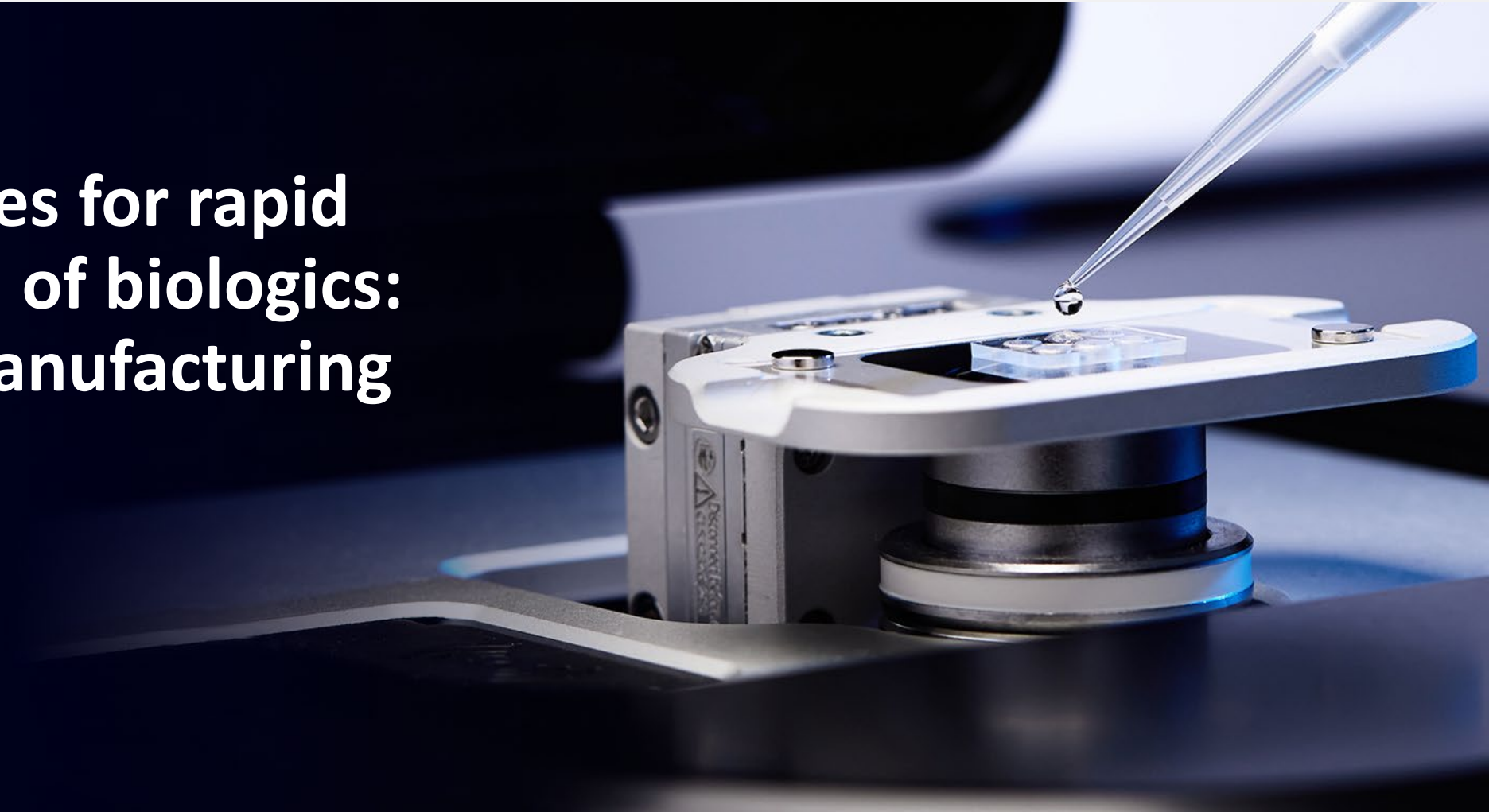
REFEYN
WEIGHING MOLECULES WITH LIGHT

New technologies for rapid characterization of biologics: From R&D to Manufacturing



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Biophysical characterization: key questions

Is the sample pure enough for the next stage of analysis?

What is the composition of the functional species?

Are the gene delivery vehicles filled?

Analysis of complexes

Sample characterization

What are the compositions of interacting species?

What conditions optimize protein production?

What is the complex stoichiometry?

Interaction studies

How does the complex form?

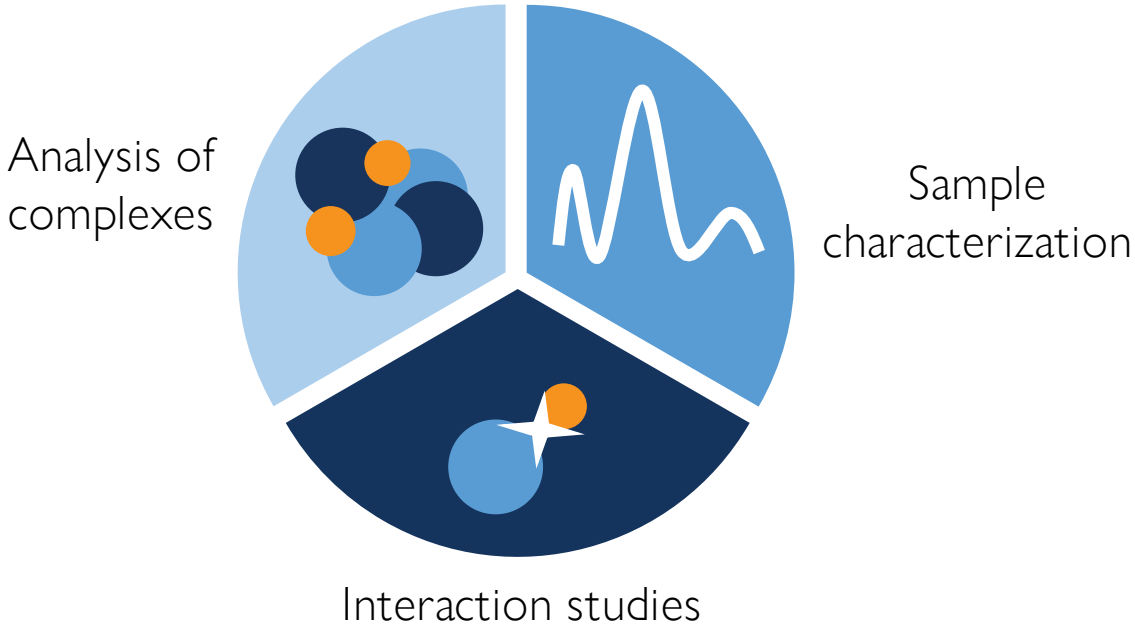
How strong are the interactions?

What is mass photometry?

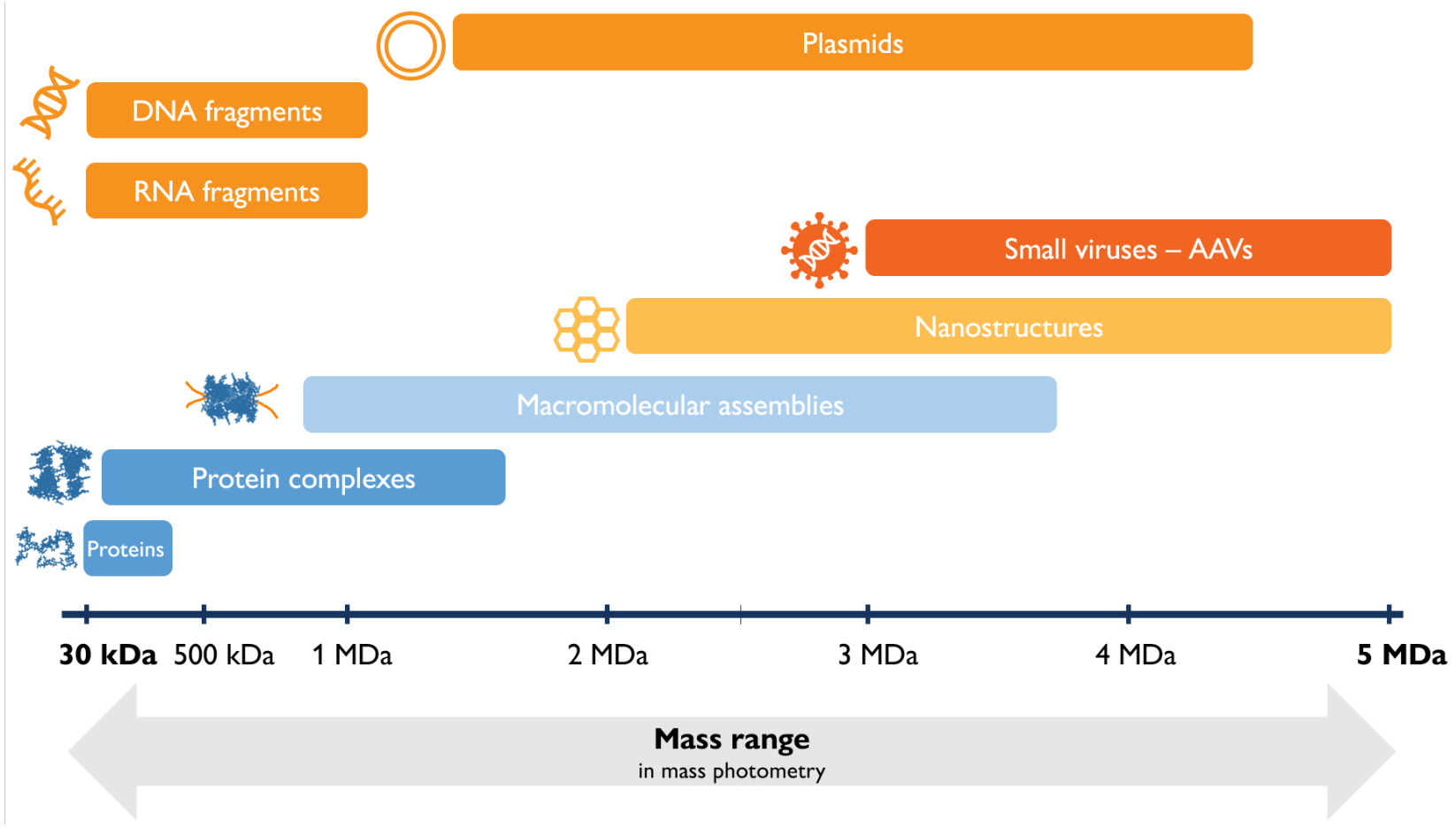
An innovative biophysical analysis tool with numerous applications

Mass photometry delivers rapid, accurate mass measurement of label-free single molecules in solution in their native state, enabling:

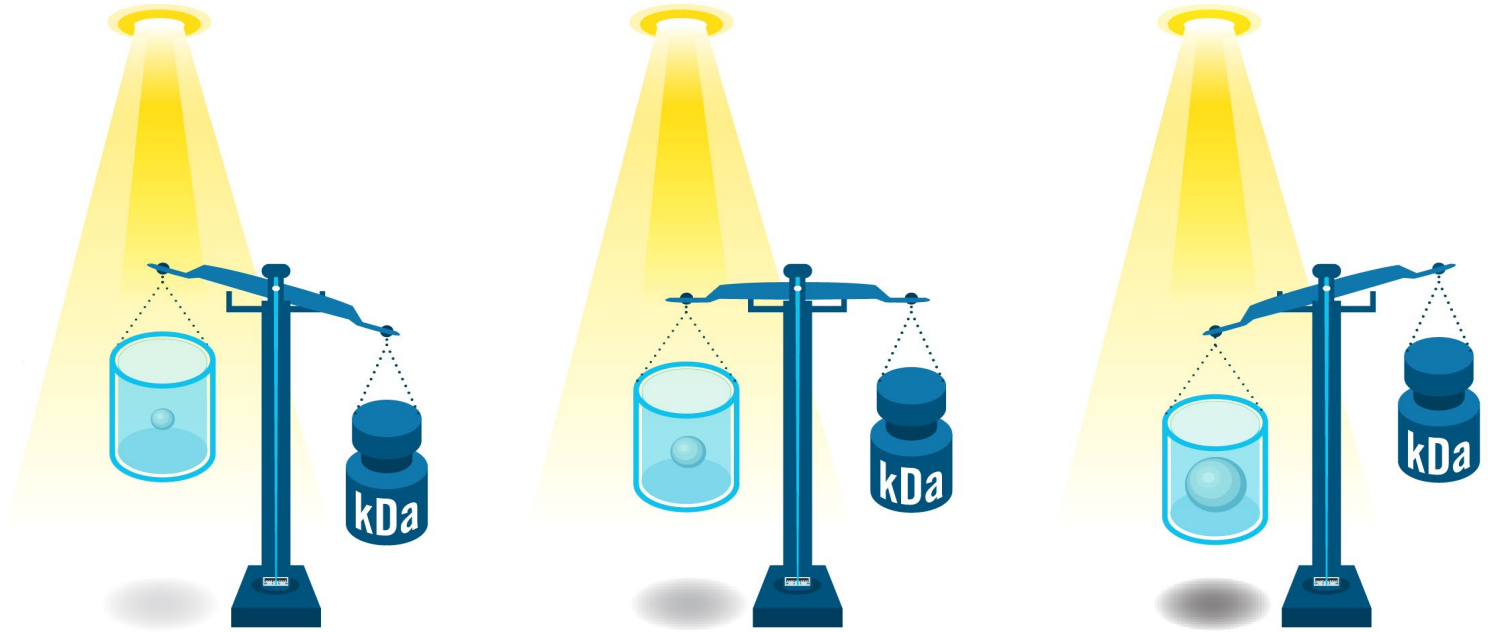
- **access to subpopulations** to determine complex stoichiometry and oligomeric state
- the monitoring of **complex, multistep processes**
- detection of **low abundance species**
- the characterization & monitoring of **sample heterogeneity**



Mass photometry measures different types of biomolecules

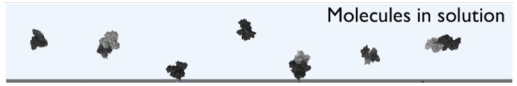


Mass photometry weighs molecules with light



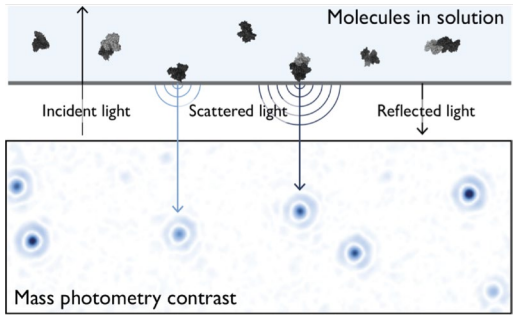
How does mass photometry work?

