Conformationally Variable Single Particles

Heterogeneity in the real world

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Workshop on Advanced Topics in EM Structure Determination:
Challenging Molecules

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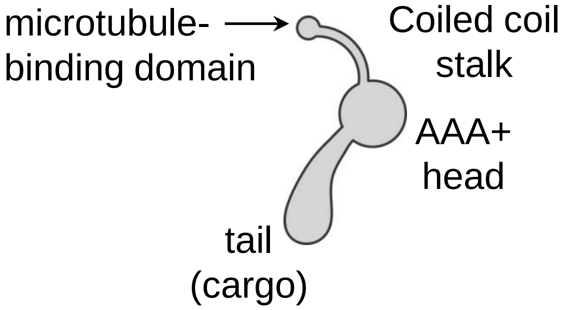
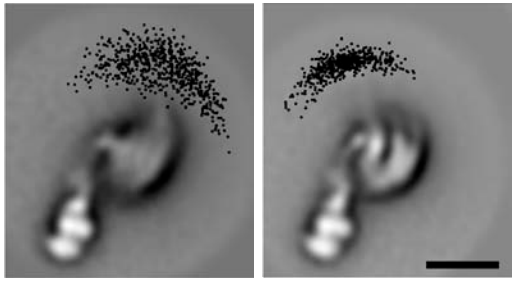
Microtubule-based motor dynein

