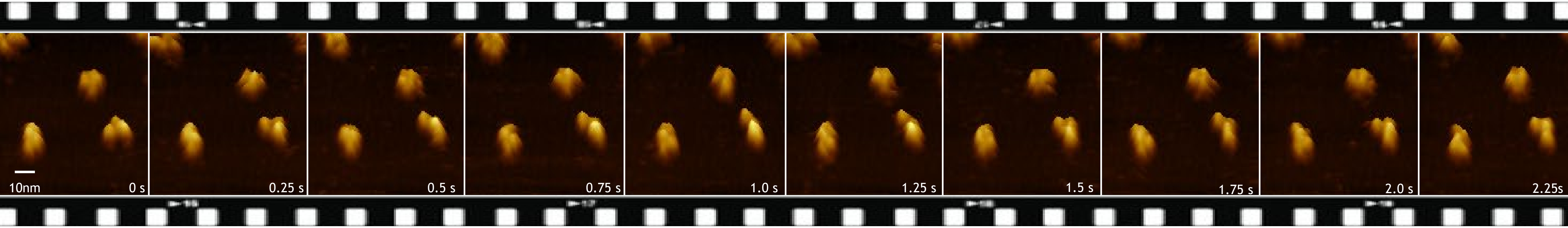
HS-AFM for Measuring Structure and Dynamics of Antibodies at the Single Molecule Level: Potential Implications on Protein Solution Stability

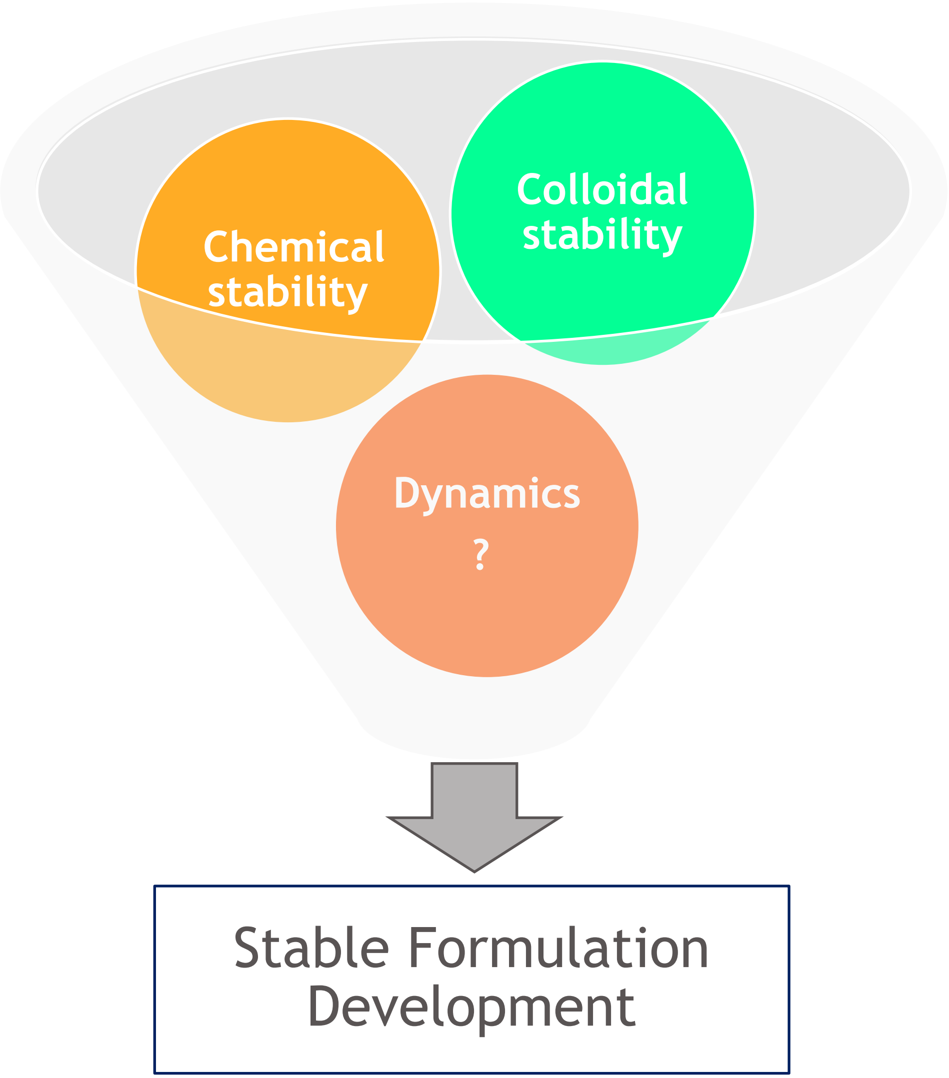
CASSS HOS 2023

Marilia Barros



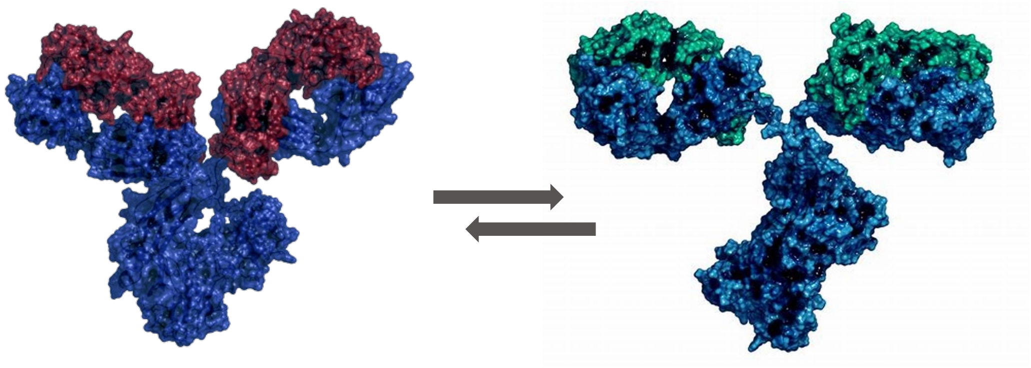
Bristol Myers Squibb™

Motivation/Opportunity Statement



Stable Formulation Development

Successful formulation of protein therapeutics requires a thorough understanding of proteins physico-chemical properties including their individual dynamic behavior



Antibodies are inherently dynamic molecules

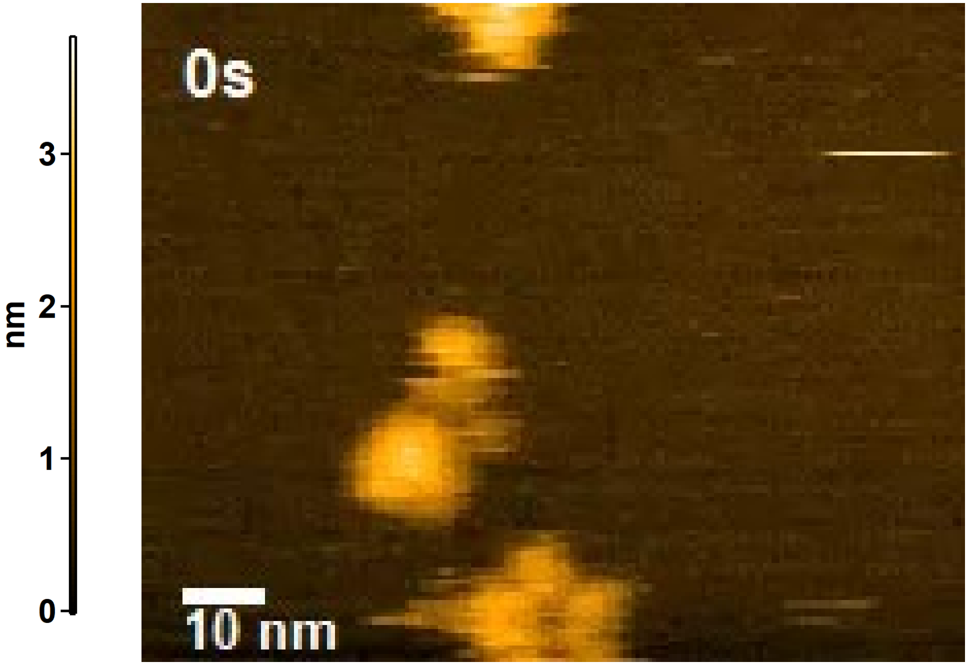
Our understanding of the complex inter-relationship between antibody intrinsic dynamics and stability is incomplete but undeniably necessary to the development of stable formulation

High-Speed AFM to bridge the Gap Between Antibody Structural Dynamics and Protein Solution Stability

Advance our understanding of the relationship between the nanoscopic observation of molecular behavior and the macroscopic stability of biopharmaceutical formulations.

Use High-Speed Atomic Force Microscopy to:

- Characterize the structural and mechanical properties of antibodies, particularly their complex conformational dynamics and flexibility
- Determine whether these properties correlate with real time and long-term stability.



Specific Goals & Experimental Plan: Feasibility Stage

I. **Investigate the effects of solution and environmental conditions on molecular flexibility (e.g. range of motions between domains), shape (e.g. compact vs. extended), molecular arrangement and molecular packing of mAbs (e.g. IgG1 vs IgG4).**

- Explore correlations between antibody dynamics and formulation stability
  - Determine if observations extend to behavior at high concentration.

II. Application to co-formulated antibodies: Investigate whether characteristic dynamics of one antibody are retained or affected in the presence of a different antibody.