1



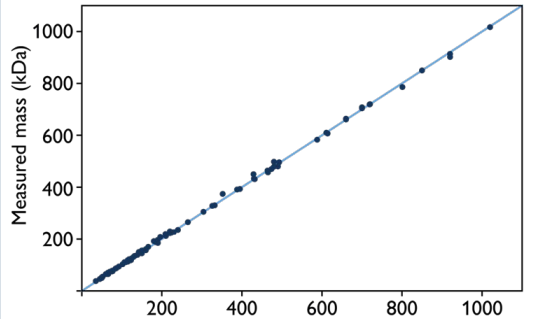
Biomolecules approaching a glass surface are illuminated by a laser

2



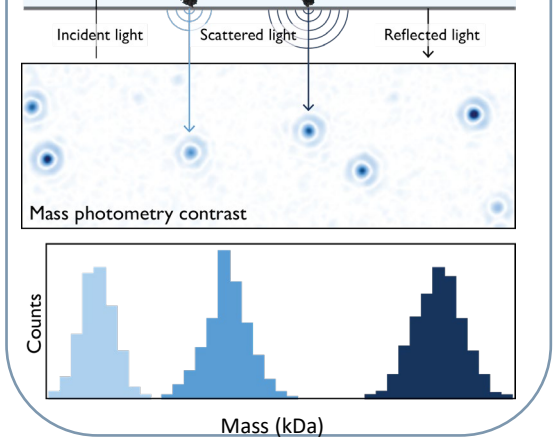
The biomolecules scatter light, which interferes with the light reflected at the interface

3



The resulting interface contrast scales linearly with the mass of the biomolecule

4



A mass histogram is generated from the single molecule measurements

What does a mass photometry measurement look like?

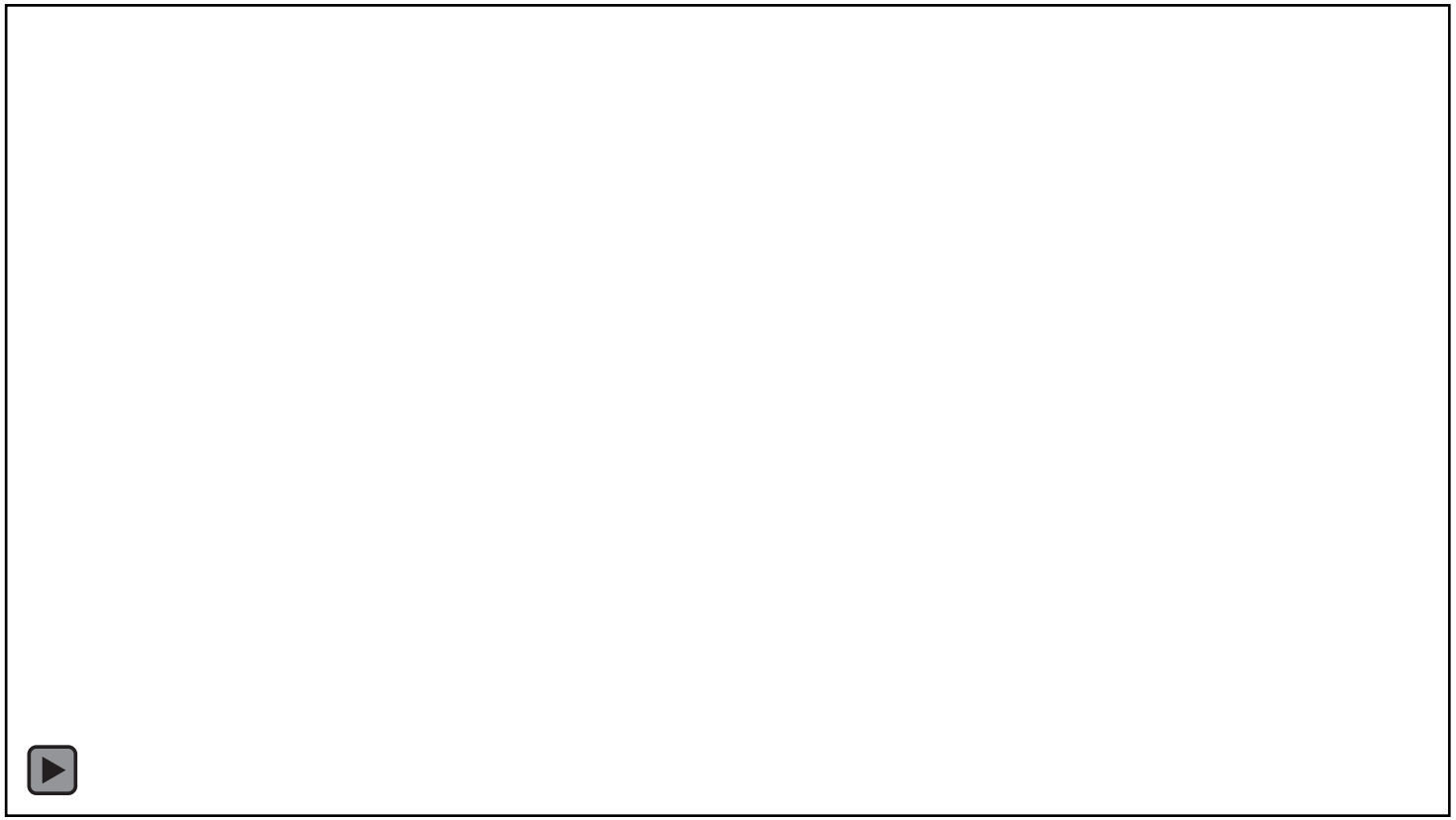
Quick sample analysis

Easy experimental procedure

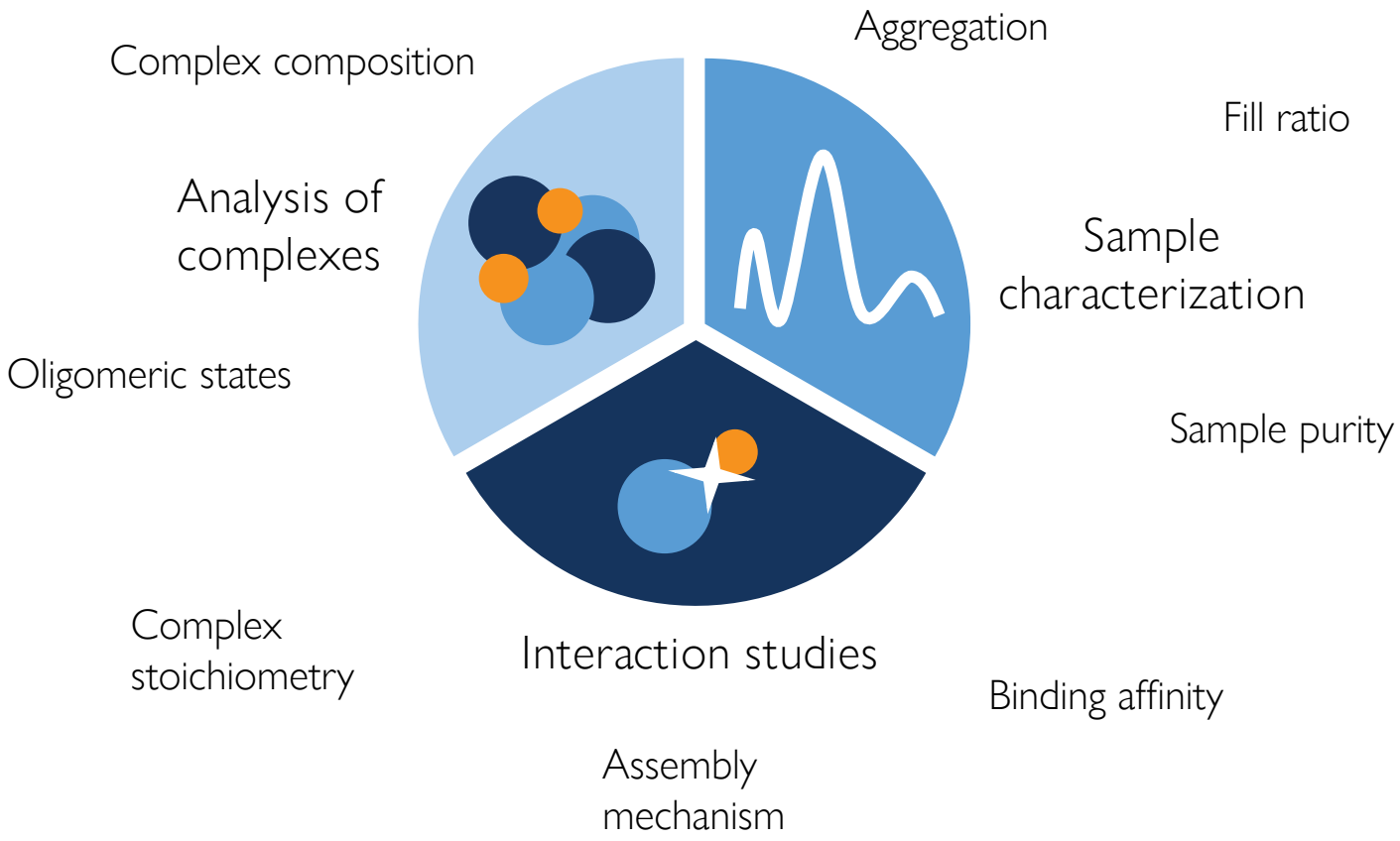
Intuitive data analysis

Low sample consumption

Small footprint



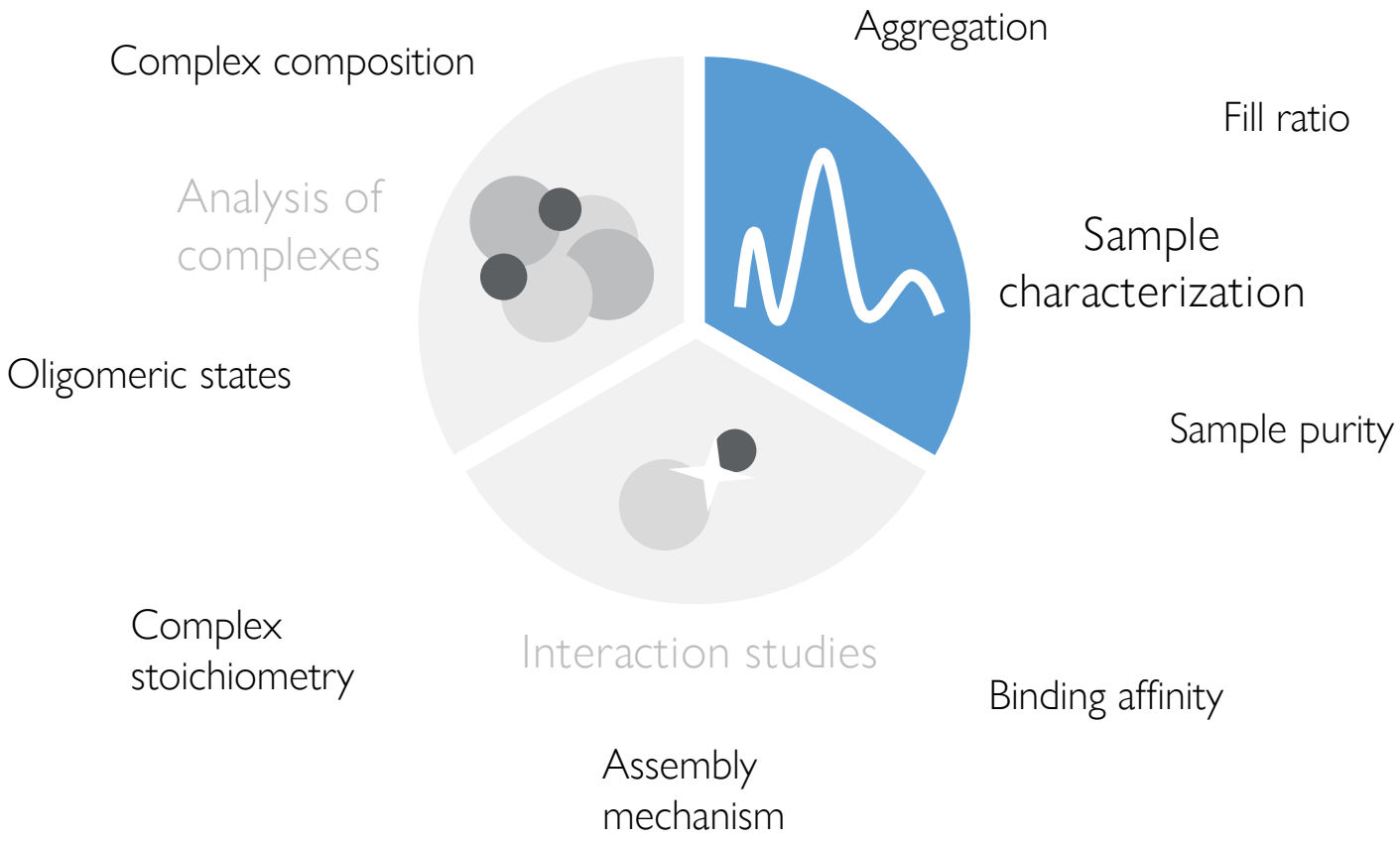
Applications of mass photometry



Biophysical characterization: key questions

Research & Discovery	Process Development	Quality Control
Understanding biomolecular interactions and behaviors	Optimizing the product and process	Assessing sample purity and quality

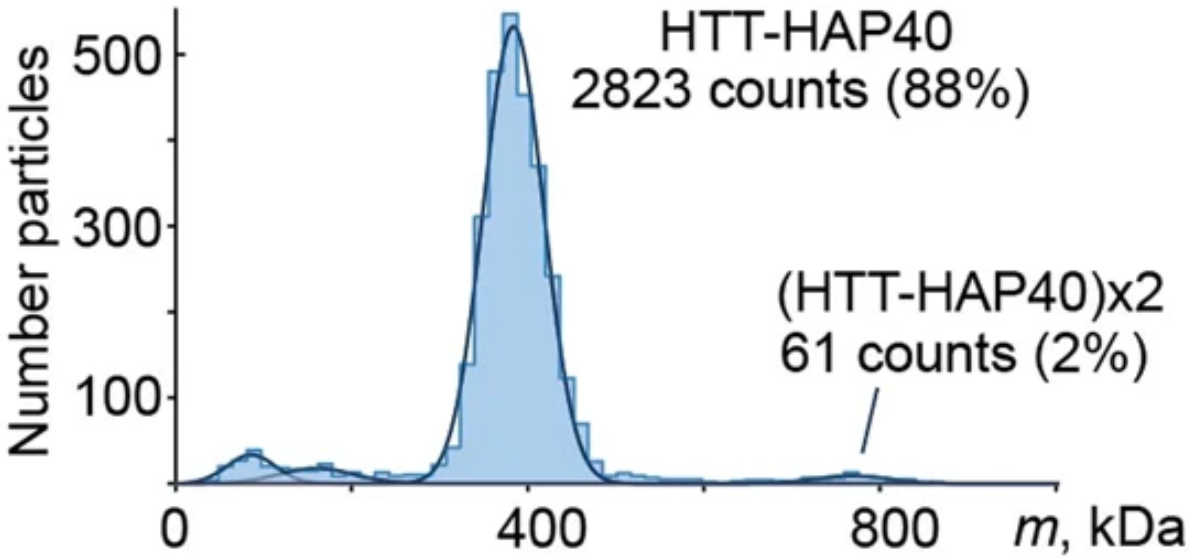
Applications of mass photometry



Mass photometry can assess sample purity

Is the sample pure enough to progress to the next stage of analysis?