Burgess *et al.* (2004) J. Struct. Biol. 146, 205–216



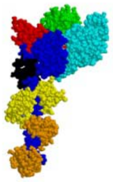
50nm

Appears flexible (stalk and tail) by negative stain EM



MTBD positions shift relative to the tail

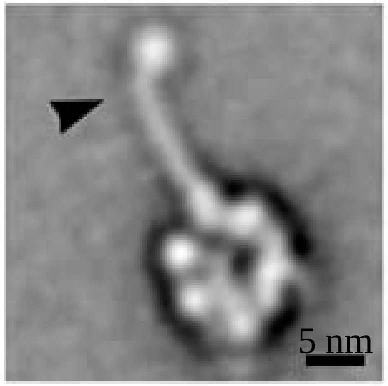
Myosin



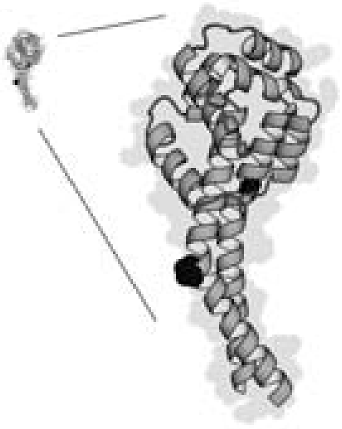
X-ray
crystallography

Negative stain
electron microscopy

Dynein

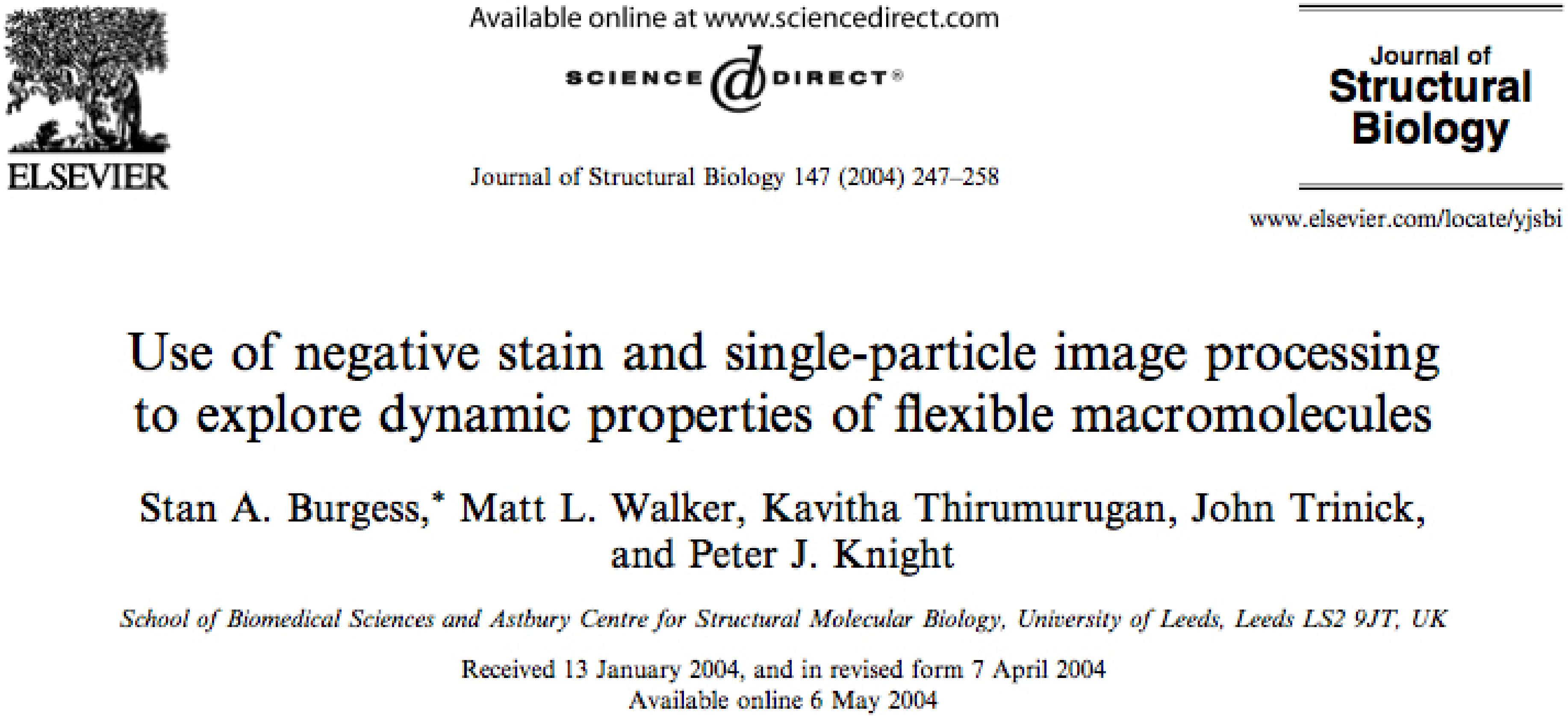


Negative stain
electron microscopy



X-ray
crystallography

Structural preservation is good in stain
Resolve small (SH3) flexible (coiled coil) domains in context of whole macromolecule



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Use of negative stain and single-particle image processing to explore dynamic properties of flexible macromolecules

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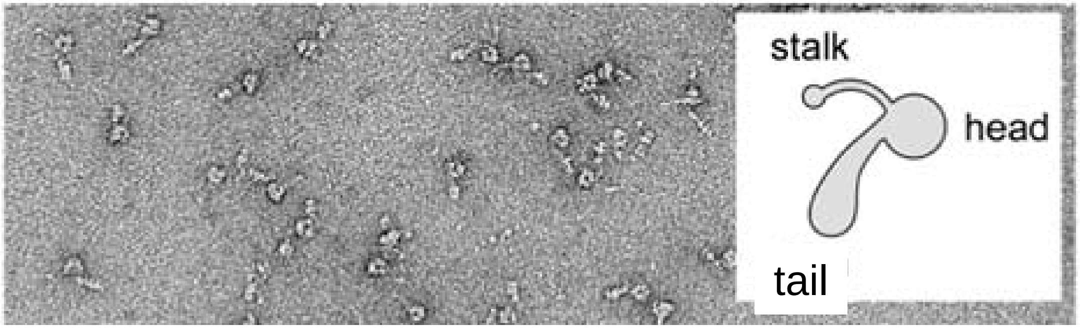
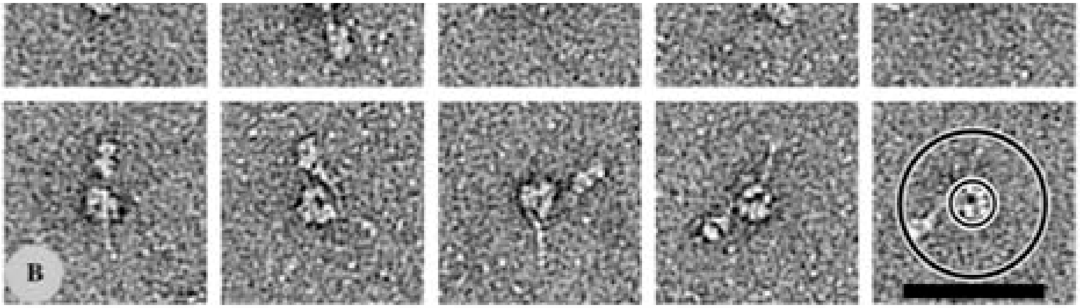


