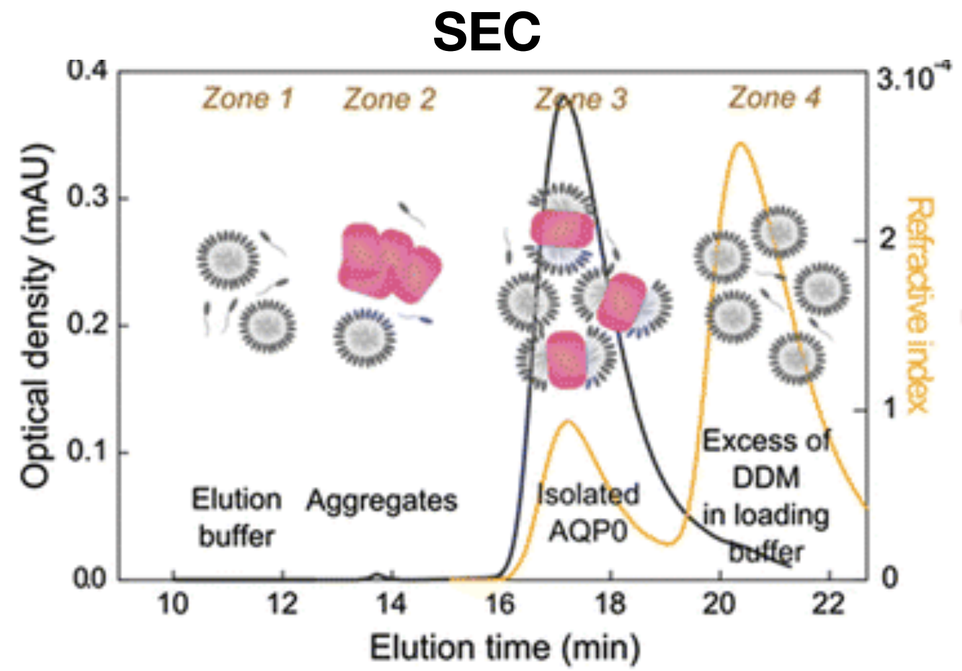
Non-canonical dimer (good fit)

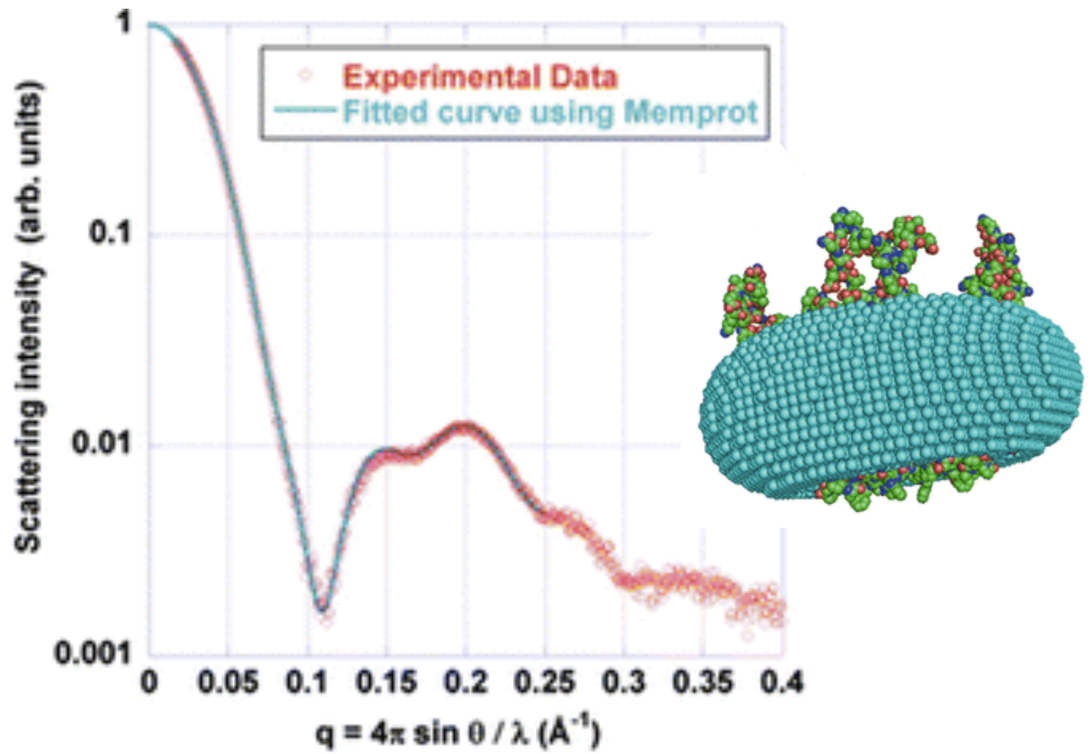
Monomer (good fit to apo)