More generally, the proposed work would provide opportunities to

- Expand the capabilities of our biophysical toolbox
- Gain invaluable knowledge and experience within the rapidly growing arena of bio-molecular imaging
- Generate opportunities to explore synergies between other imaging technologies (liquid-EM, Cryo-EM), computational approaches (molecular dynamics simulations) and biophysical tools

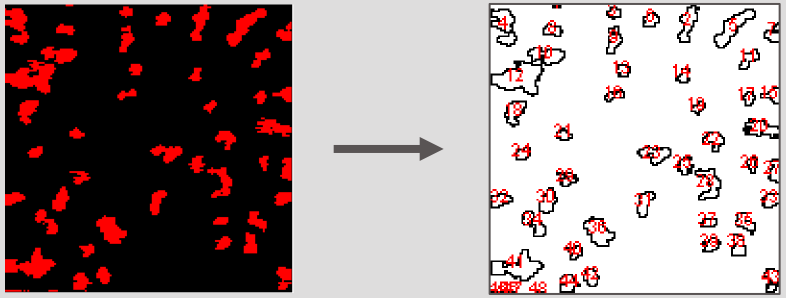
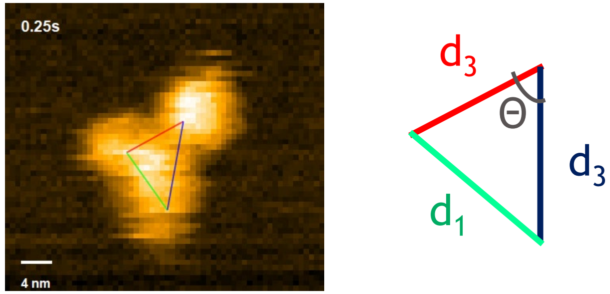
Bridging the Gap Between Antibody Structural Dynamics and Protein Solution Stability

- **Investigate the effects of solution and environmental conditions on molecular flexibility (dynamics of our antibodies)**

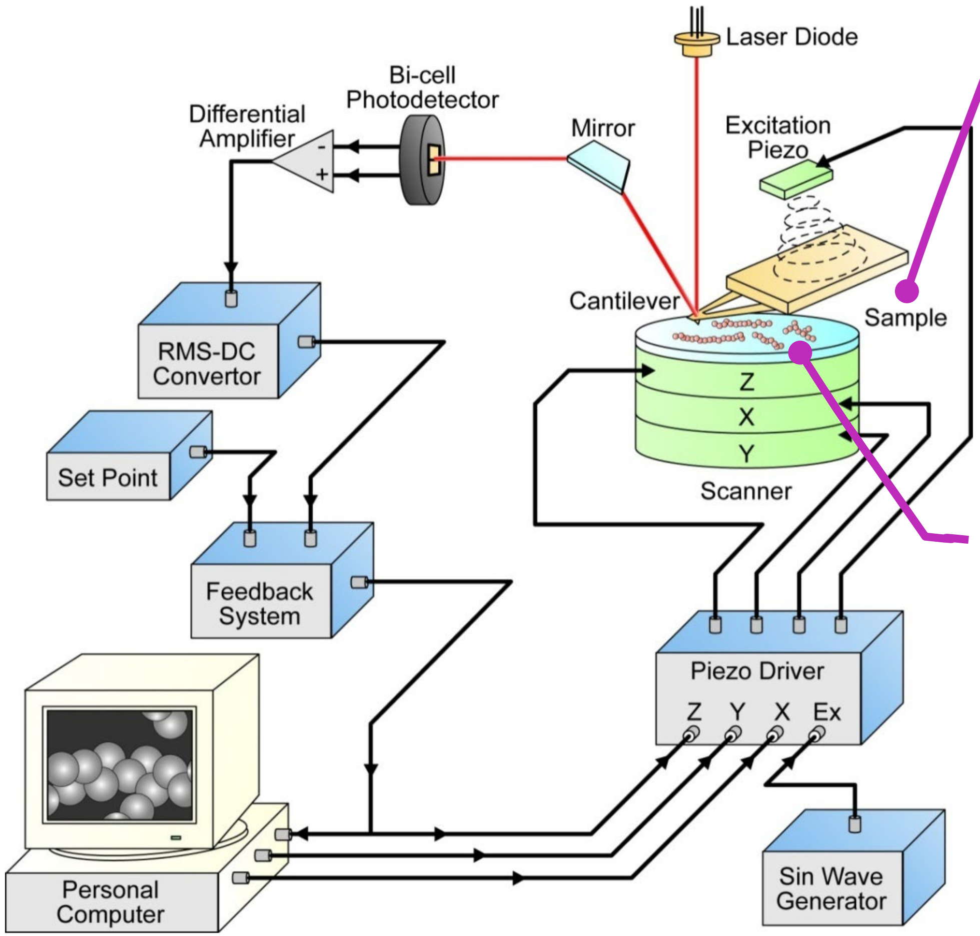
- **Explore correlation between antibody dynamics (flexibility and intrinsic motion) and formulation stability**

mAb	IgG Subclass	**Solution Property**
mAb-4A	IgG4	Viscous at High Conc, No self-association
mAb-4B	IgG4	Not Viscous, self-association
mAb-1A	IgG1	Low level of hinge clipping
mAb-1B	IgG1	High level of hinge clipping/Adsorption
mAb-1C	IgG1	High level of hinge clipping/Aggregation

List of Molecular Descriptors

Molecular Descriptors	Information	Tool
Area	Aggregation state (monomer/dimer/trimer)	ImageJ Particle Analysis / Batch Processing 
Volume	Aggregation state	
Shape	Compact vs extended	
Dwelling time	Preferential conformational state	IgorPro Single molecule Tracking 
Angle between mAb domains	Flexibility	
Distance between mAb domains	Compact vs extended	
Aspect Ratio Distribution	Flexibility	

HS-AFM Key Elements & Experimental Conditions



<5ul

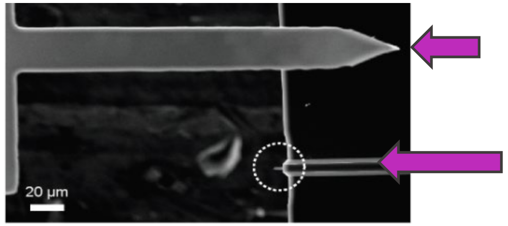
conc. 1 to 10 ug/ml

250 µl buffer reservoir

Mica: Bare surface or treated with APTES

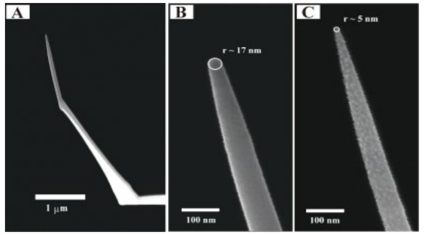
Simultaneously Reveal in situ Structure shape and Dynamic behavior of unlabeled and individual biomolecules in well controlled liquid environment