Start with a lot of molecules >10,000
Some of our studies start with 50,000 for a single construct/nucleotide state
Recent work used 230,000 molecules
Use automatic particle picking where possible

Number required hard to say, depends on
image quality,
number of views,
extent of heterogeneity

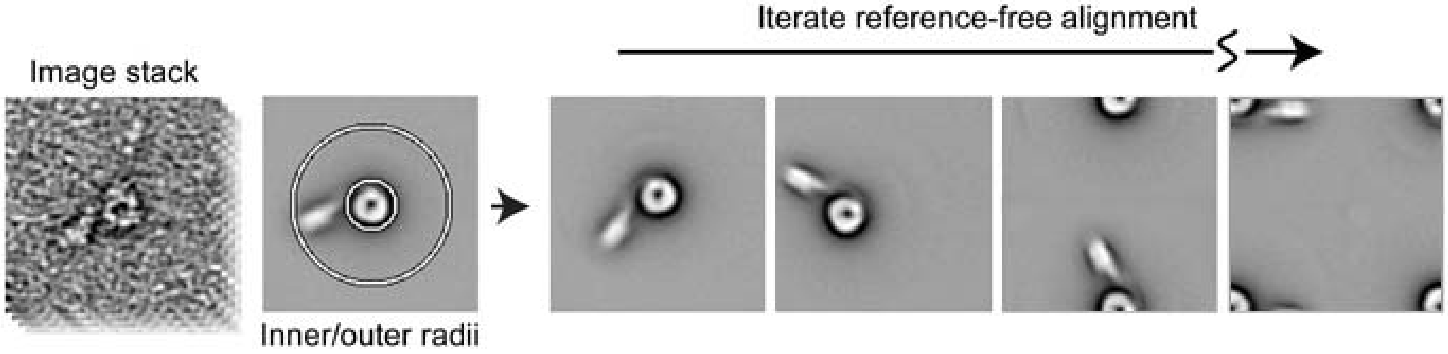
Throw away bad ones (classes, image statistics, stain quality) after initial processing

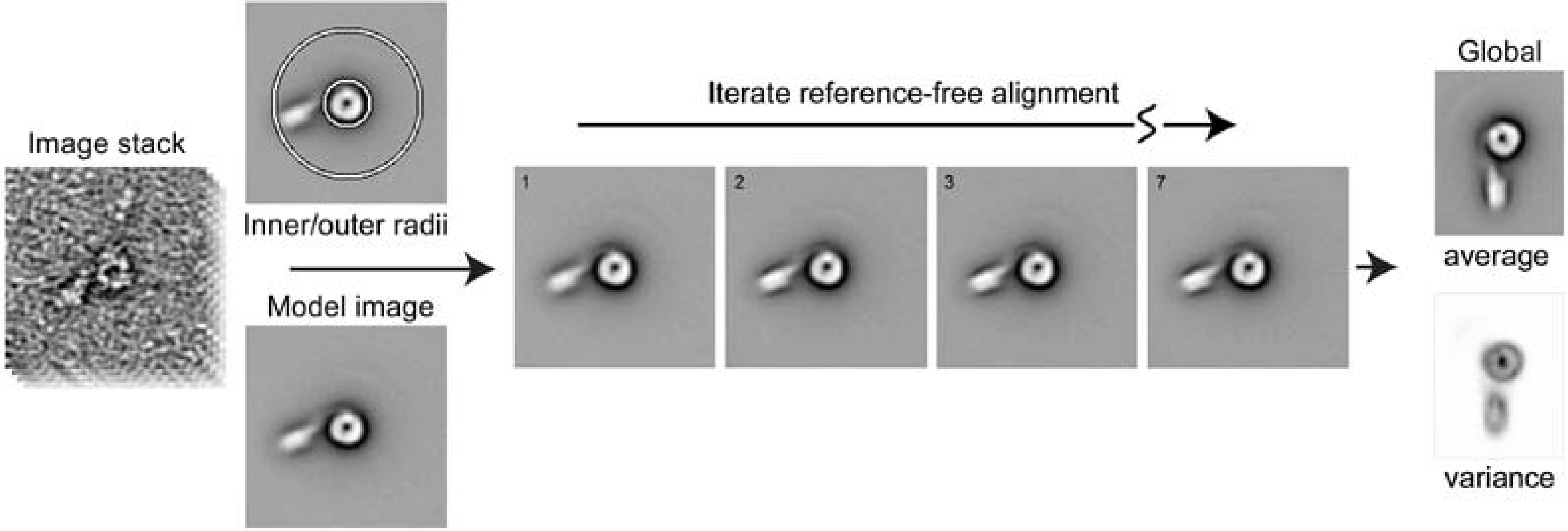
Those left should provide good statistics of heterogeneity