- Mass photometry analysis demonstrated the monodispersity of samples of the protein complex HTT-HAP40
  - HTT is altered in Huntington's disease
- Mass photometry enabled straightforward assessment of sample quality before further analysis



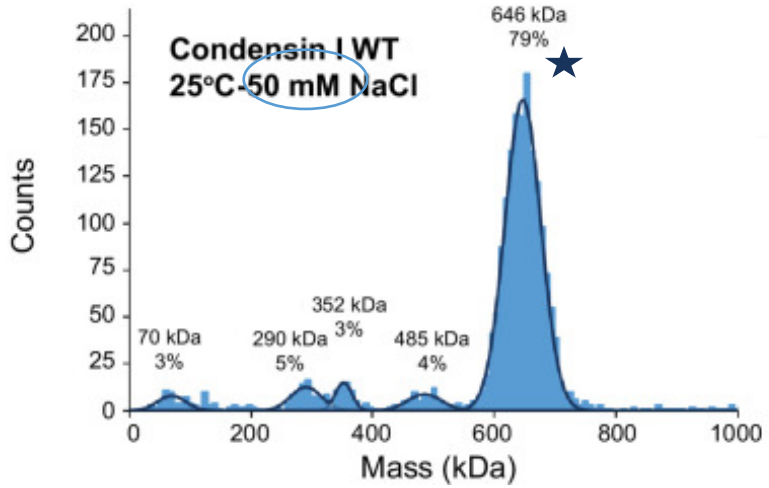
Measured on the One^{MP}

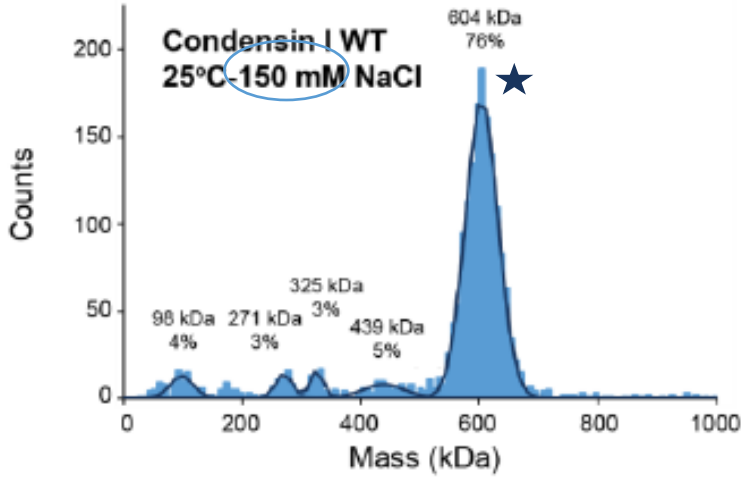
Harding, R.J., et al., Comm. Biology, 2021

Mass photometry can assess complex stability

Are the complexes stable in the buffer conditions?

- Mass photometry made it possible to quickly confirm the purity and intact nature of condensin complexes (★) before further analyses
- The mass measurements showed that the condensin complexes remained intact in the buffer conditions used for the binding assays





Measured on the One^{MP}

[Choppakatla, P et al., eLife, 2021]

Mass photometry helps optimize purification

What conditions are required to ensure optimal production of the desired protein?

- Monitoring enrichment of 700 kDa 20S proteasome

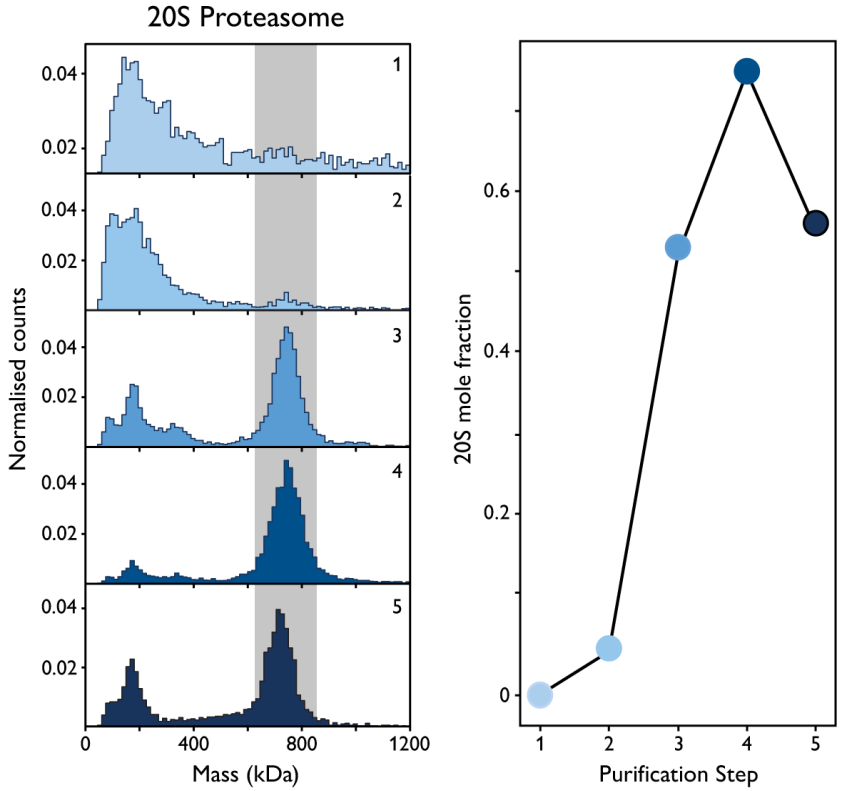
(1) Early elution gel filtration

(2) Late elution gel filtration

(3) After 2nd chromatography

(4) After buffer exchanged

(5) After last column



→ Clearly, the purest sample is after step 4

→ No need for final purification step

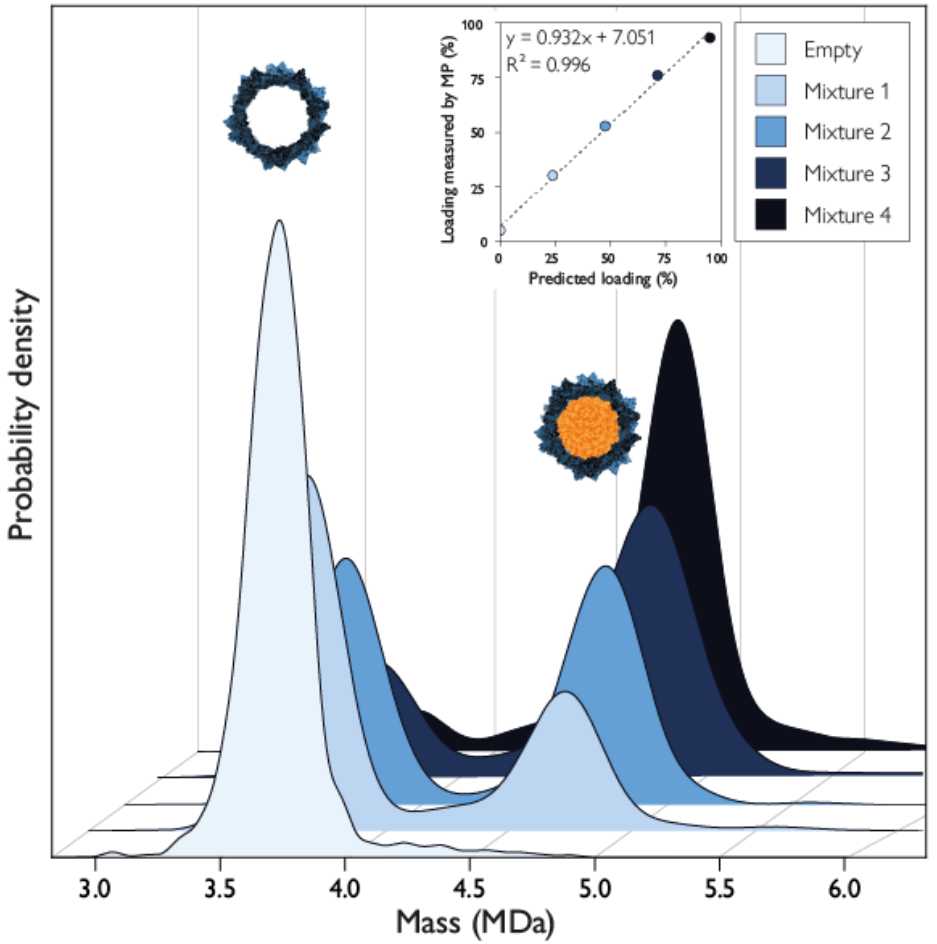
Measured on the One^{MP}

Courtesy of Michal Sharon lab @ WIS

Mass photometry quantifies AAV vector loading

What proportion of AAV capsids are fully loaded?

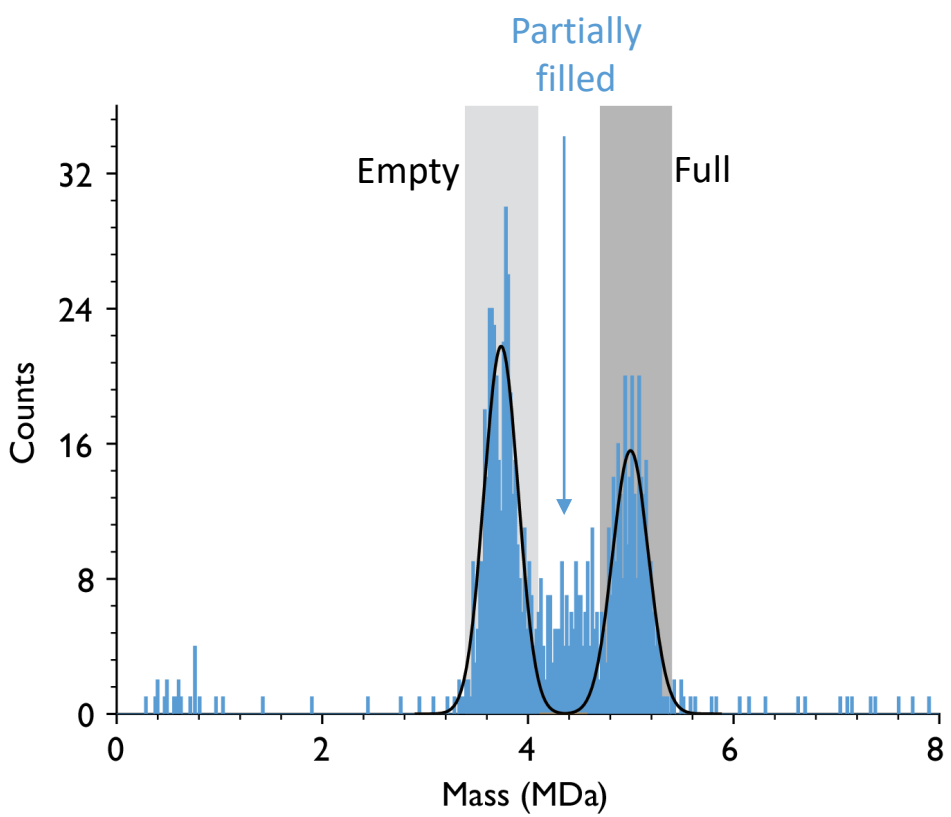
- DNA cargo increases a capsid's mass, but not its size
- These samples contained varying proportions of full AAV5 capsids
- Mass photometry accurately quantified the loaded proportions
- Only minimal sample required, with results in just a few minutes and serotype agnostic