Ultra Small Cantilever for High Speed and Soft Tapping

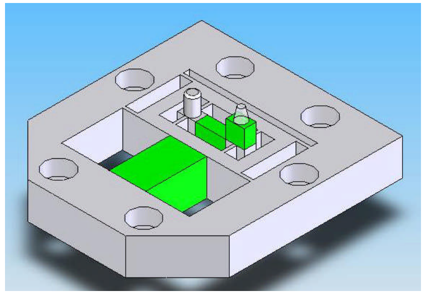


Conventional AFM cantilever

HS-AFM cantilever

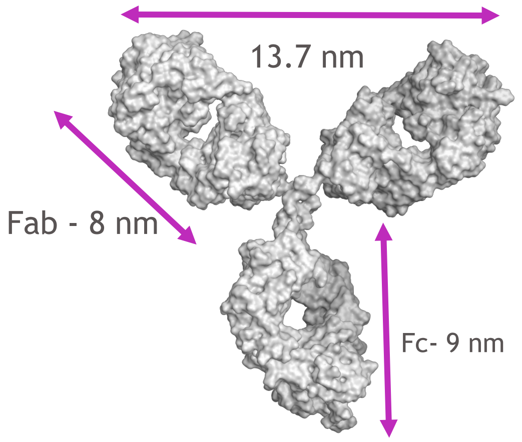


Ultra Sharp tip for High Resolution

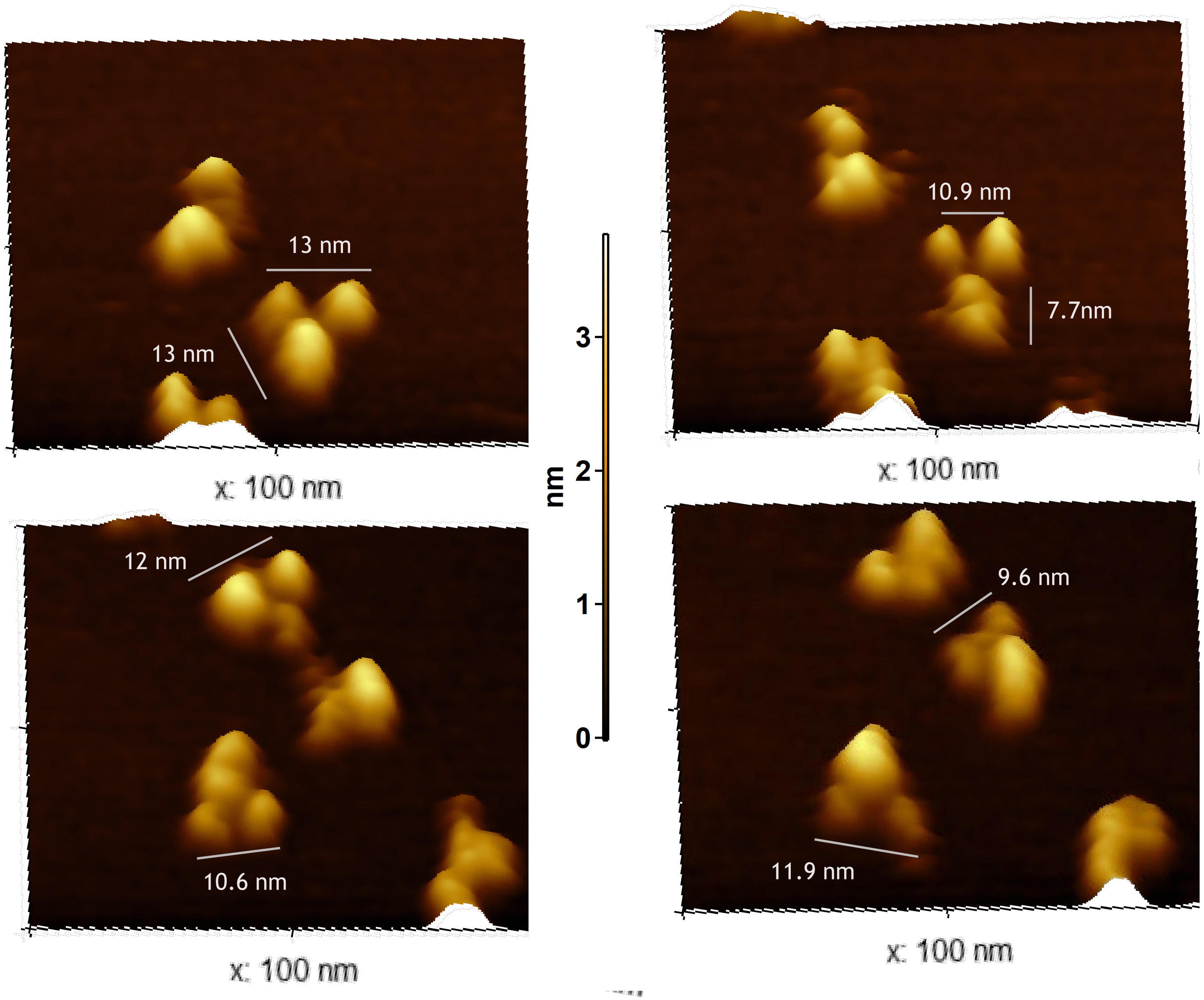


High-speed AFM Scanner

Antibody monomer Structure Morphology & Dimensions



mAb Homology Model



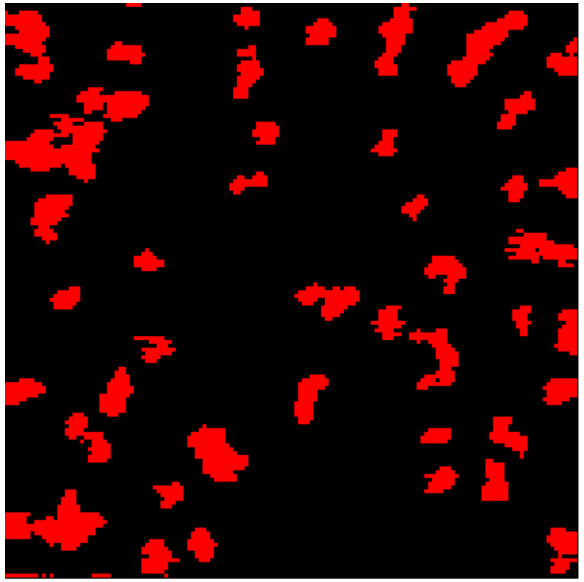
Real-time *in situ* measurements of protein dynamics in solution

High Spatial & Temporal Resolution

- 1 – 2 nm in horizontal (x,y) and 0.1 – 0.2 nm in (z) resolutions
- ms - µs dynamics

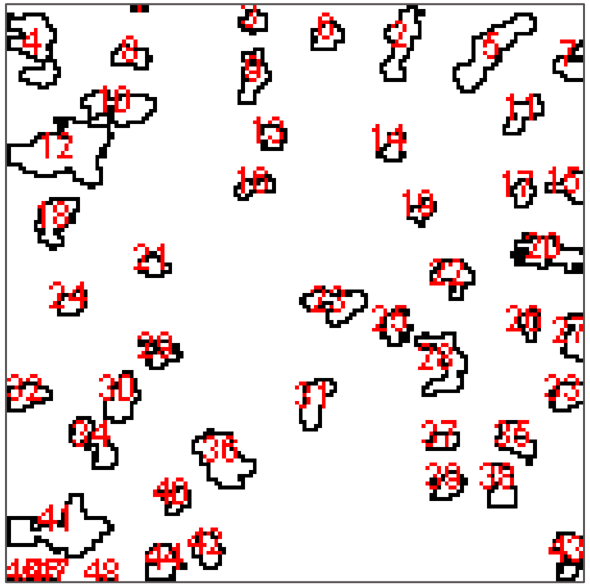
HS-AFM Images Analysis with ImageJ

Batch Processing



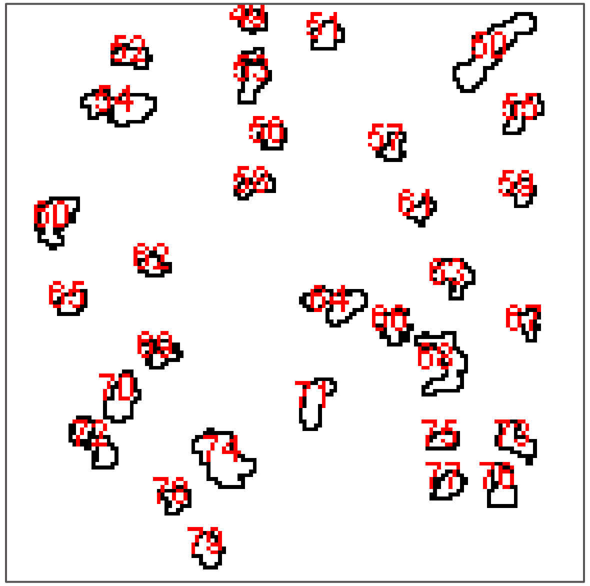
Masking

Selects particles based on
their height



Particle analysis

Area, Volume
Aspect Ratio (Shape)



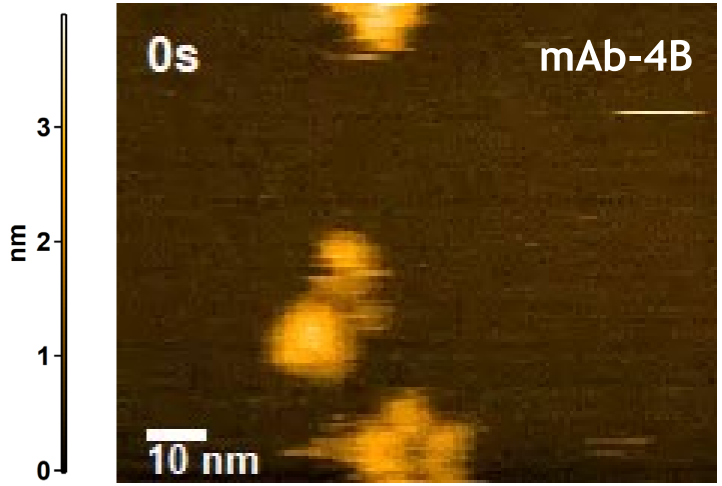
Excluded edges

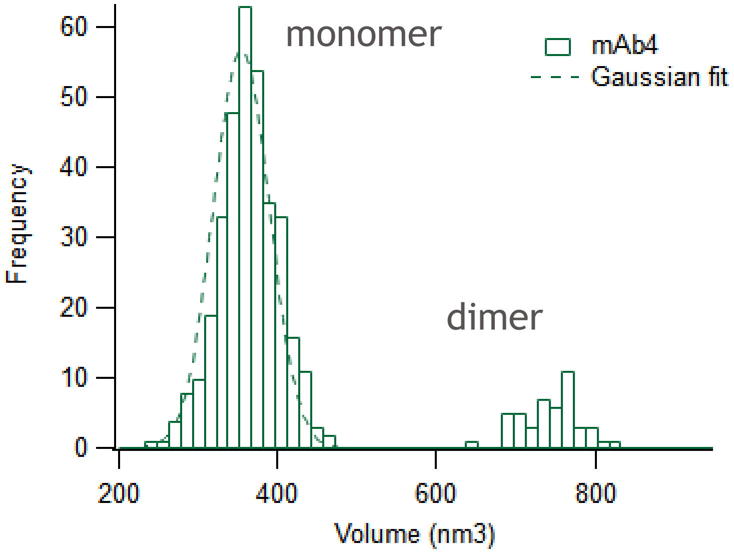
Avoid selection/analysis of
partial particles

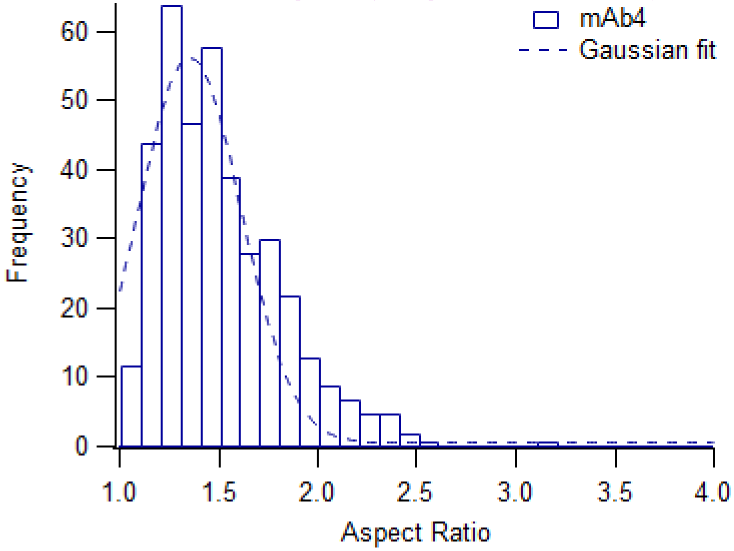
IgG4 Particle Distribution Analysis Comparison

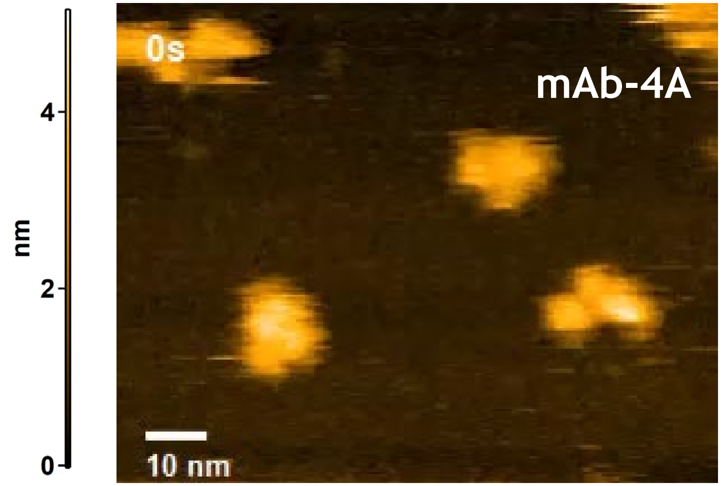
Volumetric Analysis

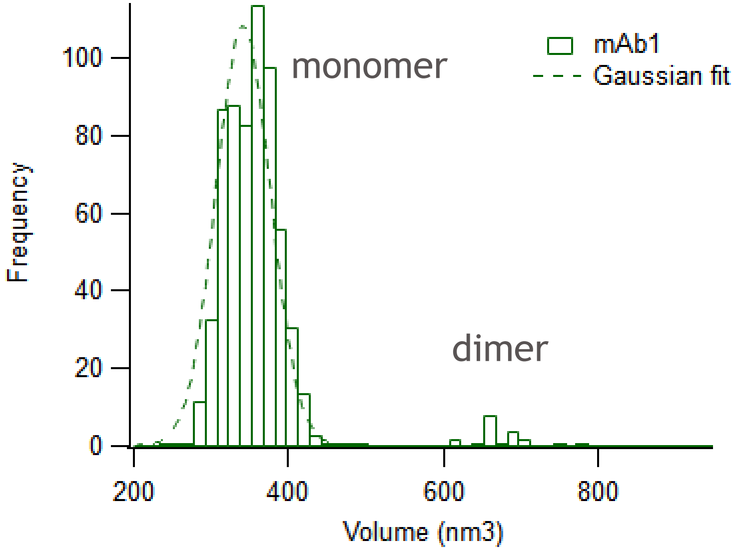
Shape (Aspect Ratio)