Parker, M. J. et al. *PNAS* 115, E4594–E4603 (2018).

SEC-SAXS of membrane proteins

- Detergent often used to solubilize membrane proteins.
- Causes problems for conventional SAXS:
  - Detergent corona scattering ~ protein scattering
  - Free micelles form spontaneously, scatter
- Studied aquaporin-0 solubilized in n-Dodecyl β-D-maltoside (DDM). Structure known from crystallography.
- SEC-SAXS was used to equilibrate detergent concentration and separate excess free micelles.
- Modeling detergent corona around aquaporin-0 produced good agreement with experiment.

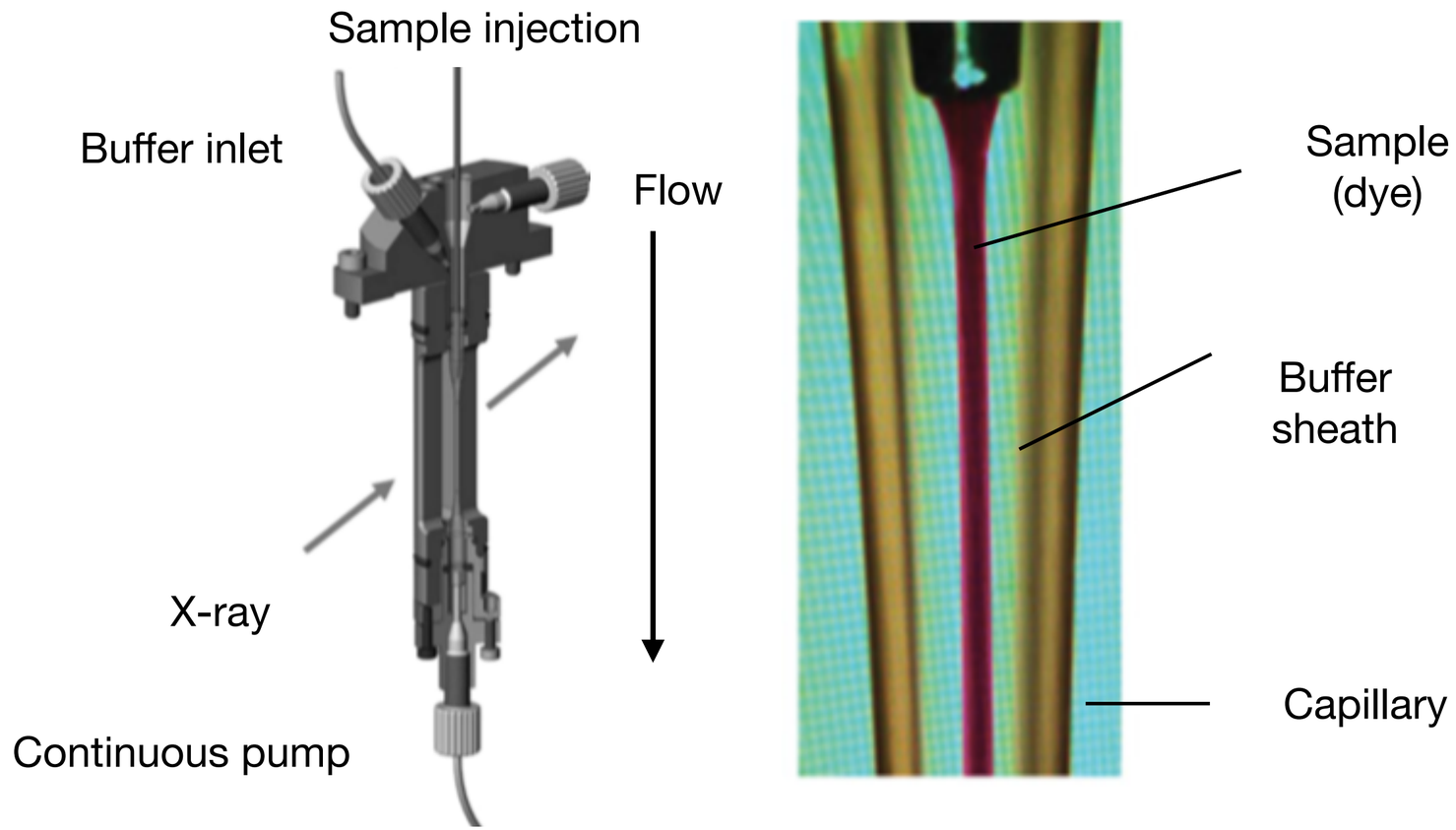




Berthaud, Pérez, & Mangenot. *JACS* 134, 10080–8 (2012).

Coflow system for high-flux data collection

- “Capillary fouling” (X-ray damage) is a particular problem at high-flux beamlines.
- Compromises data quality (although some programs can correct for it)
- Coflow system envelops sample stream in buffer sheath, so protein never touches the X-ray windows.
- Enables increased X-ray flux, both for regular and SEC-SAXS.