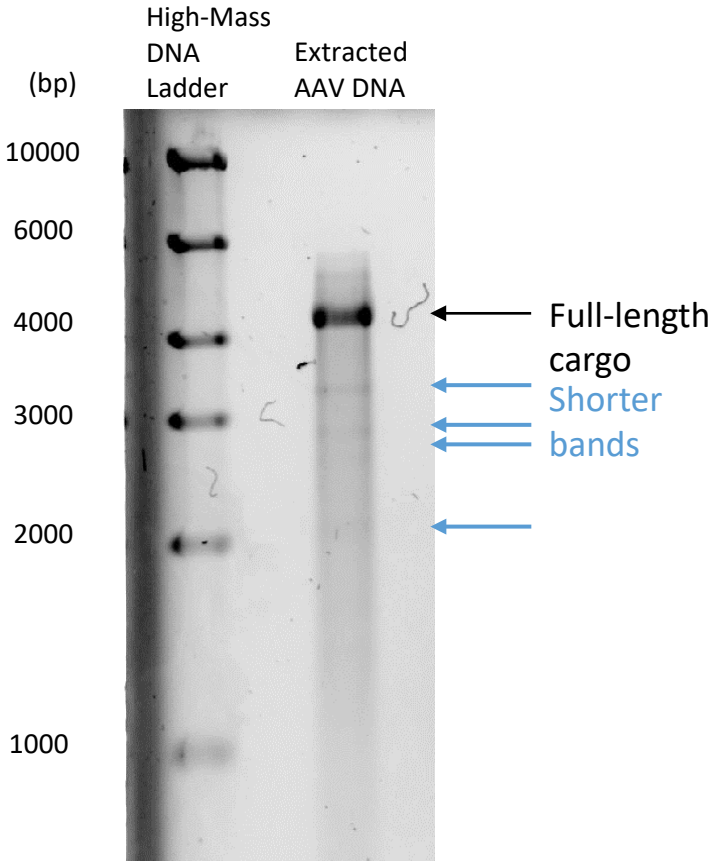
Measured on the Samux^{MP}

The Samux^{MP} can resolve partially filled AAVs

Mass photometry histogram



Extracted AAV ssDNA cargo



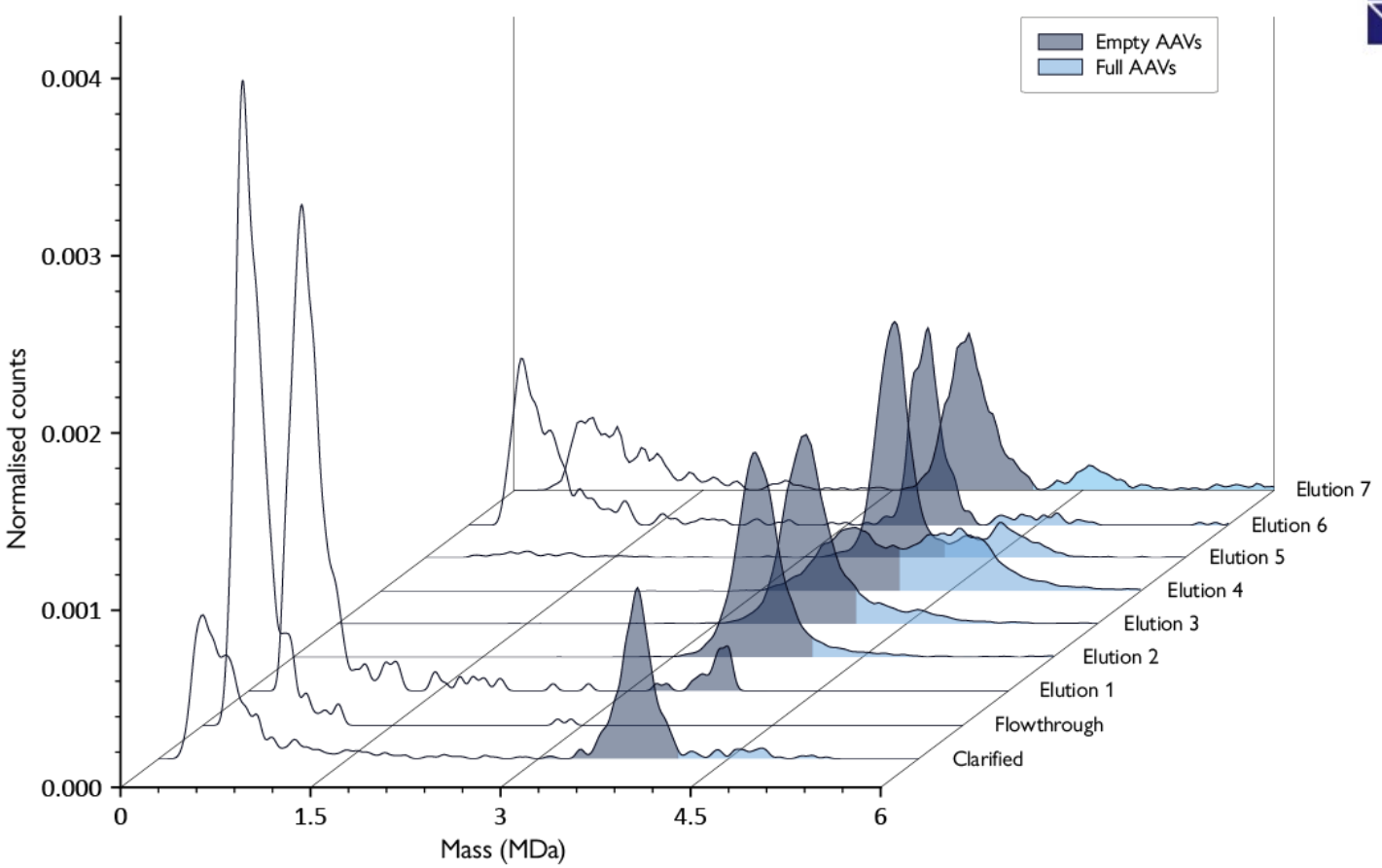
- The Samux^{MP} identified AAV9 capsids of varying mass, likely empty, full and partially filled
- Analysis of extracted AAV ssDNA showed distinct bands that were shorter than the full-length cargo
- Samux^{MP} and SEC-MALS agreed on % full capsids

Percentage of full capsids	
Mass photometry	**SEC-MALS**
34.4 %	**40.4 %**

Measured on the Samux^{MP}

Data courtesy of a biopharmaceutical company focused on innovative gene therapies

Samux^{MP} is ideal for optimizing downstream AAV purification protocols



Measured on the Samux^{MP}

康龙化成 PHARMARON

Performed at Pharmaron Gene Therapy, Liverpool, UK

AAV analytics during downstream purification

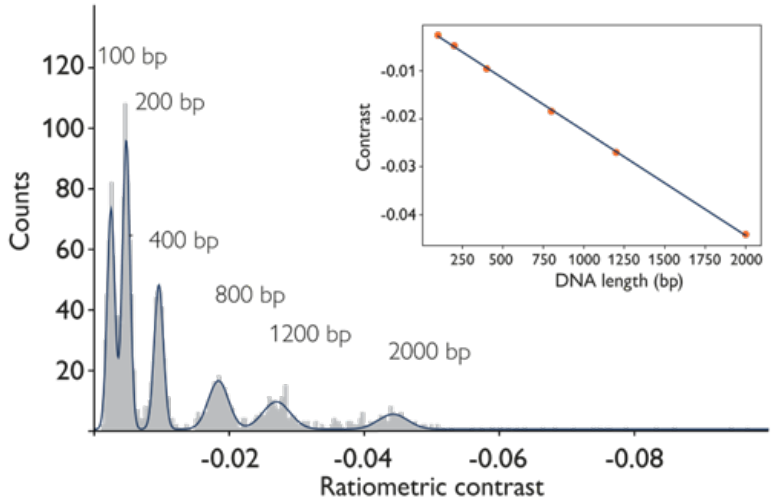
- Samux^{MP} was used to assess AAV content of sample fractions at key stages during purification
- **This test preparation is not representative of Pharmaron's platform process*
- Elution 4 = most enriched for full AAV capsids
- Samux^{MP} can help facilitate AAV purification protocol optimization due to its
  - Speed of measurement
  - Ease of use
  - Requirement for only very little sample

Mass photometry measures nucleic acids

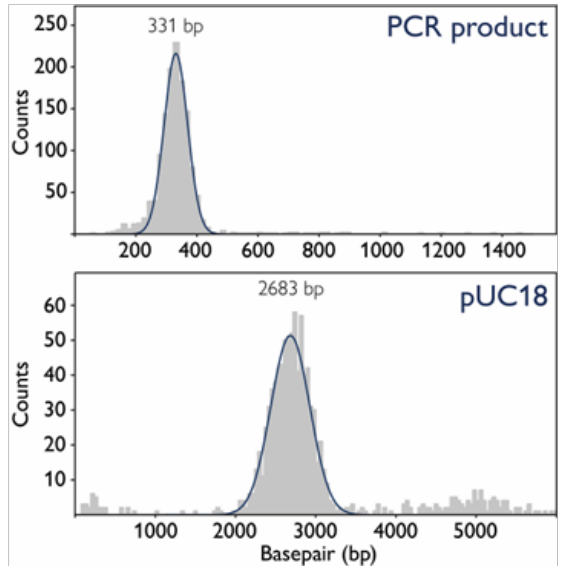
Nucleic acid sizing

- Mass photometry can be applied to nucleic acids and other biomolecules
- Same linear dependence between measured contrast & length/mass
- Here, the coverslip was coated in PLL to enable DNA binding

DNA calibration



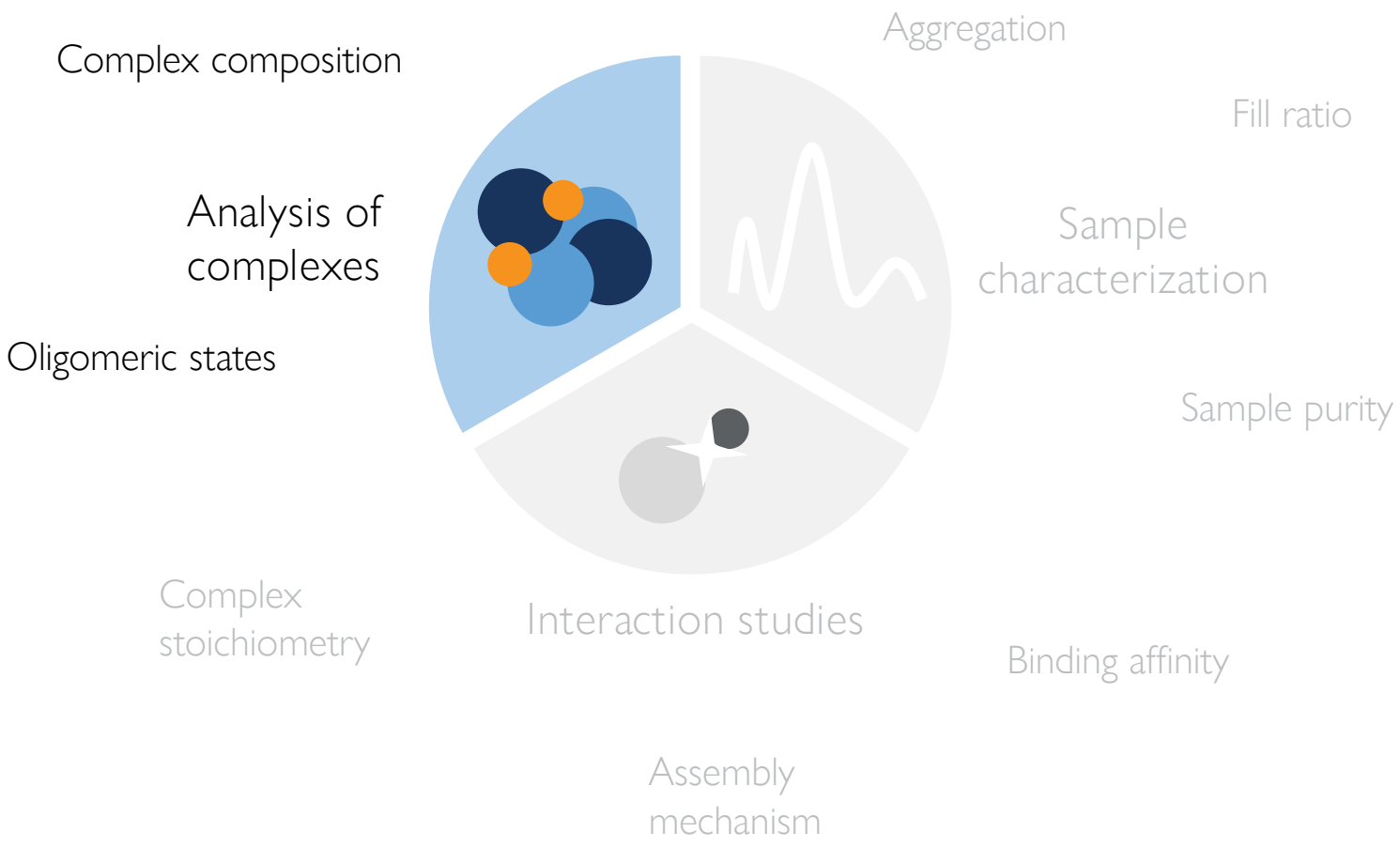
Unknown DNA lengths determined



Measured on the One^{MP}

Application Note – Mass Photometry of Nucleic Acids

Applications of mass photometry

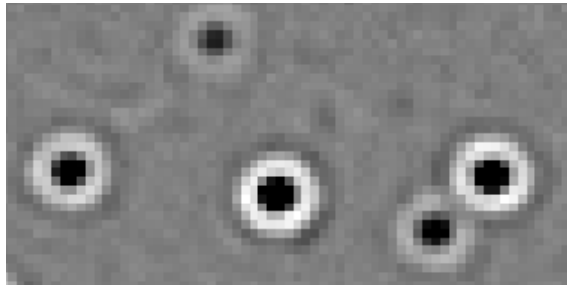


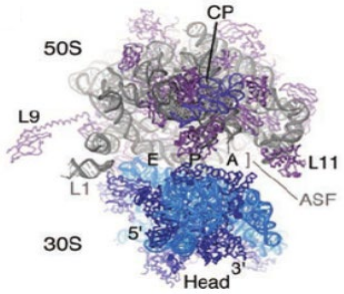
Mass photometry can measure large, complex biomolecules

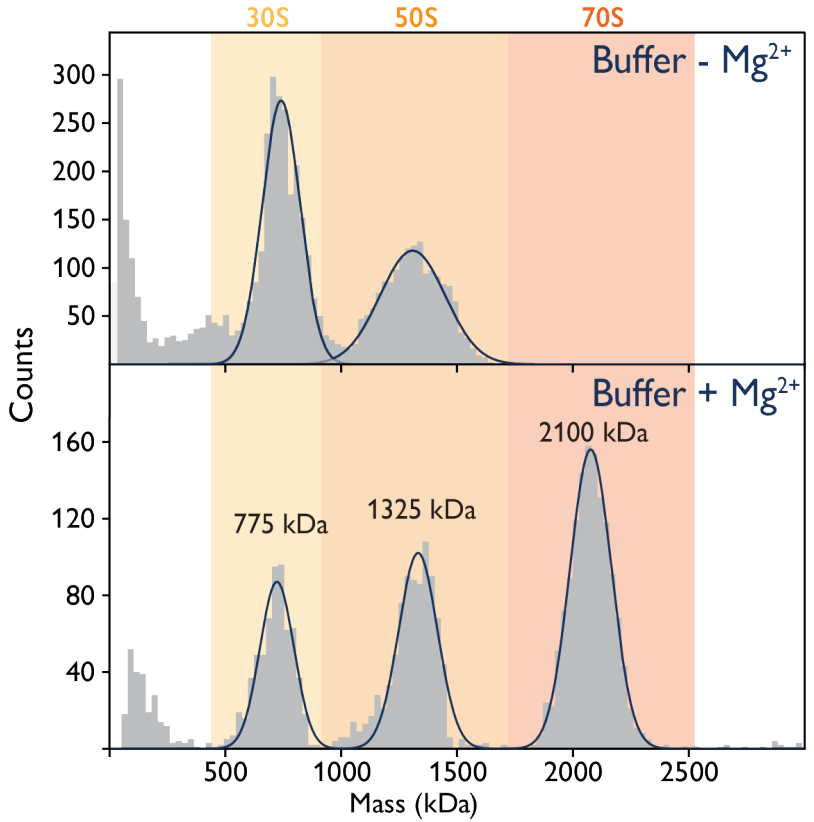
Is the ribosome complex stable?