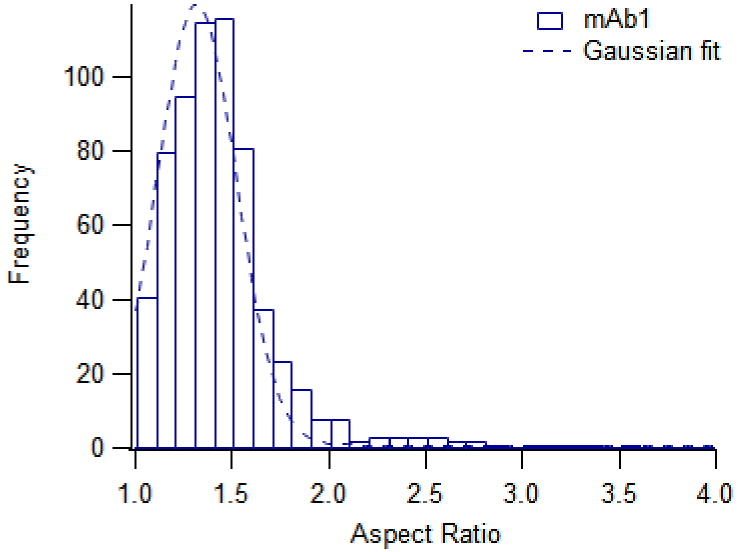






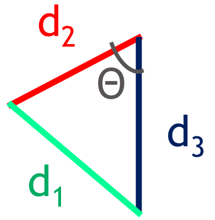


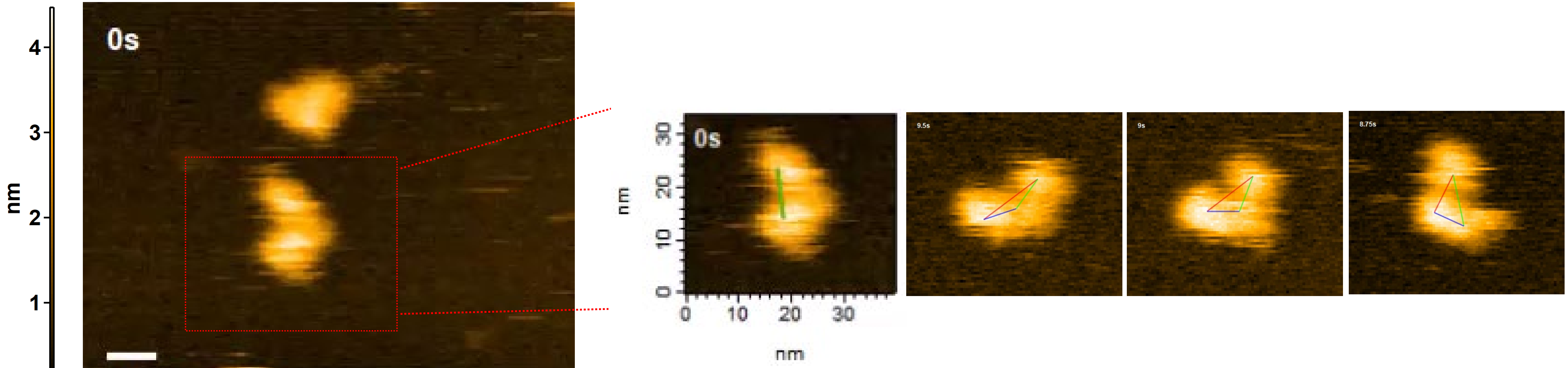




Shape distribution suggests mAb-4B assumes a more elongated (prolate) form in comparison to mAb-4A

HS-AFM: Tracking Real-Time Dynamics of Individual Molecules

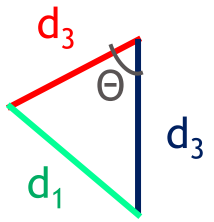




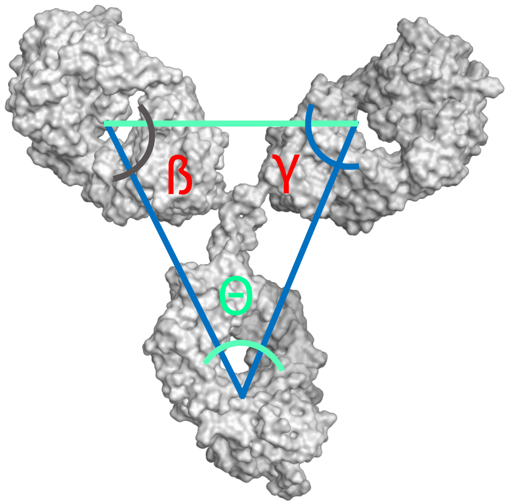
Automatically track 3 maximum points from which we can determine angle & distance between the axis

Current Approach Drawbacks: Throughput for analysis is low and does not allow for advanced image analysis and extracting (classification, categorization)

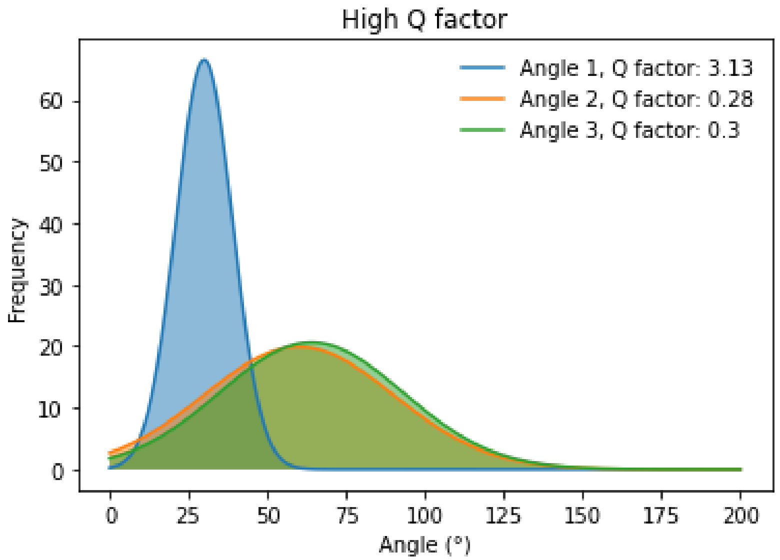
Flexibility Model for Antibodies Approach #1

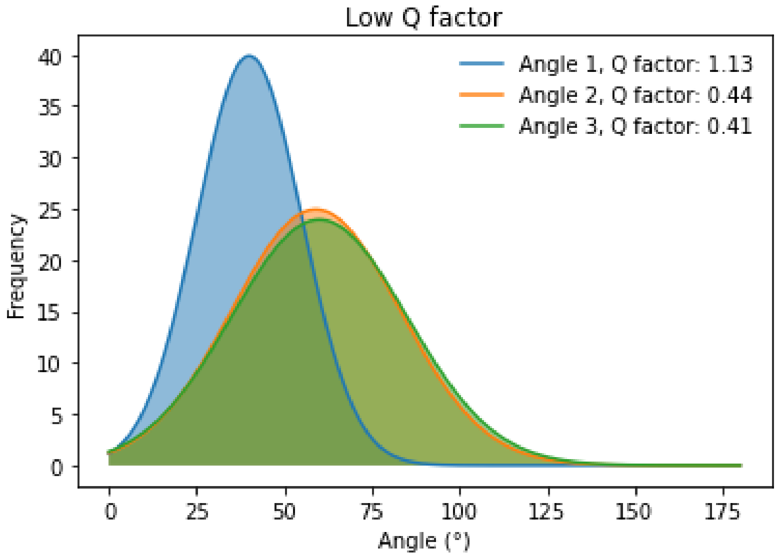


- **Plot the Angle and Distance distributions for each individually tracked molecule**
  - Based on mAb symmetry expect two distribution be similar
  - Calculate “Quality factor” as a measure of flexibility
  - Q-factor calculation: (Gaussian peak height /width)



Distance and angle distributions provide similar information about flexibility

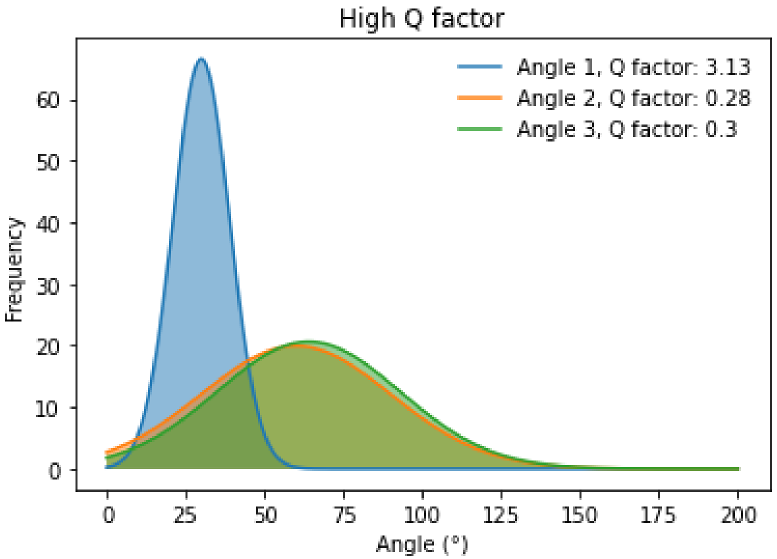




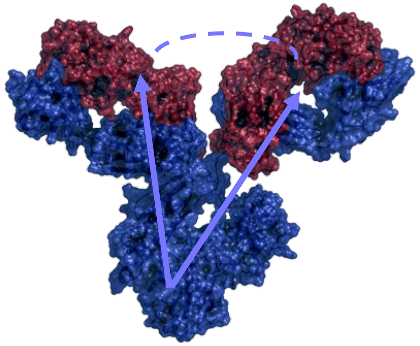
High Q-factor indicates less flexible molecule