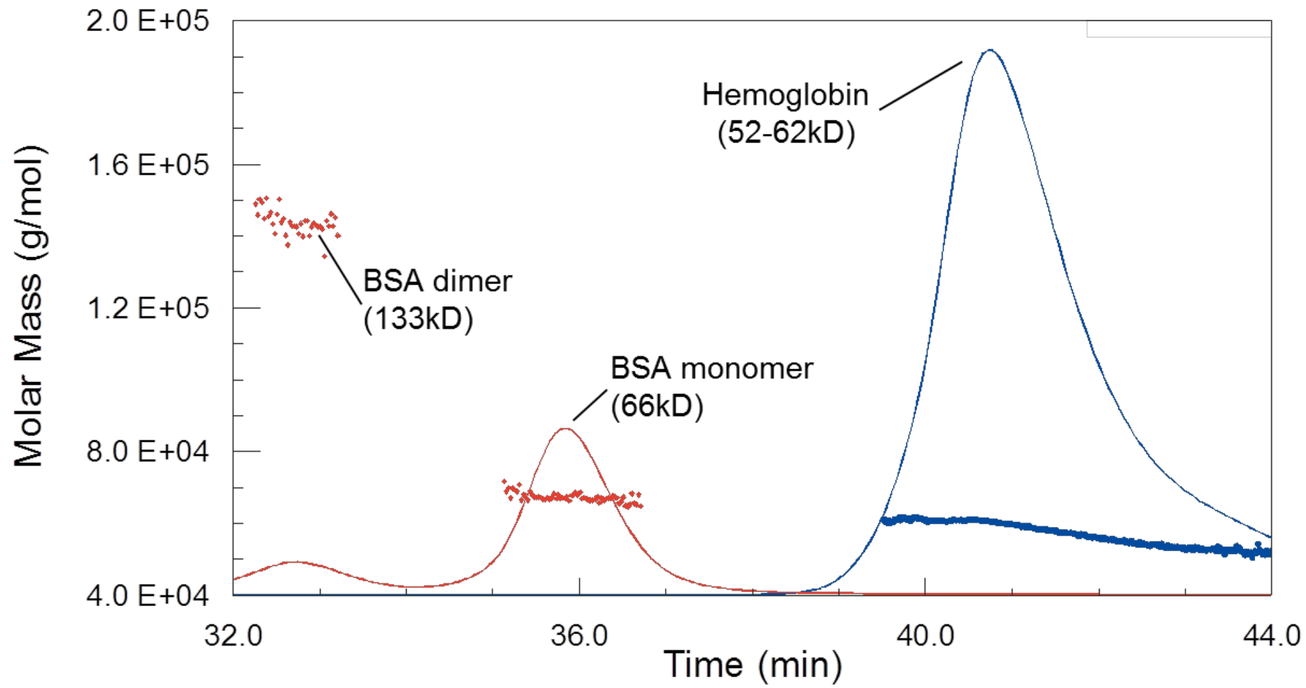


Ryan et al. J Appl Cryst 51, 97–111 (2018).
Kirby et al. Acta Crystallogr D 72, 1254–1266 (2016).

In-line Multi-Angle Light Scattering (MALS)

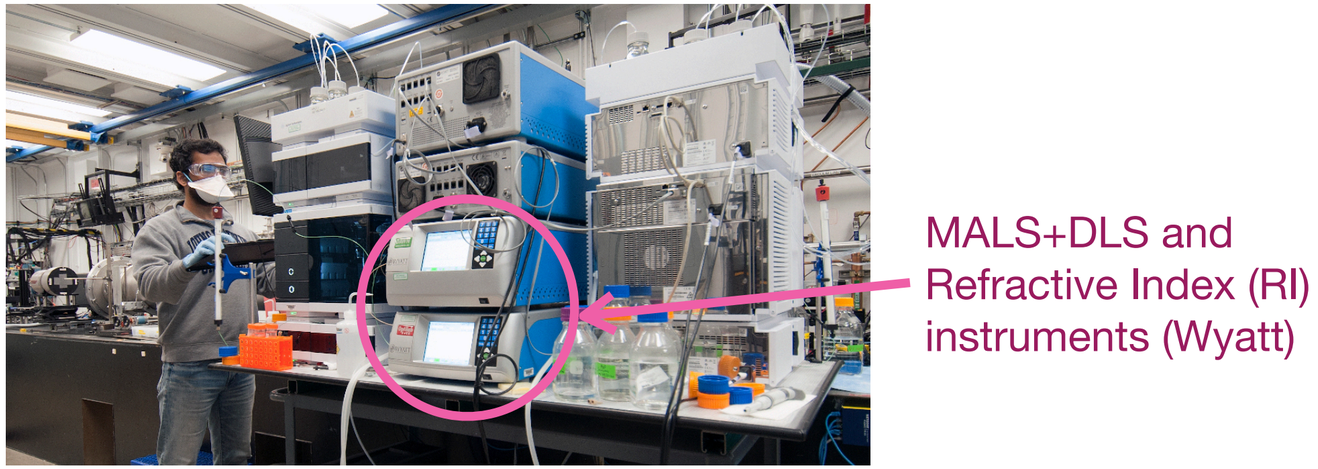
- SEC-MALS provides accurate MW readout during elution.
- Highly complementary to SAXS
- Several user facilities now offer SEC-MALS-SAXS, including:
  - APS (US), BioCAT
  - CHESS (US), ID7A
  - ALS (US), SIBYLS
  - Petra III (Germany), EMBL (P12)
  - Diamond (UK), B21
- Sample preparation, column media, and equilibration have more stringent requirements.