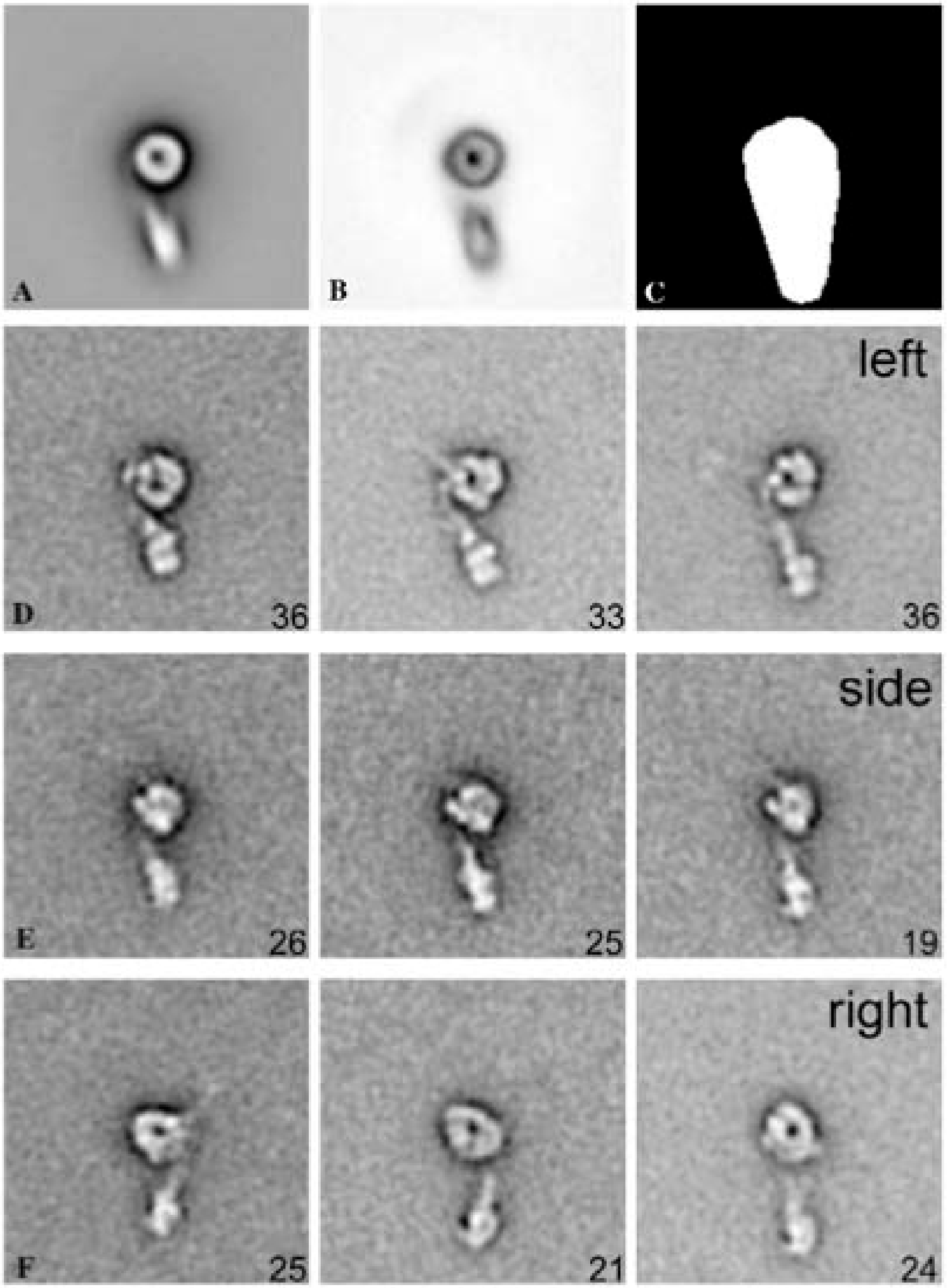


Many 1000s of molecules aligned computationally

Negatively stained dynein
(demo)