Flexibility Descriptor for Antibodies: Quality factor

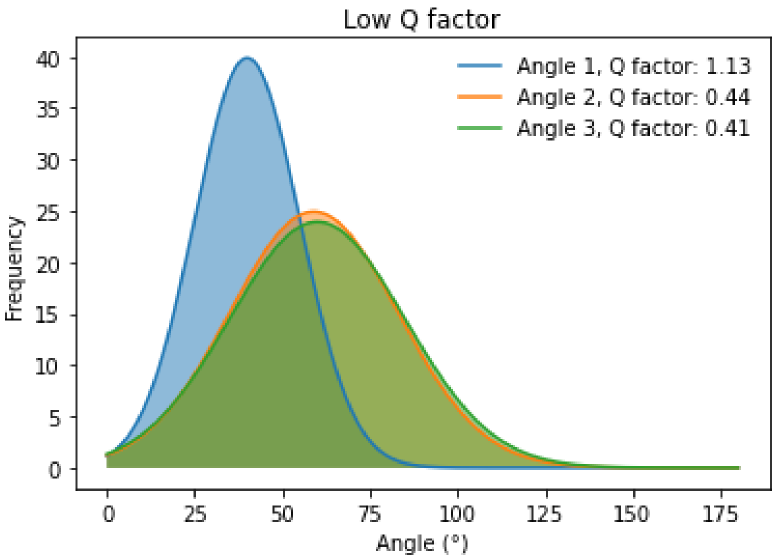
mAb-4B


High Q factor

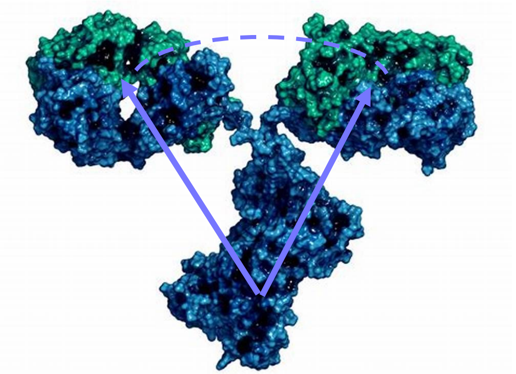
Y-shaped



mAb-4A


Low Q factor

T-shaped



High Q-factor indicates less flexible molecule

Q-factor Rank order:

* mAb-4B > mAb-4A

mAb-4B adopts a preferred Y-shape orientation while mAb-4A preferred state resembles a T-shape

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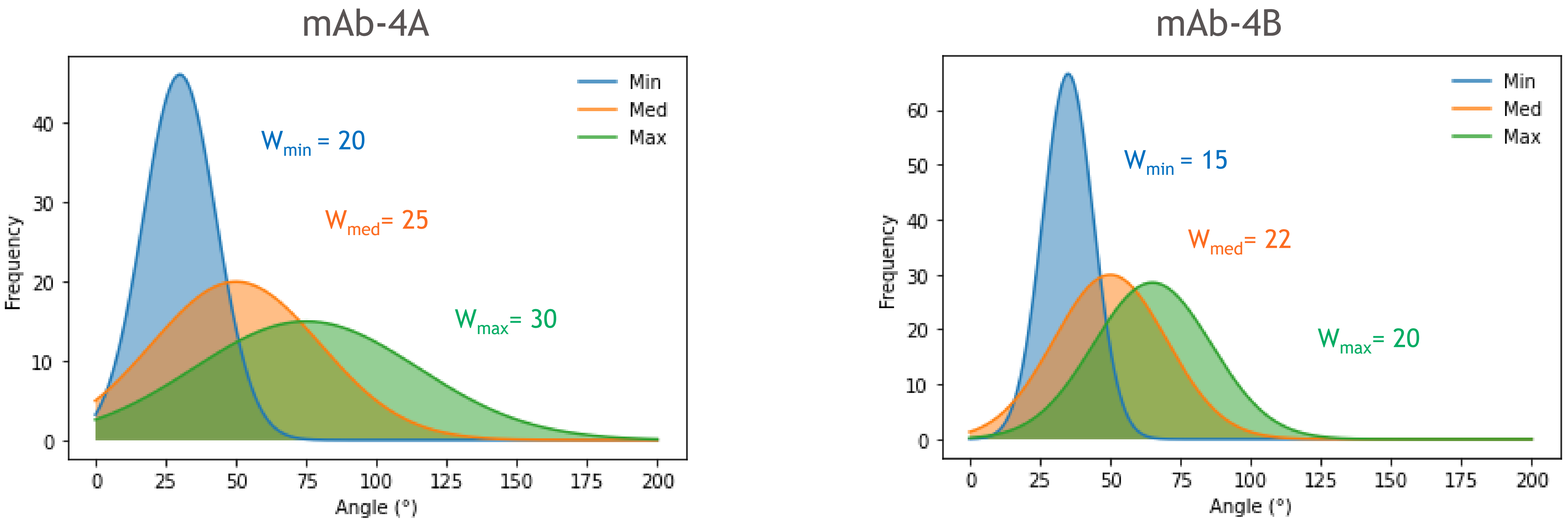
* Angular distributions and Q-factor values are not representative of the molecules shown, just for illustrative purposes

Flexibility Model for Antibodies Approach #2

Sort the 3 angles measured for individual molecules in each frame by value min to max

Plot max, median and min angle distribution



The width distribution of mAb-4A is overall larger than mAb-4B, therefore mAb-4A is more flexible than mAb-4B in agreement with approach #1

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* Angular distributions and width values are not representative of the molecules shown, just for illustrative purposes

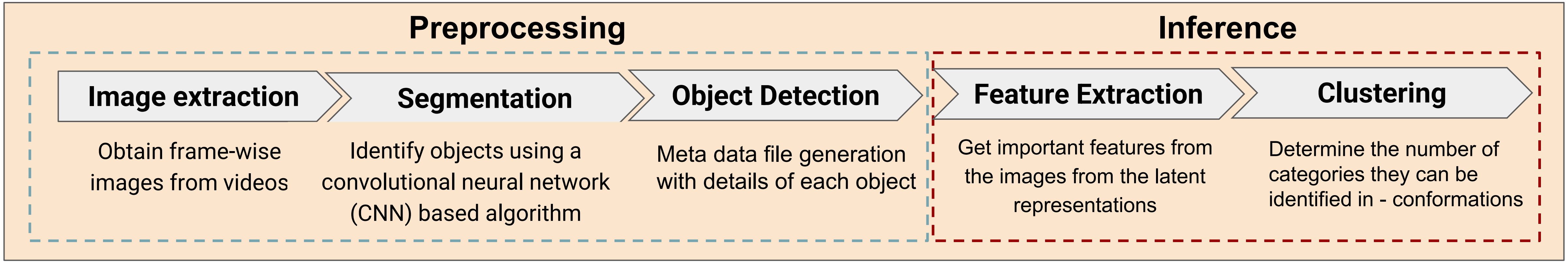
Conclusions and next steps

- The HS-AFM data enables sensitive detection of structural differences between mAbs of same IgG subtypes which may correlate with their different physical stability → Rational design of antibodies
  - Provided quantitative insights of mAbs intramolecular flexibility

- Measure multiple antibody dynamics at various formulation conditions to create a flexibility scale

- Refine flexibility model (potentially develop a physical model of torsion or flexibility to describe the data

Leverage ML to streamline image processing by automating the process of image analysis and interpretation



Preprocessing

Image extraction

Obtain frame-wise images from videos

Segmentation

Identify objects using a convolutional neural network (CNN) based algorithm

Object Detection

Meta data file generation with details of each object

Inference

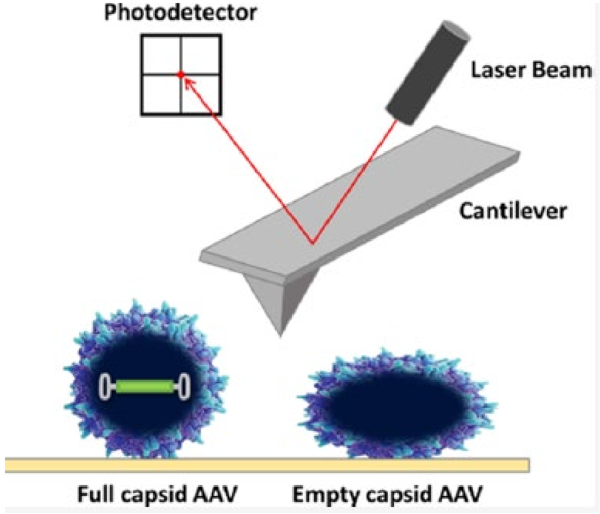
Feature Extraction

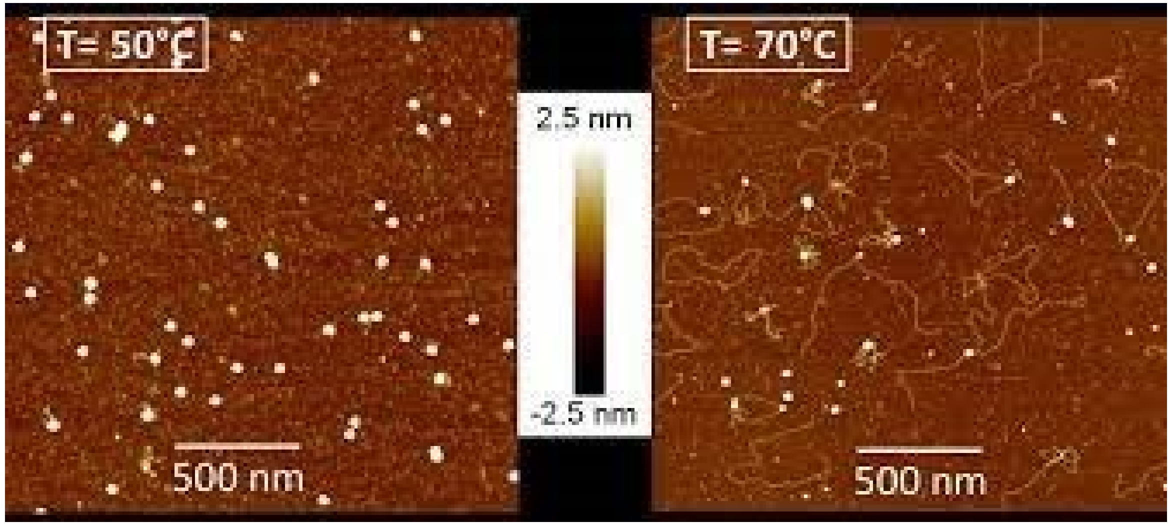
Get important features from the images from the latent representations

Clustering

Determine the number of categories they can be identified in - conformations

Forward-Looking Applications of HS-AFM for Novel Drug Product Modalities





Characterize structural and nanomechanical properties of viral vectors, lipid nanoparticles at the single particle level

- **Mechanical Properties (stiffness)**
- **Viral Genome release**

Understand the impact of solution conditions, serotype, full vs empty affect on the mechanical properties and critical quality attributes in real-time to potentially correlate with long-term stability