SEC-MALS of BSA and Hemoglobin



https://www.wyatt.com/solutions/techniques/sec-mals-molar-mass-size-multi-angle-light-scattering.html

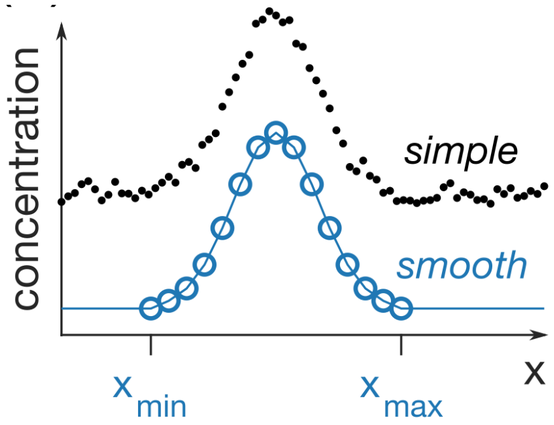
SEC-SAXS-MALS setup at BioCAT



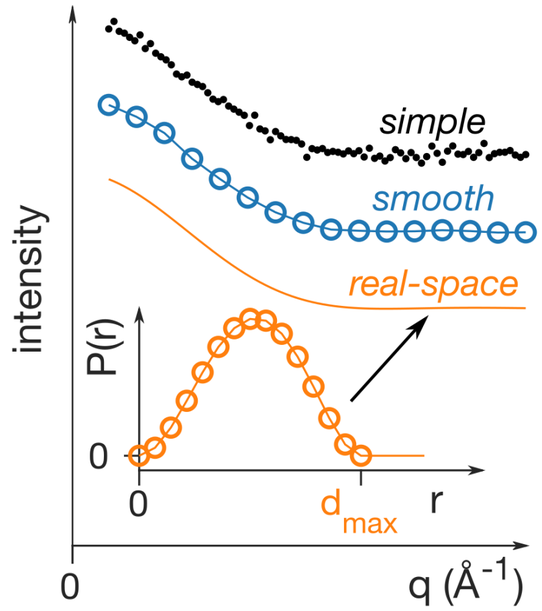
REGALS = “REGularized Alternating Least Squares”

- EFA fails if peaks are highly overlapping, or if background changes (AEX-SAXS)
- REGALS modeling adds new peak and concentration models to deconvolve physically meaningful components
- Also works on time-resolved SAXS and equilibrium titrations.
- Try it yourself!
  - https://github.com/ando-lab/regals
  - GUI in development (Jesse Hopkins)

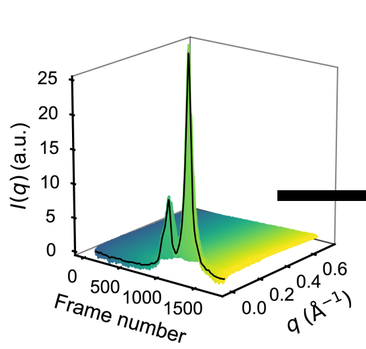
Parametric curves model components in REGALS