- Bacterial (*E.coli*) ribosomes directly visualized with mass photometry
- Measured under different buffer conditions to assess complex integrity

→ Ribosome fully assembles in the presence of Mg^{2+}





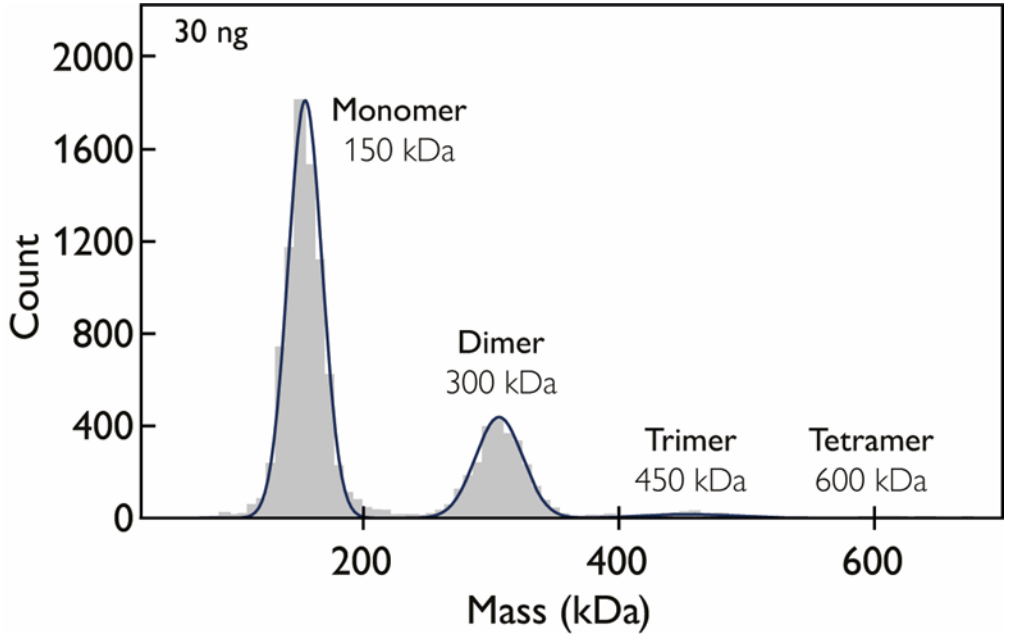


Measured on the One^{MP}

Mass photometry detects oligomer formation

Which oligomeric states does the antibody form?

- Formation of antibody oligomers identified from very little sample (20 µL @ 10 nM, 30 ng)
- Higher-order, low-abundance oligomeric states were also detected
  → Mass photometry has a broad dynamic range



Measured on the One^{MP}

Mass photometry identifies changes in oligomeric state

How does phosphorylation impact the oligomeric state of a complex?

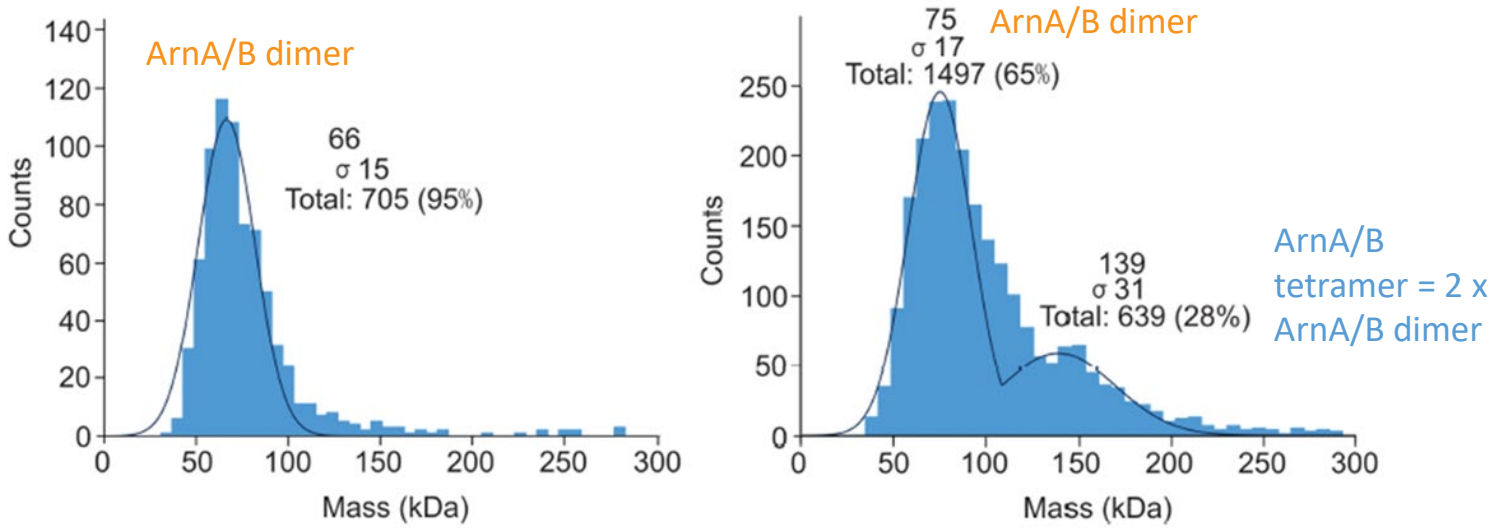
- Mass distributions of ArnA/B complex and phosphorylated ArnA/B complex were compared
- Mass photometry showed phosphorylation-dependent formation of an additional $(ArnA/B)_2$ tetrameric complex

50 nM ArnA/B complex

→ dimers

50 nM ArnA/B complex *phosphorylated*

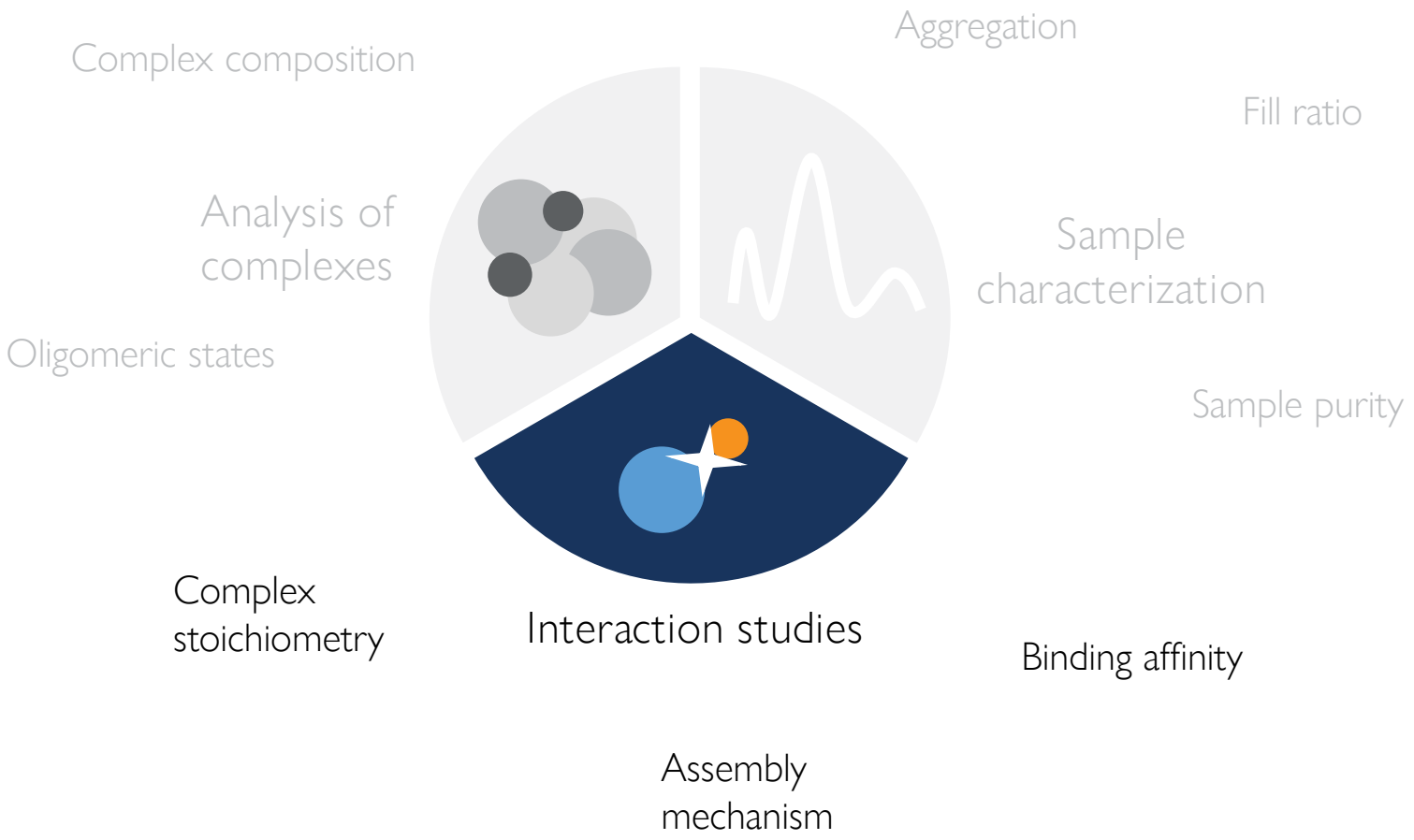
→ dimers and tetramers



Measured on the One^{MP}

Ye, X., et al., Frontiers in Microbiology, 2020

Applications of mass photometry



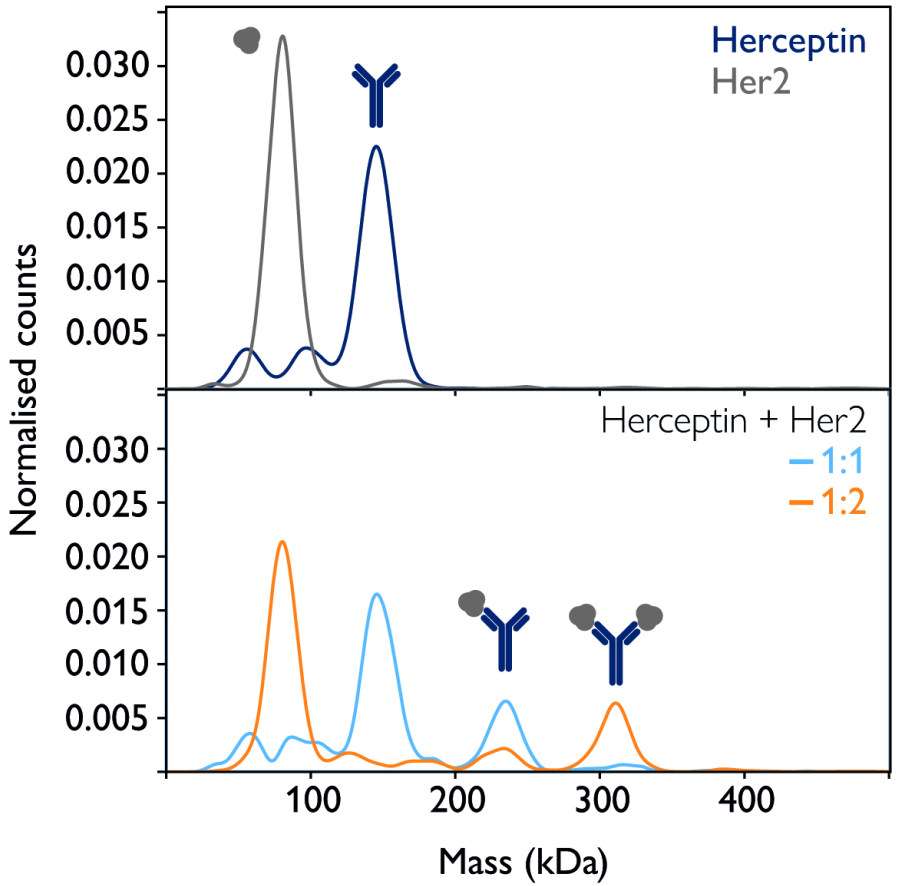
Mass photometry reveals antibody-antigen interactions

What is the complex stoichiometry of the antibody-antigen interaction?

Antibody Herceptin binds to antigen Her2

- Antibody has two binding sites for the antigen

→ Mass photometry quantifies how the occupancy of the binding sites varies depending on the relative concentrations of antibody and antigen



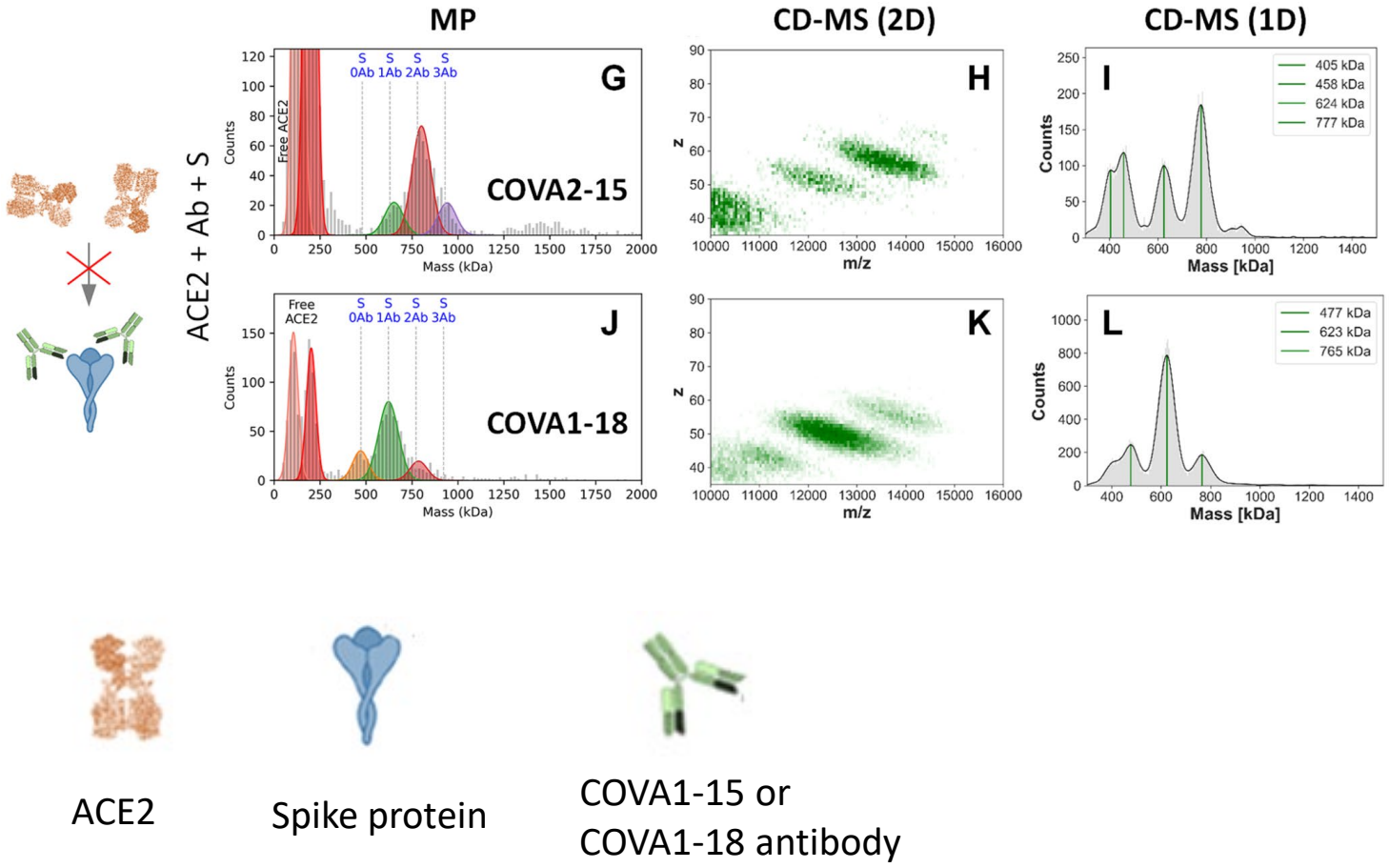
Measured on the Two^{MP}

Data courtesy of Weston Struwe, Univ. of Oxford

Mass photometry enables study of full-length antibodies

Can antibodies prevent the spike protein from binding to the receptor?