Nam et al, Langmuir 2023

Bernaud J. et al, J Biol Phys 2018

Acknowledgements

BMS Collaborators

Stephen Thomas

Steven Gossert

Joe Valente

Mark Bolgar

Weill Cornell Medicine

Motonori Imamura

Professor Simon Scheuring

Bio-AFM-Lab

Carnegie Mellon University

Natalie Pham

Gautami Kant

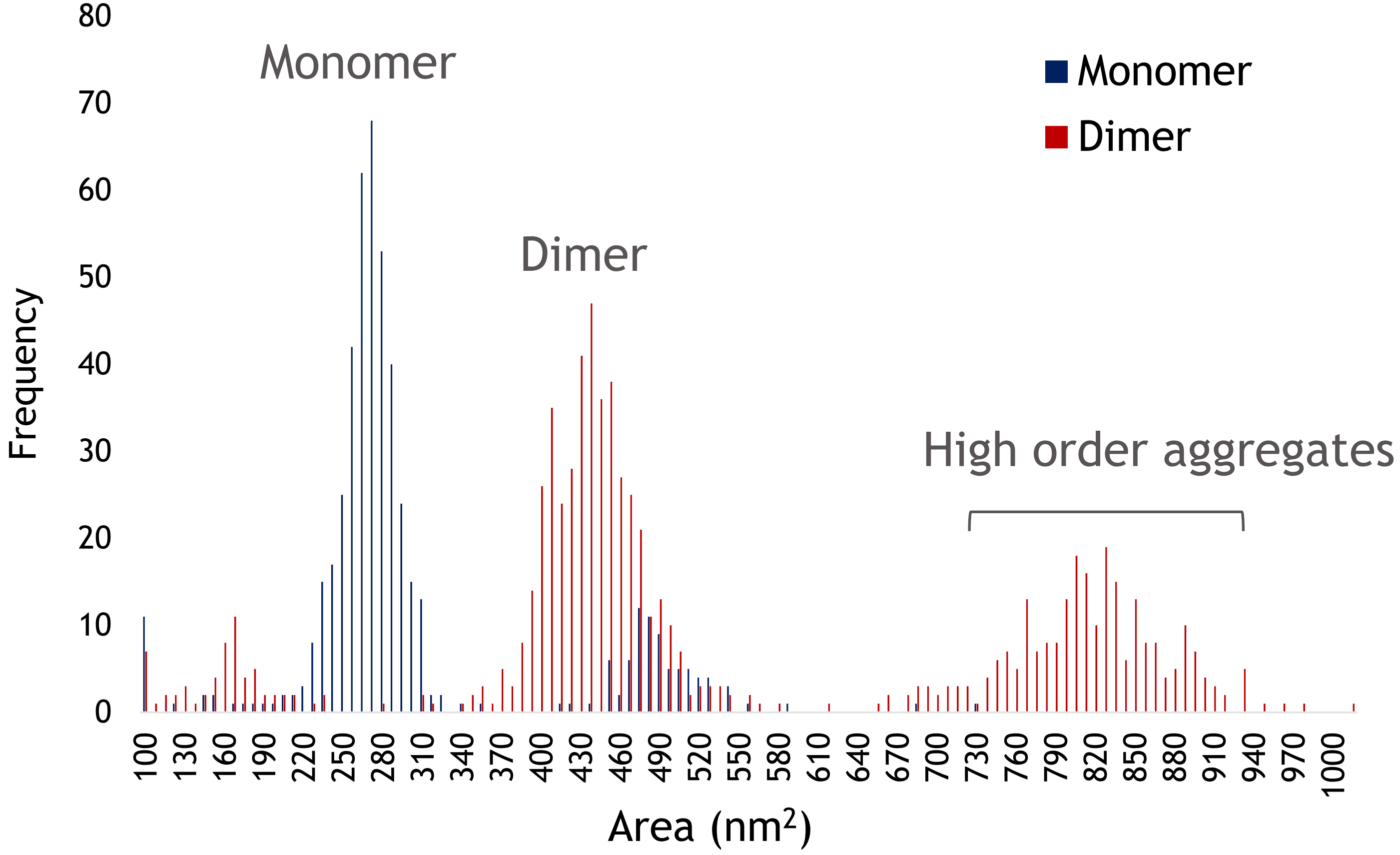
John Urbanic

Manfred Paulini

Thank you!

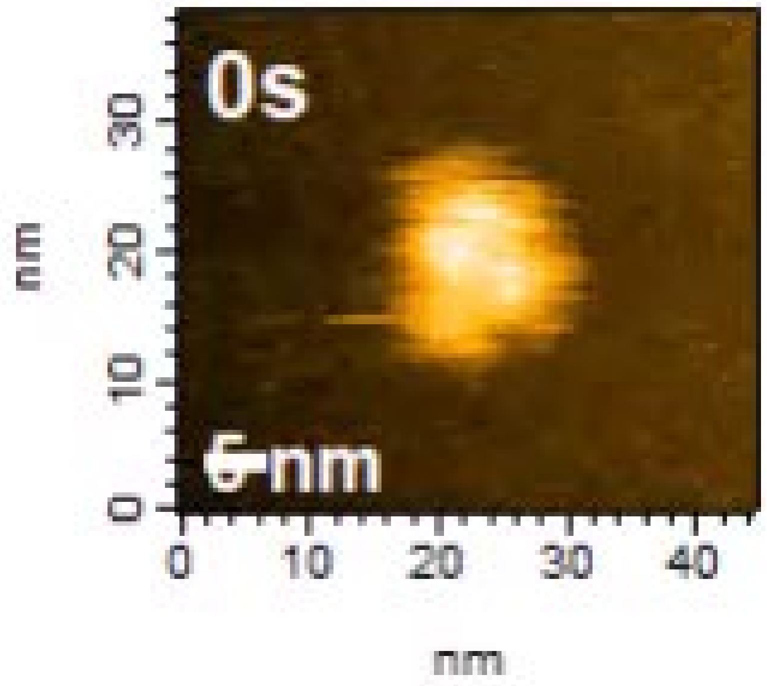
mAb8 Area Distribution Analysis

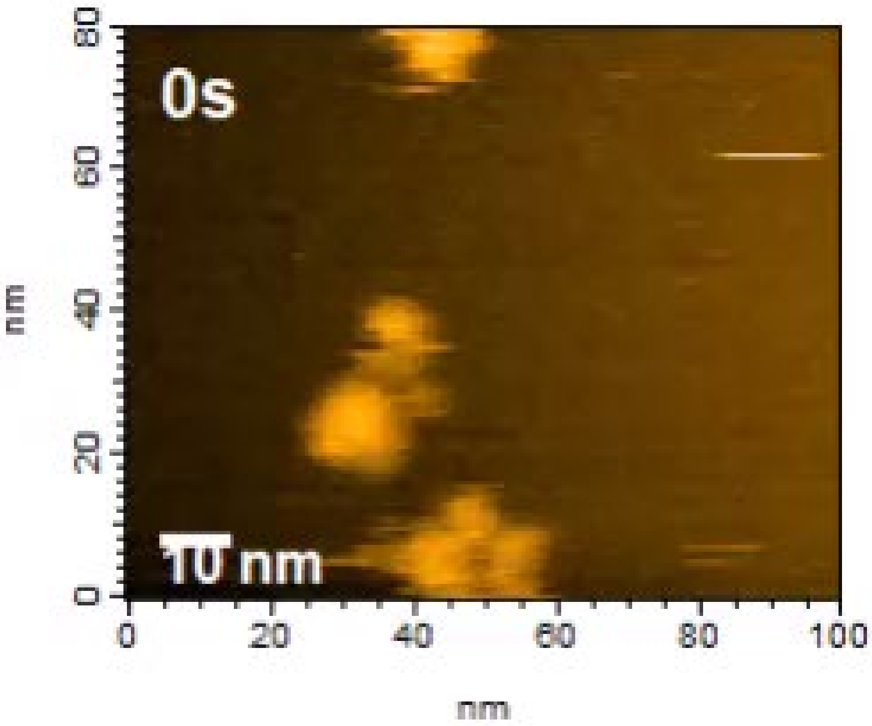
Urea Stressed Fractionated Samples in PB

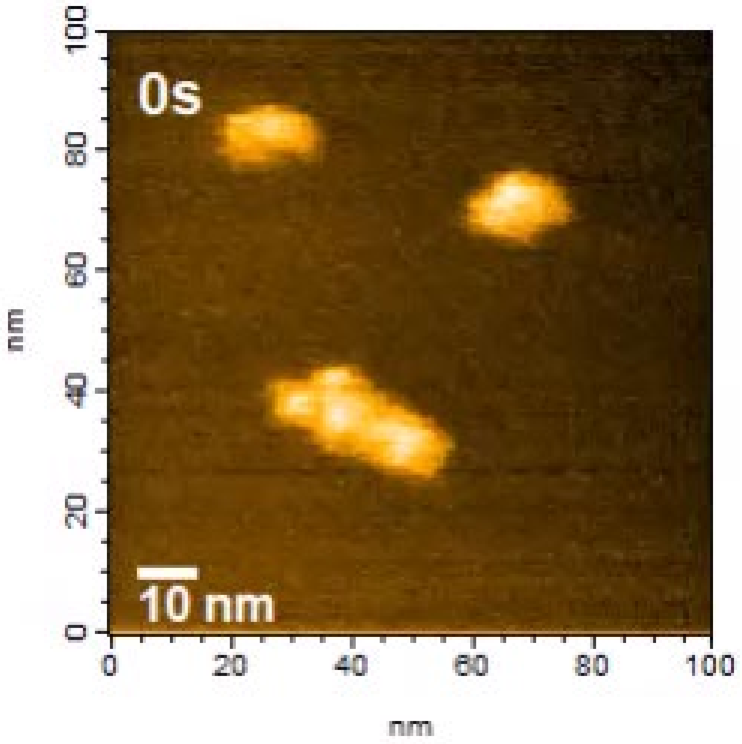


Dimer fraction is a mixture containing both dimers and bigger species

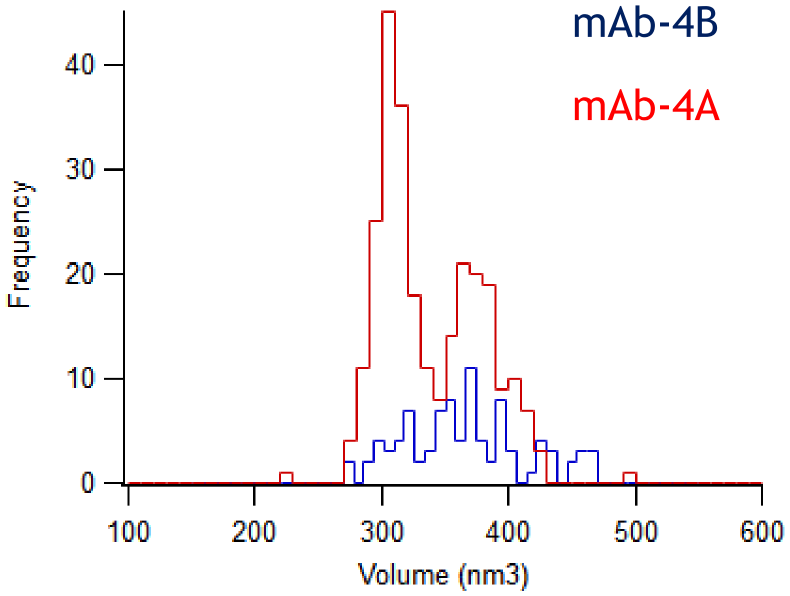
HS-AFM Image Analysis at Single Molecule Level
mAb-4A