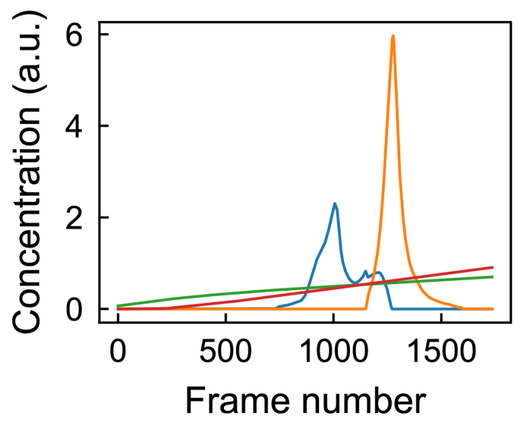


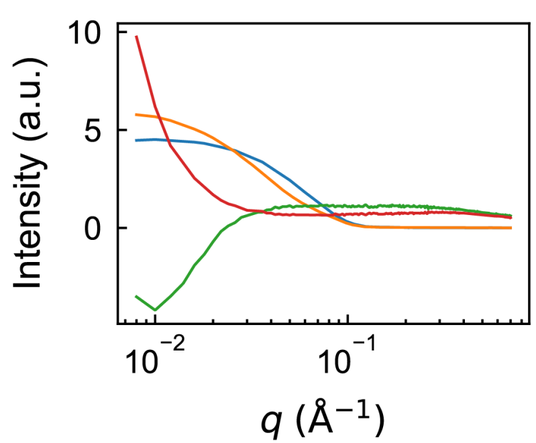
X



REGALS separates AEX-SAXS dataset on BsRNR:







Meisburger, S.P., Xu, D. & Ando, N. (2021) I*UCrJ* **8**(2).

Summary

- SEC can separate molecules based on size
- SEC-SAXS is great at removing aggregates, separating oligomers, providing a good buffer match, and increasing overall confidence in data.
- SEC-SAXS typically requires extra sample, time
- Take care: experimental variables, assess data quality
- Powerful deconvolution methods (SVD, EFA, REGALS, …)
- Exciting technical developments underway.

Questions?

Acknowledgements

Nozomi Ando Lab at Cornell
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Twitter: @AndoLab
GitHub: github.com/ando-lab/

Cornell University

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SEC-SAXS at user facilities in the US

Facility, Beamline	Link	Modes	Chromatography system	Columns
CHESS, BioSAXS/ HP-Bio (ID7A)	https://www.chess.cornell.edu/users/biosaxs-hp-bio-beamline	SEC-SAXS, SEC-MALS-SAXS; **High pressure (HP) SEC-SAXS.**	Akta Pure FPLC (4 deg C), WYATT MALS, HPLC pumps for HP mode.	User-supplied columns; GE Superdex 200 10/300; GE Supderdex 200 5/150
APS, BioCAT (18ID)	https://www.bio.aps.anl.gov/pages/about-saxs.html	SEC-SAXS, SEC-MALS-SAXS. **Coflow.**	Akta Pure FPLC (4-50 degC), Agilent 1260 series, Wyatt MALS, QELS, RI	User-supplied columns; GE Superdex 200 Increase 5/150 and 10/300; GE Superdex 75 Increase 10/300 and 5/150; GE Superose 6 Increase, 10/300
ALS, SIBYLS (12.3.1)	https://bl1231.als.lbl.gov/htsaxs/instructions/secsaxs	SEC-MALS-SAXS	Agilent 1260 series HPLC, Wyatt MALS, QELS, RI	Shodex KW-802.5; Shodex KW-803; Shodex KW-804
SSRL, SMB (4-2)	https://www-ssrl.slac.stanford.edu/smb-saxs/content/documentation/sec-saxs	SEC-SAXS	Thermo Fisher Scientific UltiMate 3000 UHPLC, RI	User-supplied columns; Superdex 200 Increase PC 3.2/300; Superdex 75 PC Increase 3.2/300; Superose 6 Increase PC 3.2/300
NSLS-II, LiX (16-ID)	https://sites.google.com/view/lixbeamline/	SEC-SAXS	Shimadzu HPLC (column box at 15-30 degC), UV and RI.	User-supplied columns; GE Superdex 200 Increase 5/150; GE Superdex 200 10/300 GL;