- Mass photometry rapidly assessed the binding of full-length neutralizing antibodies to SARS-CoV-2 spike protein
- None of the antibodies bound the S trimers in a full (3:1) stoichiometry, but they still prevented spike-ACE2 binding.
- Mass photometry results were comparable to charge-detection MS (on Thermo Fisher Q Exactive UHMR)
  - However, mass photometry requires much less effort & costs less than CD-MS



ACE2 | Spike protein | COVA1-15 or COVA1-18 antibody

Measured on the One^{MP}

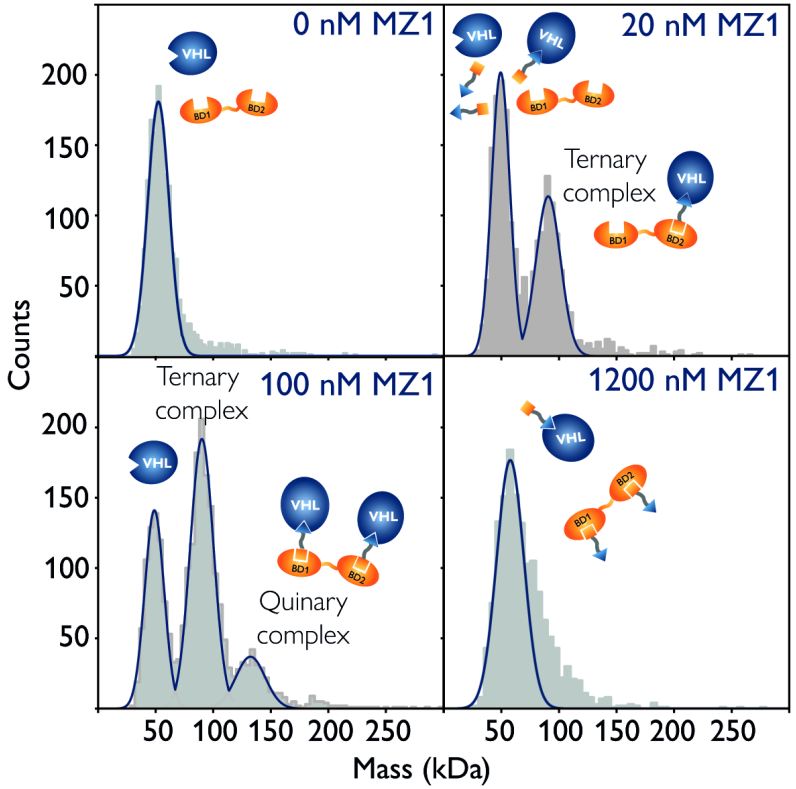
[Yin et al., ACS Centr. Sci, 2021]

Mass photometry enables PROTAC characterization

How does the ternary complex form?

- Characterization of small bifunctional molecule (PROTAC) binding
- Ternary complex formation measured as a function of MZ1 (PROTAC) concentration

1. Target protein (with binding sites BD1 & BD2) + E3 ligase (VHL)



2. PROTAC binds E3 ligase to binding site on target protein

→ ternary complex forms

3. PROTAC **binds** E3 ligases to both binding sites on target protein

→ quinary complex forms

4. Saturation of binding sites at target protein & ligase

→ inhibition of complex formation at 1200 nM MZ1

Measured on the One^{MP}

[Refeyn Application Note - PROTACs]

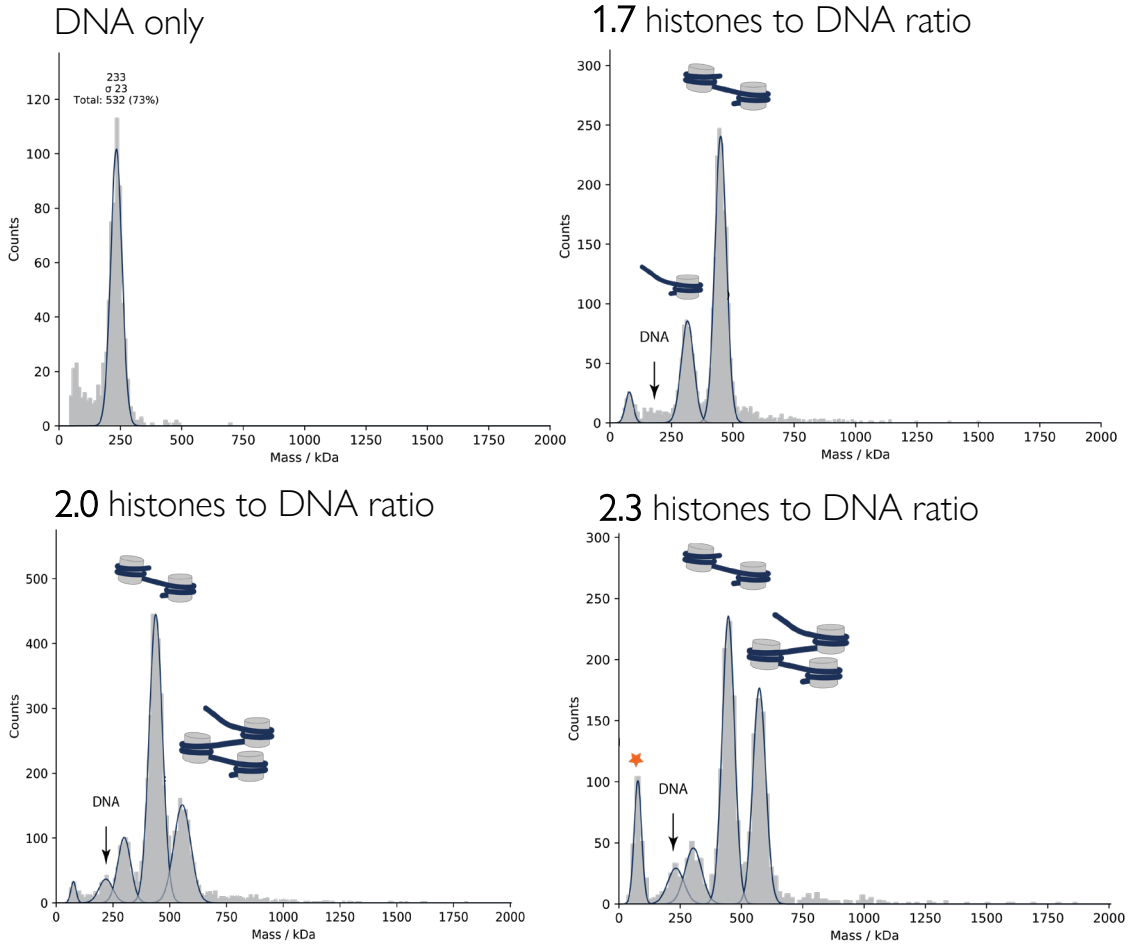
Mass photometry reveals protein-DNA interactions

How does the nucleosome assemble?

- Determine how many proteins bind to a DNA sequence
- Perform titration of increasing ratio of histone:DNA
- Small changes in protein/DNA ratio clearly observable & can also detect free histones

→ It is straightforward to assess how the DNA-protein complex forms

- Possible to study nucleosomes remodelling and chromatin organisation



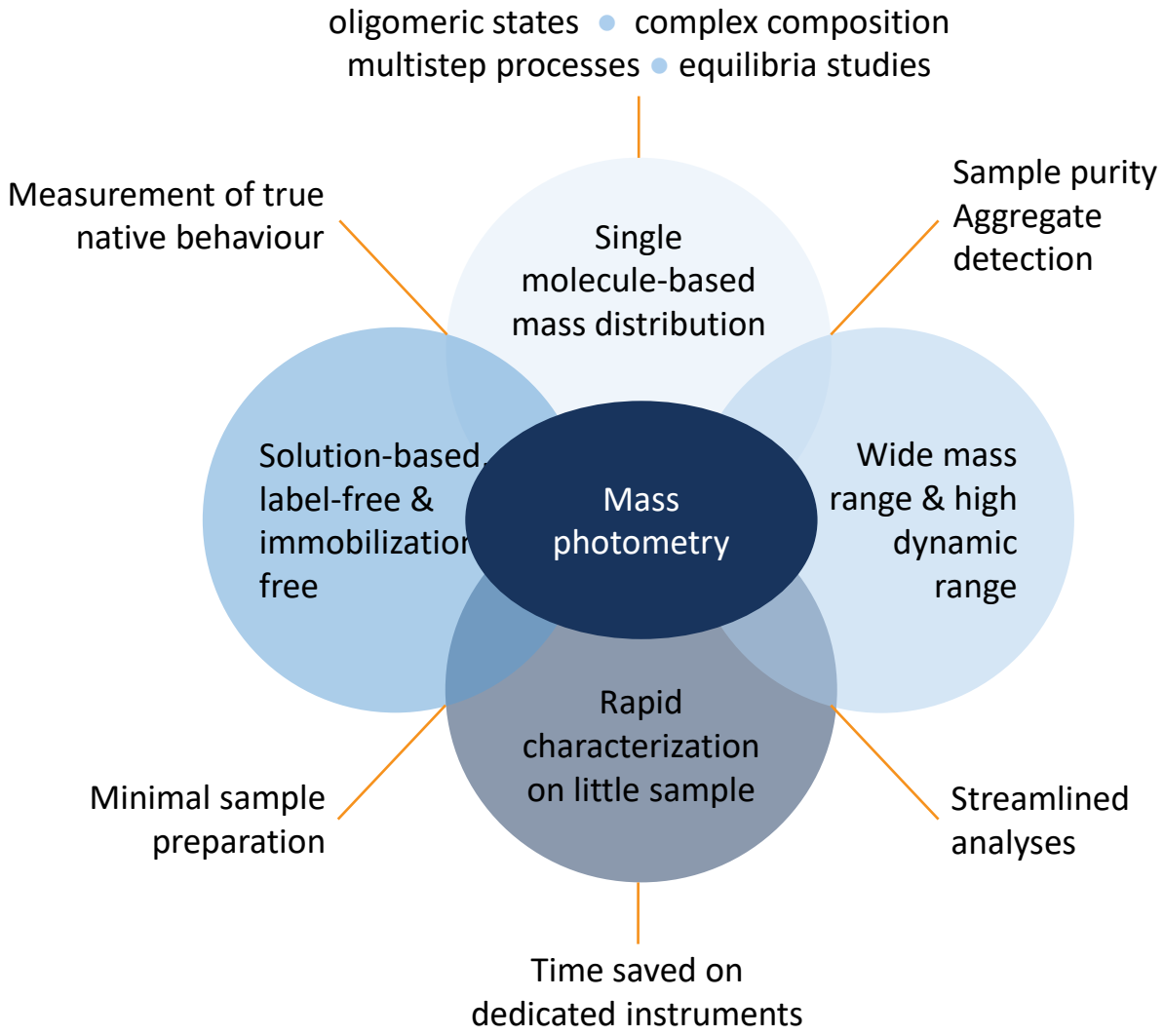
Measured on the One^{MP}

Summary

Mass photometry in biophysical characterization

Single-molecule counting over a wide mass range & high dynamic range of **label-free** biomolecules in their native state

- Applicable to many biological questions
- Quick & easy to use
- Cost effective



Mass photometry in biophysical characterization

Meet the family!

Two^MP Auto

Automated mass characterization

Samux^MP

optimized for AAV characterization

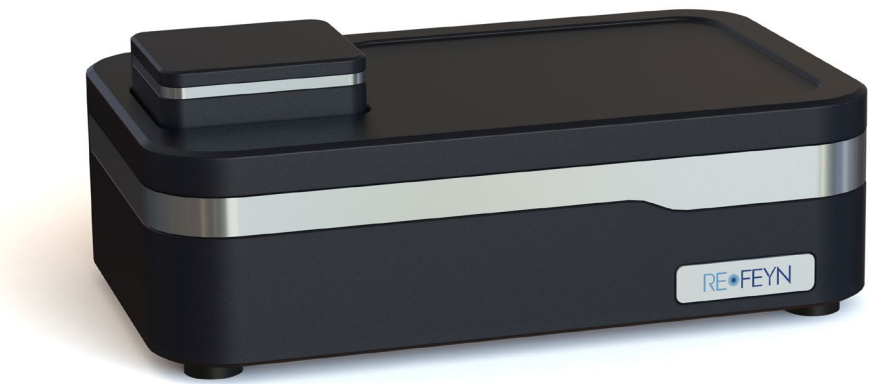
Two^MP

Second-generation mass photometer for single-molecule mass measurements

Two^{MP}

A versatile mass photometer with a wide range of biophysical applications

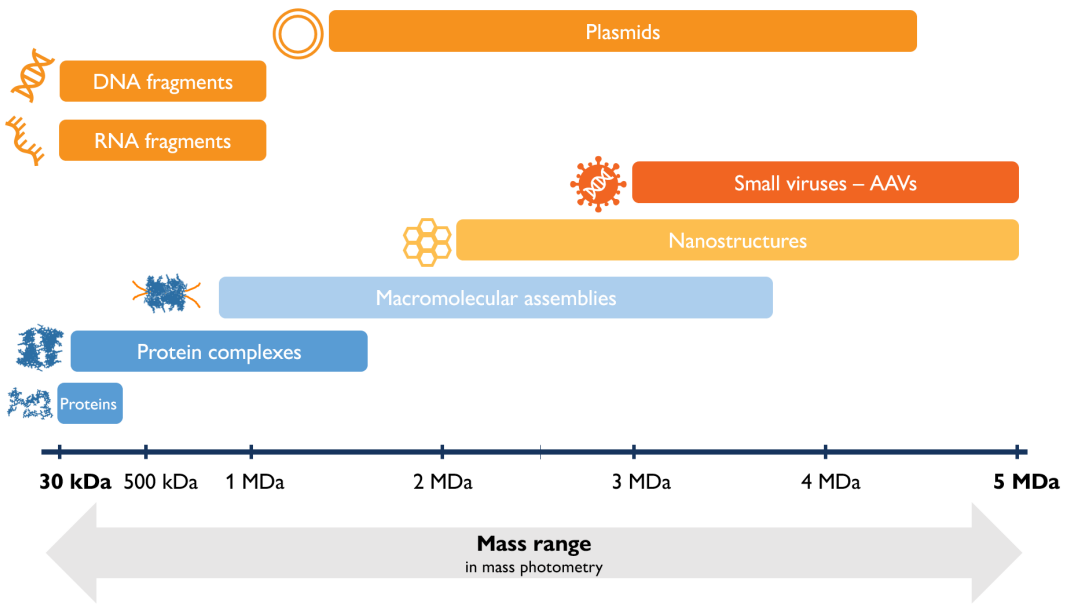
Detect **low abundance species** and verify **sample purity**
Characterise **sample heterogeneity** and determine **filled ratios**
Determine **oligomeric states** & characterise **complexes**
Monitor **complex, multistep processes** & quantify **biomolecule interactions**



with a rapid, single-molecule mass measurement of
label-free biomolecules in their native state, over a
wide mass range with minimal sample consumption