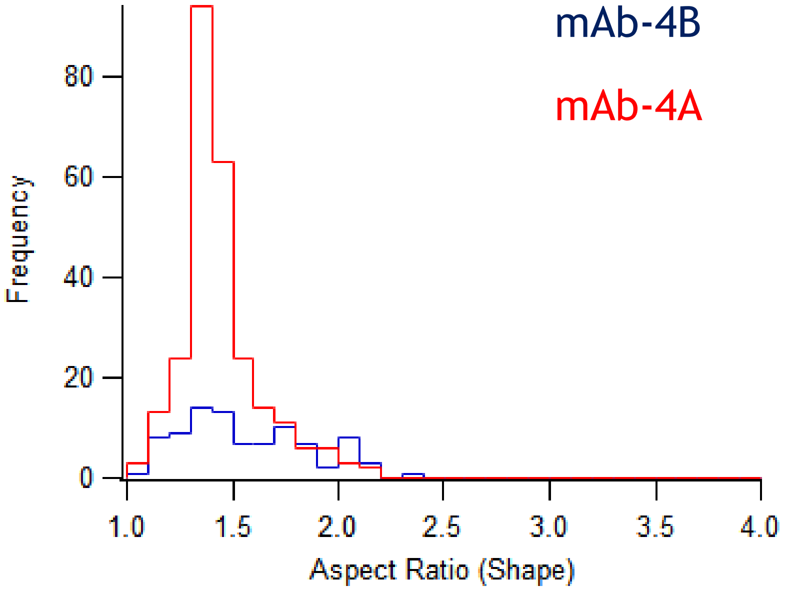






Summary Volume/Shape Distribution Analysis

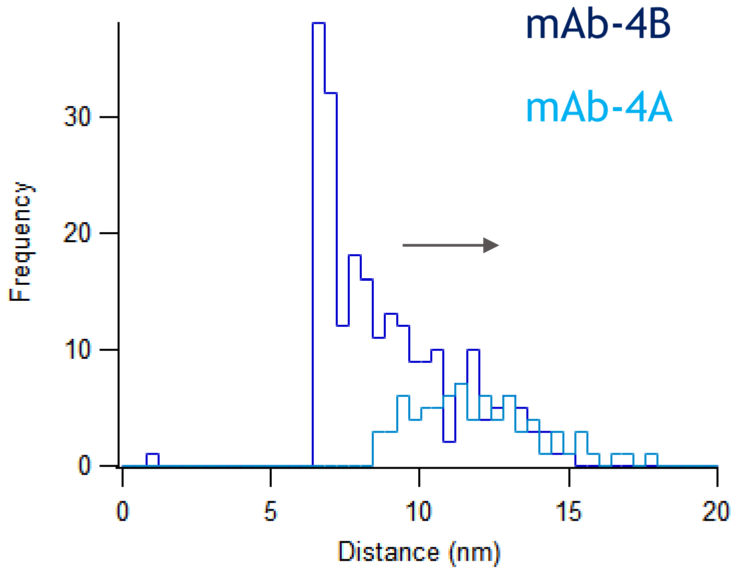


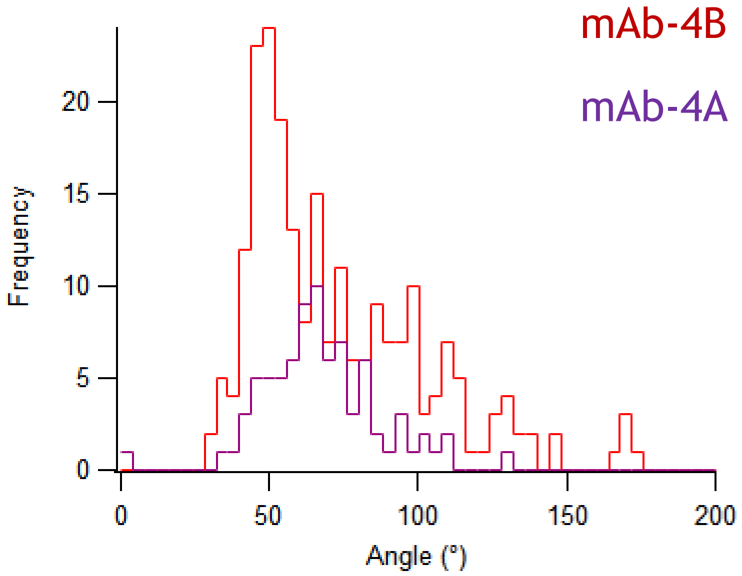


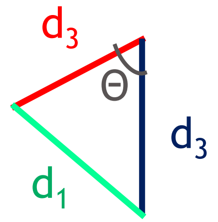
	IgG4		IgG1
Molecular Descriptors	mAb-4A	mAb-4B	mAb-1C
Area (nm2)	167 ± 8 208 ± 18	223 ± 48	284 ± 4
Volume (nm3)	305 ± 18 370 ± 31	355 ± 64	340 ± 65
Shape (Aspect Ratio)	1.2 ± 0.1	1.5 ± 0.5	*1.7 ± 0.4

*Presence of aggregates may have contributed to higher value

Comparison of IgG4 Angle/Distance Distribution Analysis





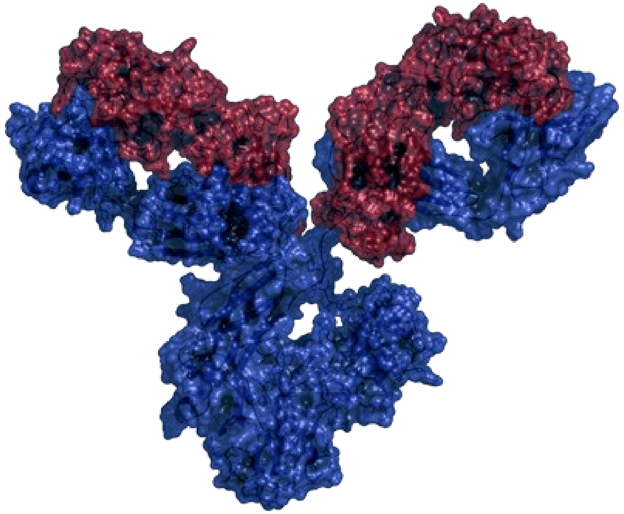


	IgG4	
Molecular Descriptors	mAb-4A	mAb-4B
Angle (°)	49 ± 13	62 ± 21
Distance (nm)	6.5 ± 0.5 multimodal	11 ± 3

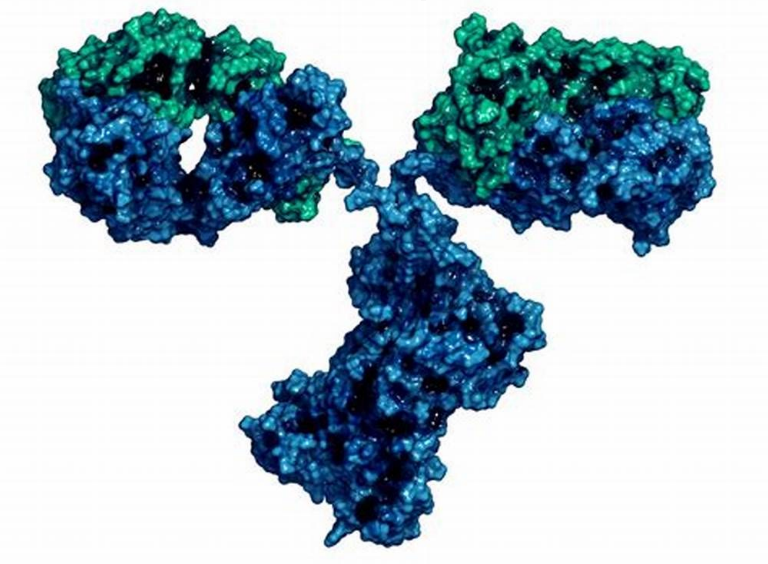
Shift in the angle and distance distribution suggests mAb-4B prefers to be in a more extended state than mAb-4A