SEC-SAXS at user facilities outside the US

Facility, Beamline	Link	Modes	Chromatography system	Columns
Australian Synchrotron, SAXS/WAXS	https://www.ansto.gov.au/user-access/instruments/australian-synchrotron-beamlines/saxs-waxs	SEC-SAXS. **Coflow**.	Shimadzu HPLC, (column box at 6-60 degC)	User-supplied columns;
Petra III (Germany), EMBL (P12)	https://www.embl-hamburg.de/biosaxs/sample.html#sec	SEC-MALS-DLS-SAXS	Agilent 1260 Infinity Bio-Inert HPLC/FPLC (ambient temp.), Wyatt MALS, QELS, rEX, DLS	User-supplied columns; GE Superdex 200 Increase 10/300 and 5/150; GE Superdex 75 Increase 10/300 and 5/150; GE Superose 6 Increase 10/300 and 5/150; Wyatt WTC-015S5; Wyatt WTC-030S5;
Diamond (UK). B21	https://www.diamond.ac.uk/Instruments/Soft-Condensed-Matter/small-angle/B21.html	SEC-SAXS, SEC-MALS-SAXS	Agilent 1200 HPLC, Wyatt MALS	GE Superdex 200 3.2/300; GE Superose 6 3.2/300; Shodex KW-402.5; Shodex KW-403; Shodex KW-404; Shodex KW-405;
SOLEIL (France), SWING	https://www.synchrotron-soleil.fr/fr/lignes-de-lumiere/swing	SEC-SAXS	Agilent HPLC	User-supplied columns; Agilent Bio-Sec 3-300; Agilent AdvBioSec 2.7-300; Agilent BioSec 5-1000; Agilent BioSec 5-2000;
ESRF (France), BM29	http://www.esrf.eu/home/UsersAndScience/Experiments/MX/About_our_beamlines/bm29/beamline-setup/hplc.html	SEC-SAXS	Shimadzu HPLC	

References

Topic	
Review	• Pérez, J. & Vachette, P. A Successful Combination: Coupling SE-HPLC with SAXS. in Biological Small Angle Scattering: Techniques, Strategies and Tips. 183–199 (Springer, 2017).
Ion exchange-SAXS, REGALS	• Parker, M. J. et al. An endogenous dAMP ligand in Bacillus subtilis class Ib RNR promotes assembly of a noncanonical dimer for regulation by dATP. PNAS 115, E4594–E4603 (2018). • Hutin, S., Brennich, M., Maillot, B. & Round, A. Online ion-exchange chromatography for small-angle X-ray scattering. Acta Crystallogr D Struct Biol 72, 1090–1099 (2016). • Meisburger, S.P., Xu, D. & Ando, N. (2021) *REGALS*: a general method to deconvolve X-ray scattering data from evolving mixtures. *IUCrJ* **8**(2).
Coflow	• Ryan, T. M. et al. An optimized SEC-SAXS system enabling high X-ray dose for rapid SAXS assessment with correlated UV measurements for biomolecular structure analysis. J Appl Cryst 51, 97–111 (2018). • Kirby, N. et al. Improved radiation dose efficiency in solution SAXS using a sheath flow sample environment. Acta Crystallogr D Struct Biol 72, 1254–1266 (2016).
EFA	• Meisburger, S. P. et al. Domain Movements upon Activation of Phenylalanine Hydroxylase Characterized by Crystallography and Chromatography-Coupled Small-Angle X-ray Scattering. JACS 138, 6506–6516 (2016). • Hopkins, J. B., Gillilan, R. E. & Skou, S. BioXTAS RAW: improvements to a free open-source program for small-angle X-ray scattering data reduction and analysis. J Appl Crystallogr 50, 1545–1553 (2017).
US-SOMO	• Brookes, E., Vachette, P., Rocco, M. & Pérez, J. US-SOMO HPLC-SAXS module: dealing with capillary fouling and extraction of pure component patterns from poorly resolved SEC-SAXS data. J Appl Cryst 49, 1827–1841 (2016).
Membrane Proteins	• Berthaud, A., Manzi, J., Pérez, J. & Mangenot, S. Modeling Detergent Organization around Aquaporin-0 Using Small-Angle X-ray Scattering. JACS 134, 10080–10088 (2012).
SVD	• Chen, L., Hodgson, K. O. & Doniach, S. A Lysozyme Folding Intermediate Revealed by Solution X-ray Scattering. Journal of Molecular Biology 261, 658–671 (1996).