TwoMP

A versatile mass photometer with a wide range of biophysical applications



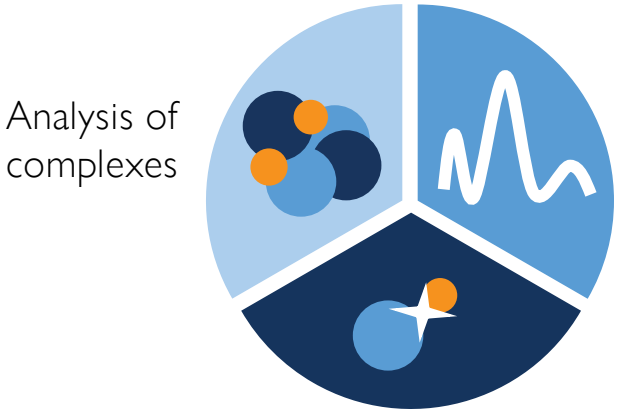
Mass range	30 kDa – 5 MDa
Concentration range	100 pM – 100 nM
Sensitivity	<< 1 ng of protein
Resolution (FWHM)	25 kDa @ 66 kDa
	60 kDa @ 660 kDa
Mass precision	± 2%
Mass error	± 5% (single measurement)

Two^{MP} Auto

Automated mass characterization of biomolecules

- Greater data confidence
- Reduced operator time
- Ideal for screening and titration assays

+ standard mass photometry applications



with rapid, autonomous measurement of multiple samples and increased data reproducibility

- Autonomous measurement of 24 samples in as little as 90 minutes
- Compatible with existing One^{MP} & Two^{MP} systems
- Same specifications as Two^{MP}*

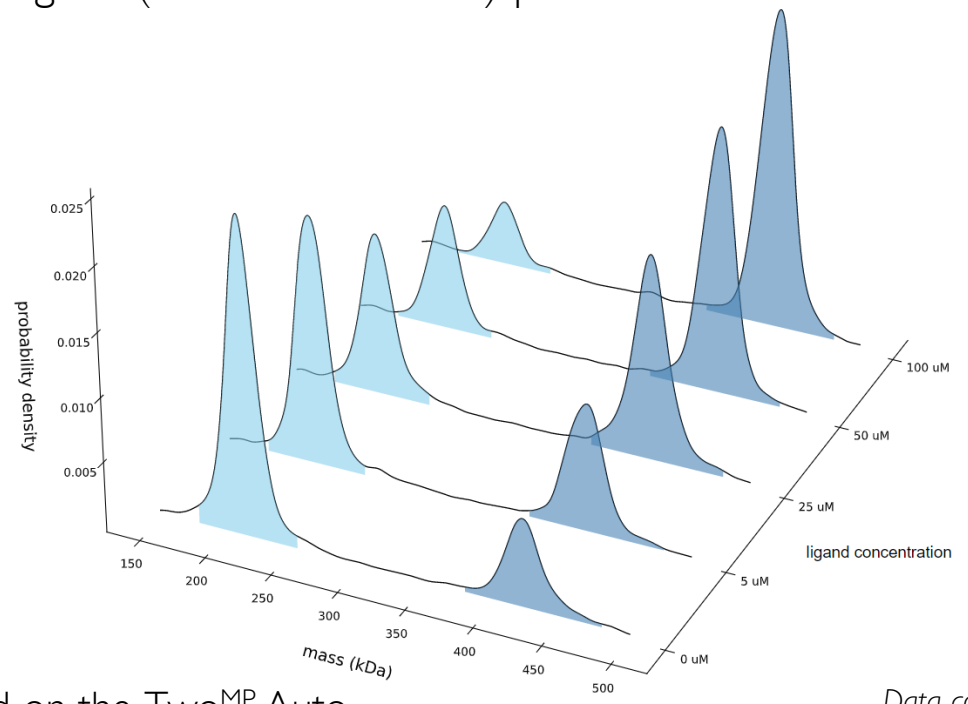
**or One^{MP} if an upgraded system*

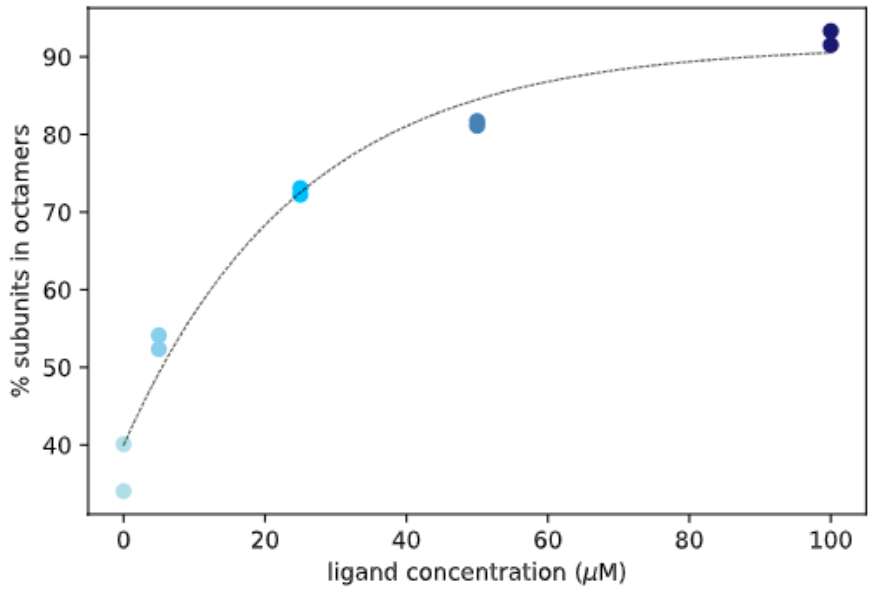
Two^{MP} Auto

Automated mass photometry – ideal for monitoring protein oligomerization

Ligand titration series performed using Two^{MP} Auto

- Protein (*undisclosed*) forms tetramers (catalytically active)
- The ligand (a small metabolite) promoted octamer formation





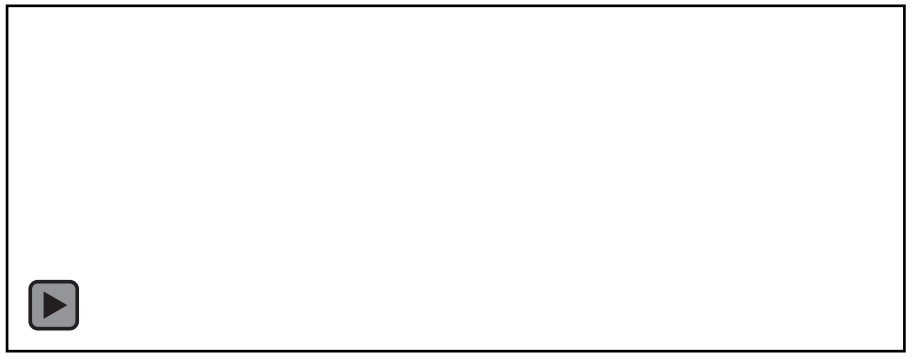
MAX PLANCK INSTITUTE FOR TERRESTRIAL MICROBIOLOGY

Measured on the Two^{MP} Auto

Data courtesy of Pietro Giammarinaro, Prof. Dr. Gert Bange and Dr. Georg Hockberg, Max Planck Institute, Marburg

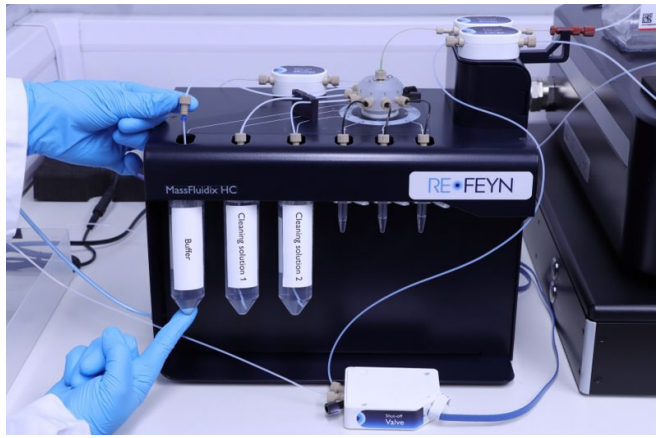
Why MassFluidix HC (High Concentration)?

- **Challenge** - Feedback from >250 customers: need to measure concentrated samples with mass photometry
  - If samples are too concentrated, the observation window get saturated



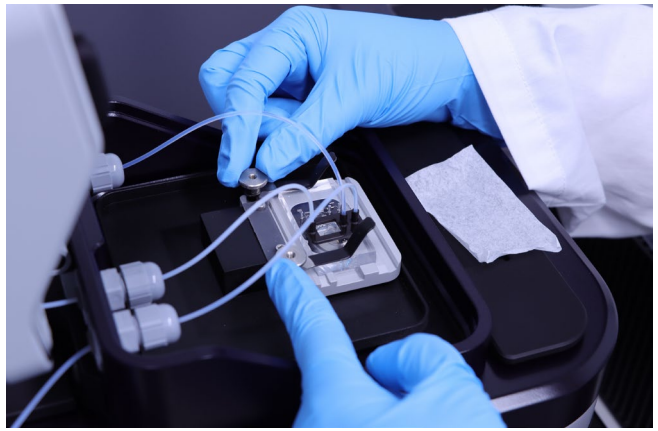
- **Solution** - Rapid dilution and continuous flow measurement
- We have developed the **MassFluidix HC** add-on to expand the sample concentration range the mass photometer can access (up to tenths of micromolar)
  - Compatible and retrofittable with One^{MP} and Two^{MP}
  - Also compatible with static measurements with easy transition between the two modes
  - Wide mass range 50 kDa – 5 MDa

Components of the MassFluidix HC system



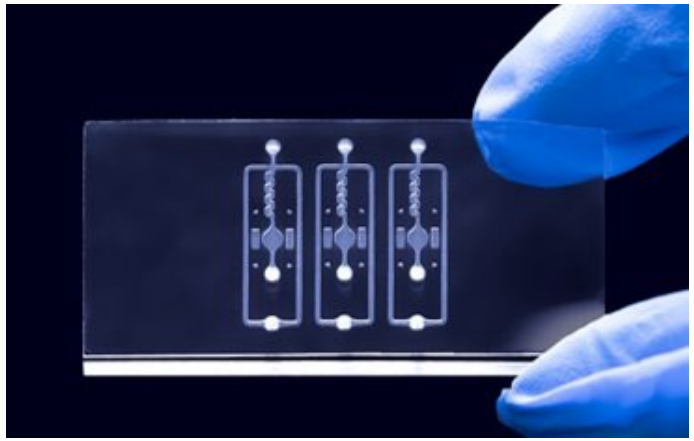
MassFluidix HC system

- 3 sample lines
- 1 buffer line
- 2 cleaning solution lines
- Connected to a main MP
- Compatible with OneMP and TwoMP



MassFluidix HC lid and stage

- Allow fluidic and static measurements
- Easy transition between both measurement modalities



MassFluidix HC chip

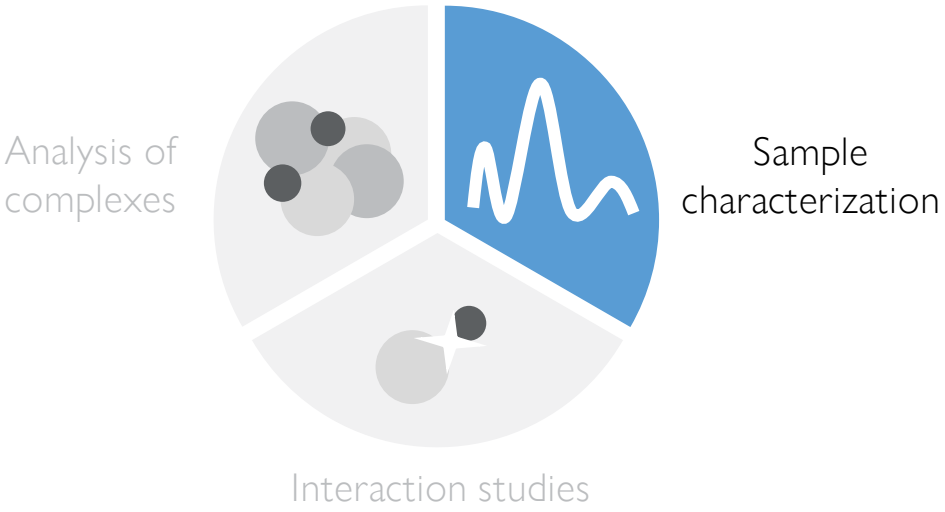
- Single-use PDMS chips
- 3 months shelf life
- 5 chips per pack
- 3 channels per chips

Included: A 1-day product training for rapid technology adoption

Samux^{MP}

A mass photometer optimized for AAV characterization

- Easily and accurately assess empty-full AAV ratios
- Quantify partially filled & overfilled particles

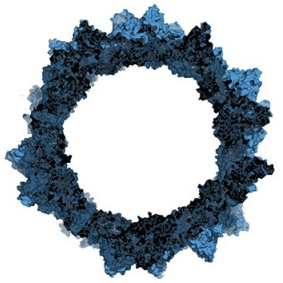


with rapid, single particle, serotype-agnostic
mass measurement of capsids in their native state, using
minimal sample consumption and with low operational costs

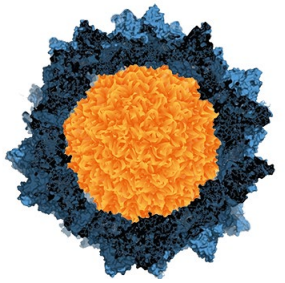
Samux^{MP}

A mass photometer optimized for AAV characterization

Mass range	500 kDa – 6 MDa
Optimal concentration	10^{11} particles/mL
Sample volume	10-20 µL
Resolution (FWHM)	235 kDa @ 3700 kDa
Measurement time	< 5 minutes
Laser wavelength	488 nm