Flexibility Approach #1

Y-shaped

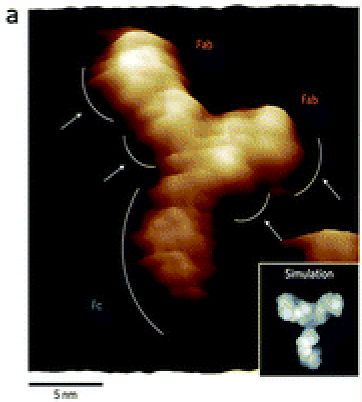


T-shaped

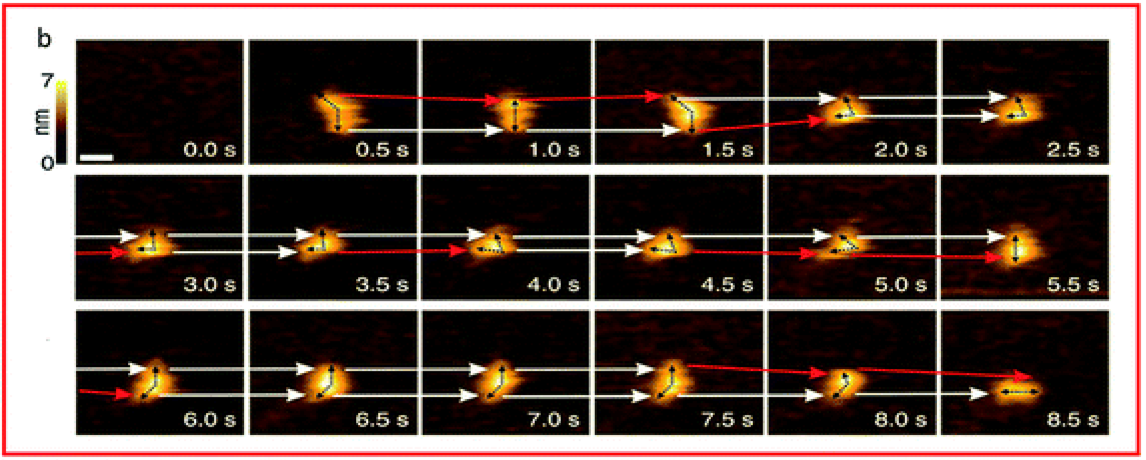


HS-AFM Case Studies

Antibody structure and locomotion on virus surface

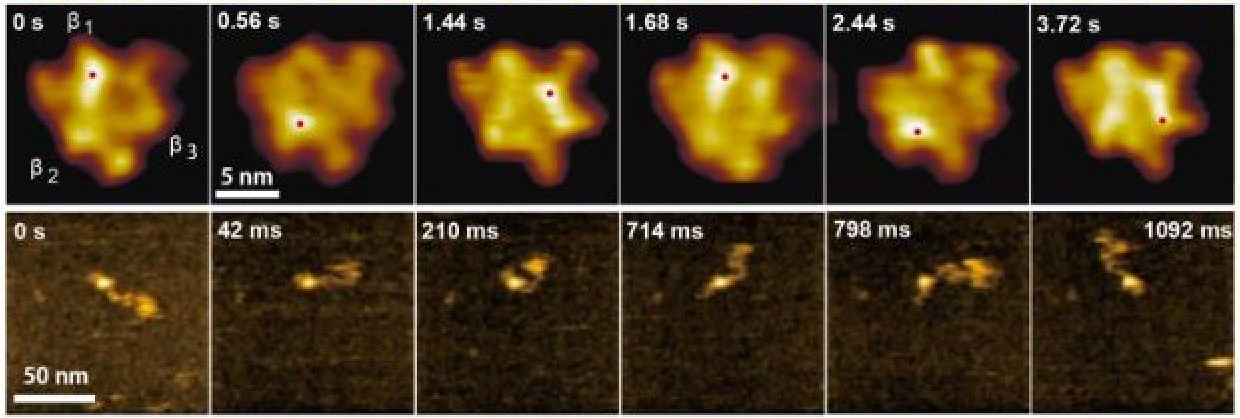


Yamada et al, Nat Materials 2014



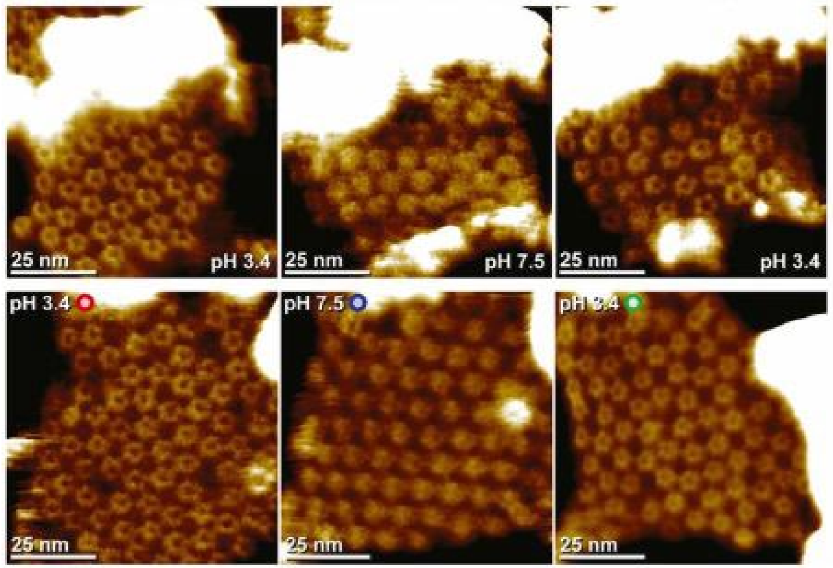
Preiner et al, Nat Commun 2014

Conformational changes of complex subunit and Intrinsically disordered protein wiggling motion



Ando et al, Biophys Rev 2017

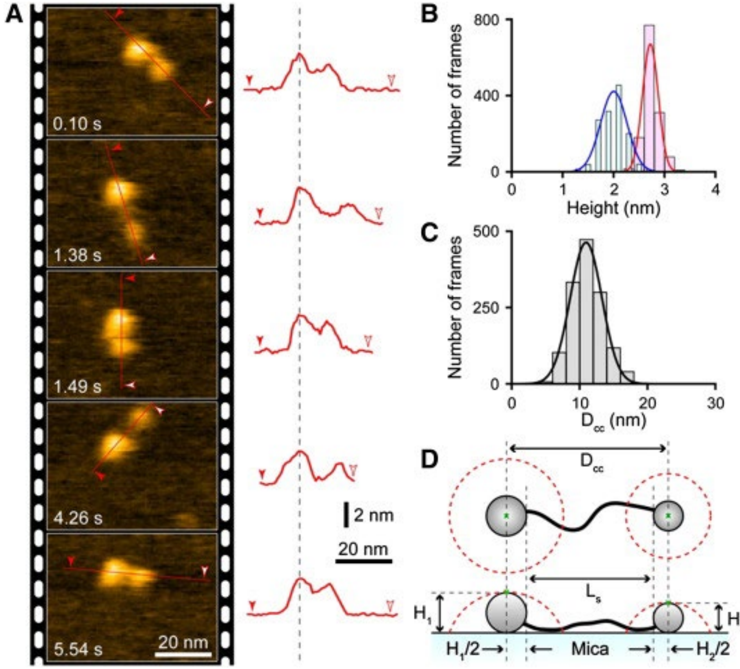
In situ dynamics of a protein channel upon pH titration



Ruan et al, PNAS 2018

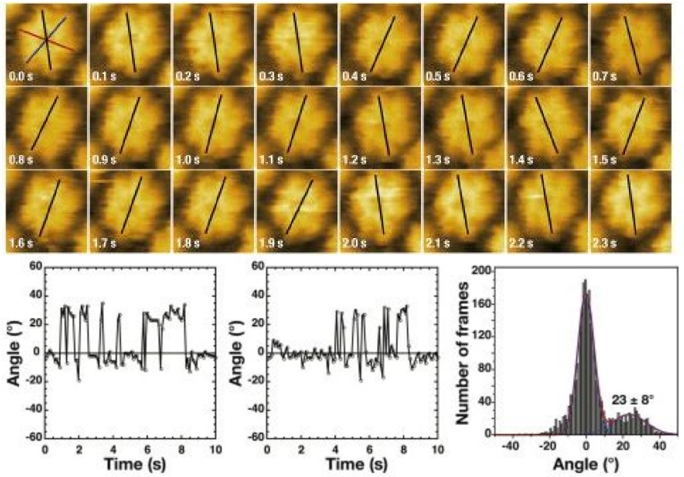
HS-AFM Imaging Analysis

Intrinsically disordered protein Height distribution and center to center distance analysis

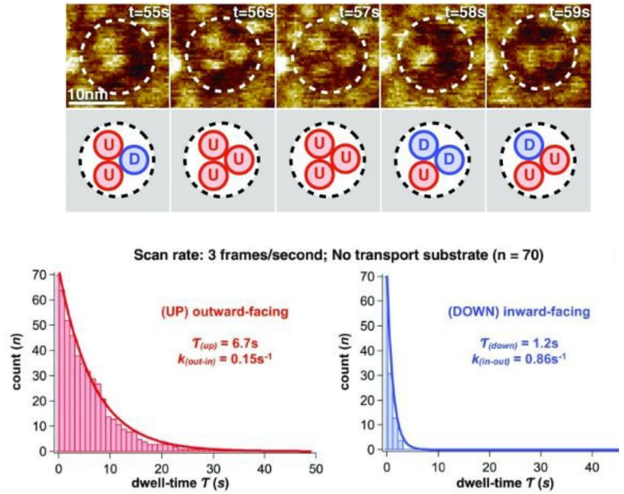


Ando et al, Biophys Rev 2017

Rotational movements of Hexameric protein

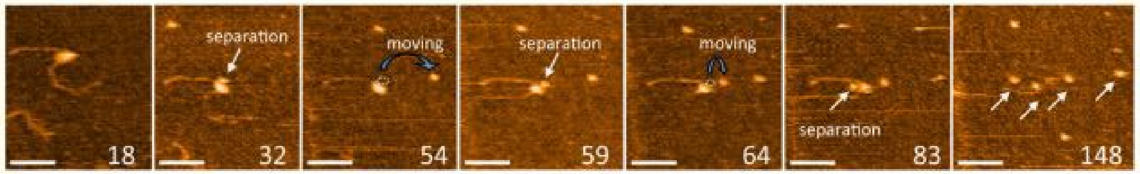


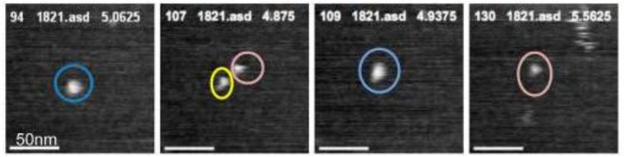
Quantification of the elevator domain dynamics

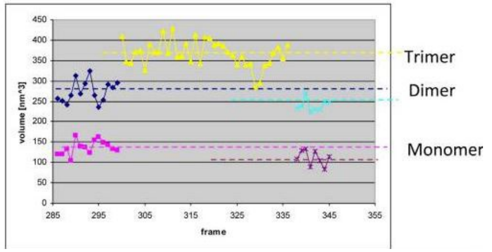


Ruan et al, PNAS 2017

Volumetric Analysis of protein during association and dissociation processes







Background

- Antibodies in solution are inherently dynamic molecules of marginal stability which present unique challenges when developing stable liquid formulations

- Global structural flexibility and intramolecular conformation fluctuations are dynamic processes that may impact protein solution quality attributes and stability properties (self-association, aggregation, viscosity, fragmentation)

- ***Our understanding of the complex inter-relationship between antibody intrinsic dynamics and stability is incomplete but undeniably necessary to the development of a stable formulation***

- HS-AFM provides information on antibody dynamics at the single-molecule level that may advance our understanding of the relationship between the nanoscopic observation of molecular behavior and the macroscopic stability of biopharmaceutical formulations.