Empty AAV particle



Full AAV particle

Refeyn offering for GMP-regulated environments

Using the Samux^{MP} (Auto) for AAV analytics in GMP-regulated environments

21 CFR 11 compliant software

For easy data integrity and traceability

User authentication

Access control

Audit trails

Electronic signatures at export

System qualification and support

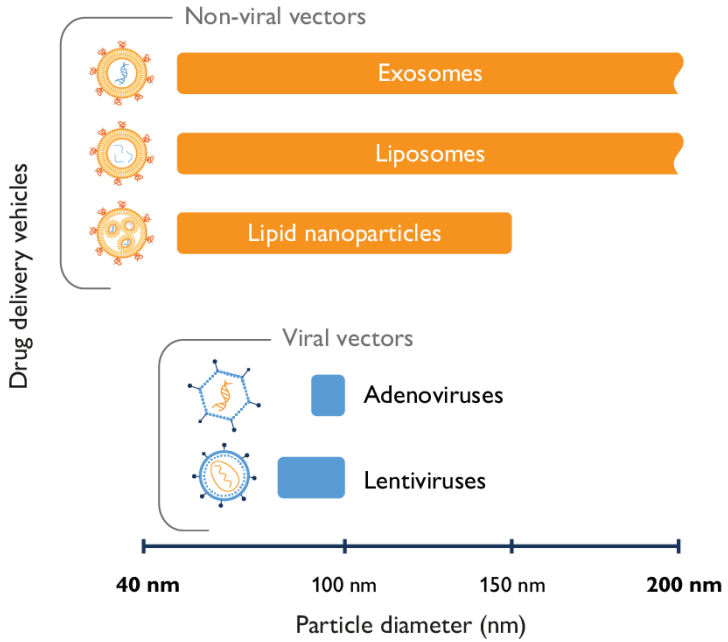
For an easy systems validation

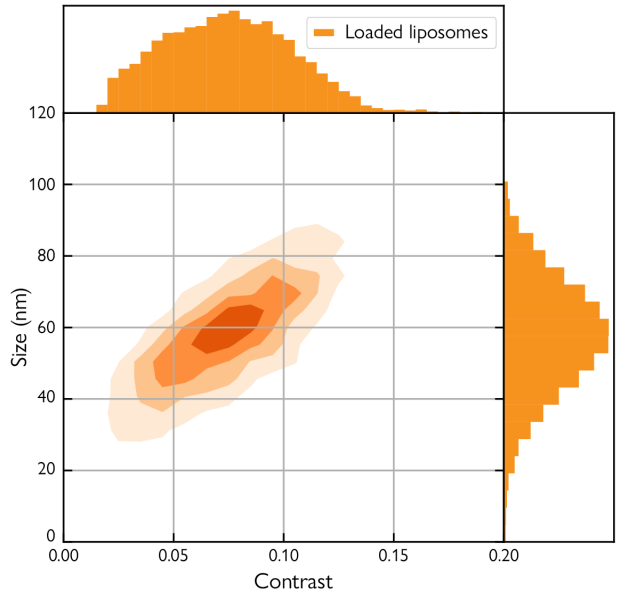
Comprehensive training and documentation

IQ/OQ

Summary

The Karitro^{MP} offers new analytical capabilities





New Product!

- Unique characterization of vectors: Size-contrast correlation
- Critical insights: Population differentiation, payload distribution, sample purity and more
- Convenient and efficient analysis: User-friendly instrument needing little sample, minimal sample prep

The Karitro^{MP} offers new analytical capabilities

- Unique characterization of vectors
  - Applies to LNPs, liposomes, exosomes and large viral vectors
  - Correlates particle size and mass
- Critical insights to guide R&D
  - Population differentiation based on size and density
  - Insights into payload distribution and surface functionalization
  - Assessment of sample purity and stability
- Convenient and efficient analysis
  - User-friendly benchtop instrument
  - Simple sample prep, little sample needed
  - Label free, in solution

Liposome observations reveal unexpected population

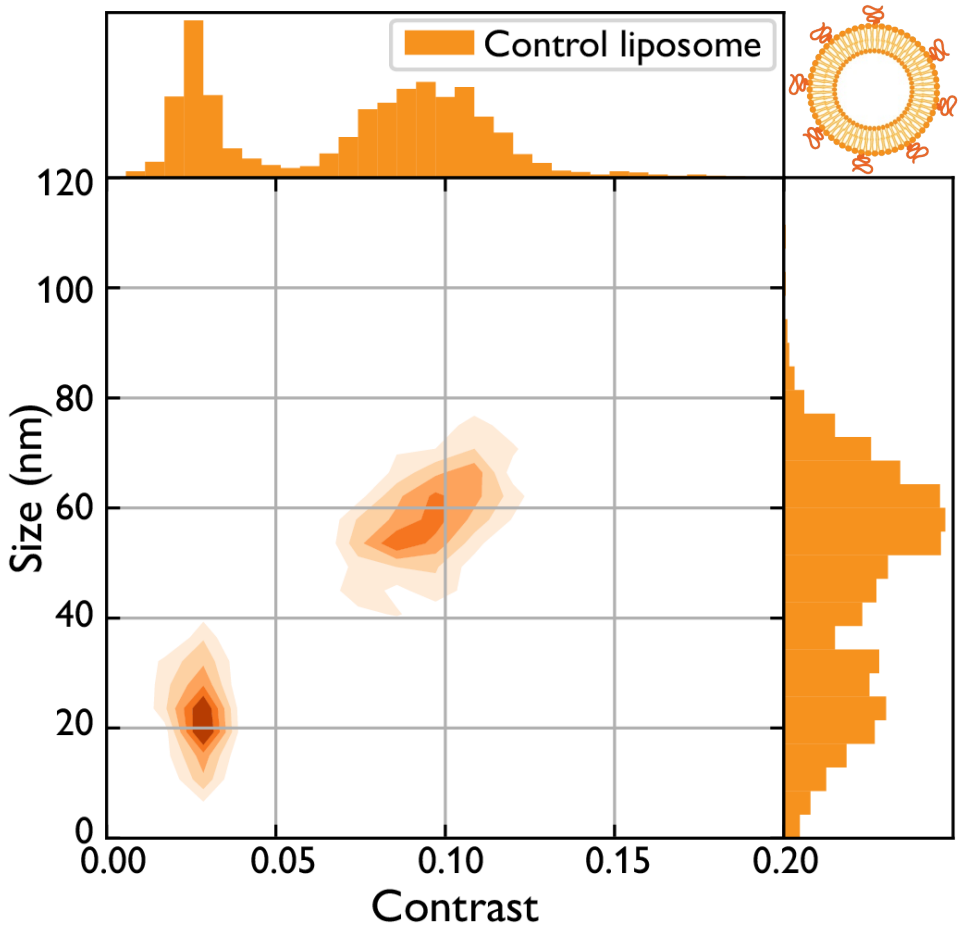
Expected:

- One population of empty liposomes
- Control sample with same lipid formulation as doxorubicin-loaded (same supplier)

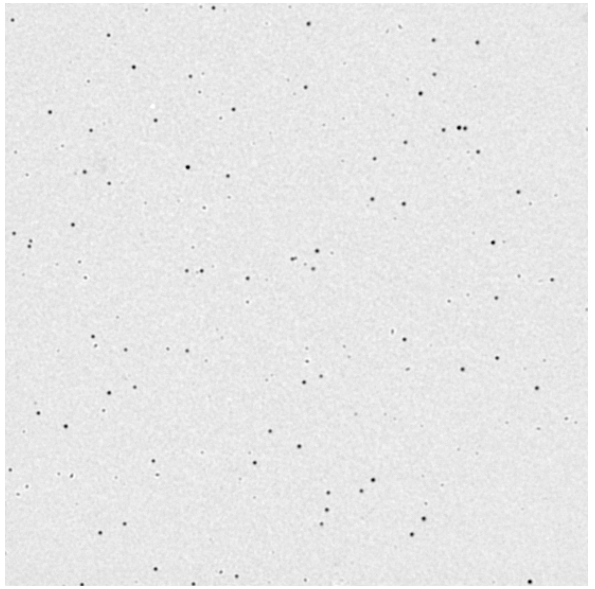
Observed:

- Two distinct populations
- Broad size distributions with two overlapping size peaks, shoulder under main peak
- Two narrow contrast distributions

A formulation issue?



Measured average size: 63.8 nm
CoA size (DLS): 96.8



Measured on the Karitro[MP]

Introducing a dedicated consumables solution for mass photometry!

Hassle-free measurements, with reduced sample preparation time & greater data confidence

Mass Photometry Starter Kits

- Ready-to-use sample carrier slides
- Sample well cassettes
- Alignment assist package:
  alignment tool, tweezers, magnetic slide holders

Sample Preparation Packages

- Ready-to-use sample carrier slides
- Sample well cassettes

Sample Carrier Slide-only Package

- Ready-to-use sample carrier slides

Summary

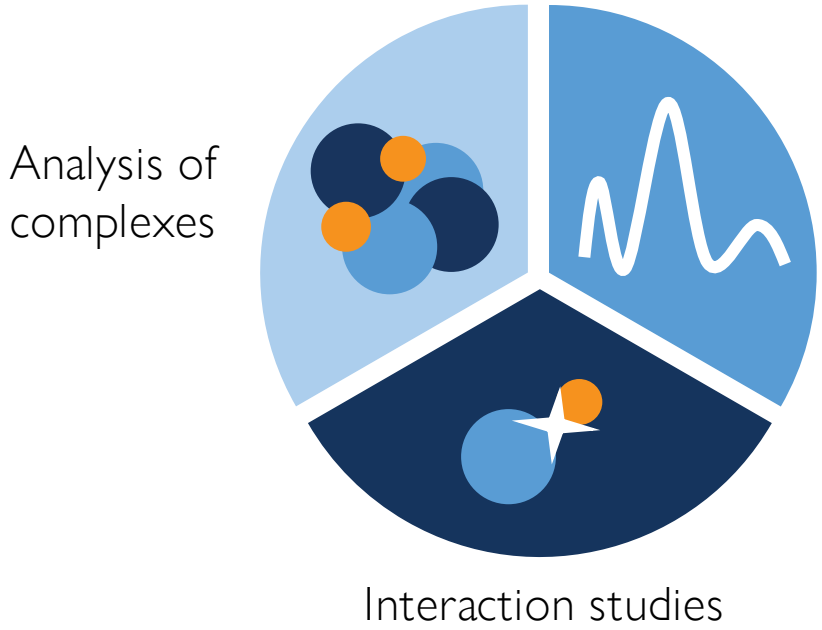
Mass photometry in biophysical characterization

Single-molecule counting over a wide mass range & high dynamic range of label-free biomolecules in their native state

In just four years, mass photometry is already making a big impact on furthering understanding of biomolecular behavior

- Over 400 mass photometers installed within academia and industry worldwide
- More than 150 peer-reviewed papers published featuring mass photometry to date – and counting!

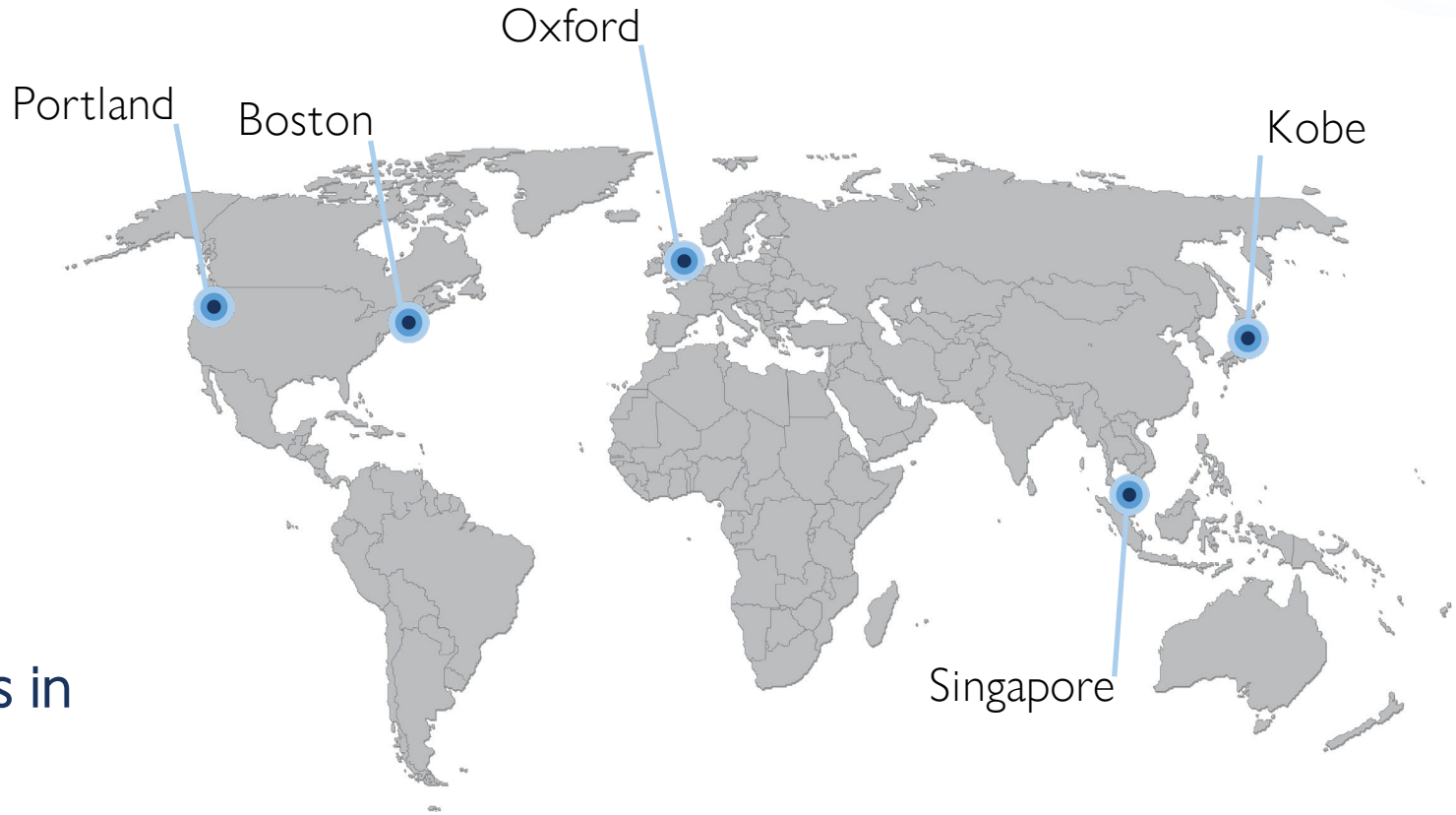
How will mass photometry advance *your* research?



Learn more about mass photometry

https://www.refeyn.com/ — Resources
- Featured publications
- Application notes
- Webinars
- Blogs

Refeyn's YouTube channel — Playlists
- Mass photometry basics
- Webinars
- Tutorials



Our mission

To transform analytical workflows in the life sciences, biopharma and beyond, using the power of light.

REFEYN

WEIGHING MOLECULES WITH LIGHT

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