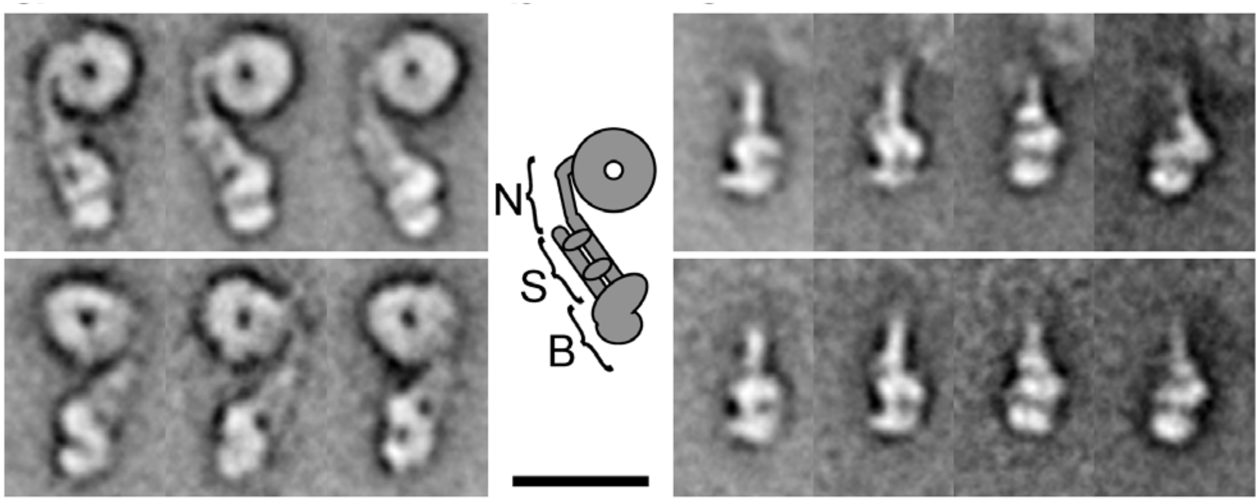

Iterate reference-free alignment
Image stack
Inner/outer radii


Iterate reference-free alignment
Image stack
Inner/outer radii
Model image
1
2
3
7
Global
average
variance


A
B
C
left
D
36
33
36
side
E
26
25
19
right
F
25
21
24

Describing the structure/flexibility of a domain

1



Torsional variability (flexibility?)

1 Head + Tail alignment
Segregate LEFT and RIGHT views
Classify tails only (structural detail in tail)
2 Realign these molecules again centred now on tails
(Determine tail positions in raw images from positions after alignment and re-window)
Classify all tails
Segregate tails again according to previous LEFT/RIGHT segregation
See same tail appearances in LEFT and RIGHT views- Torsional flexibility

Describing the structure/flexibility of a domain

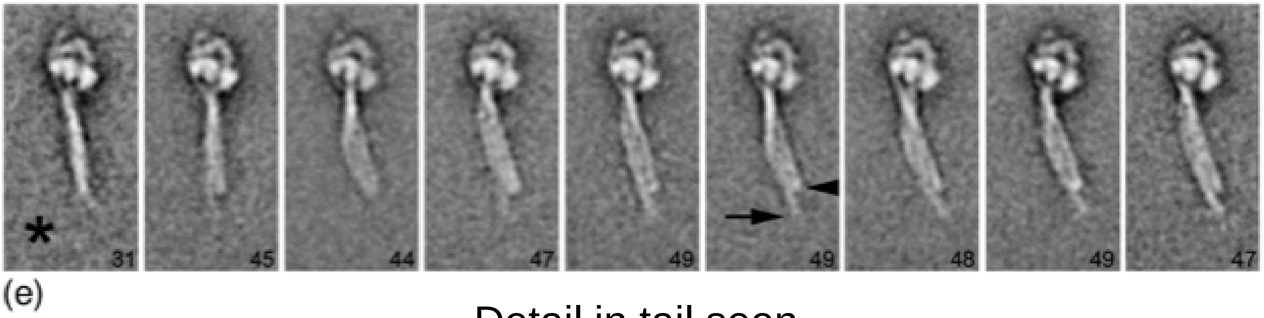
3

A second example of ‘head tail’ flexibility

(Myosin motor molecule from smooth muscle)

Another example:
Smooth muscle myosin molecule (also has Head and Tail domains)
Coiled-coil folded back on itself twice (3 coiled coil bundle)
Coiled coil bundle ~50nm long
Whole molecule alignment shows detail in tail
Head alignment and tail classification shows flexibility

Whole molecule
Aligned
(head and tail
flexing)



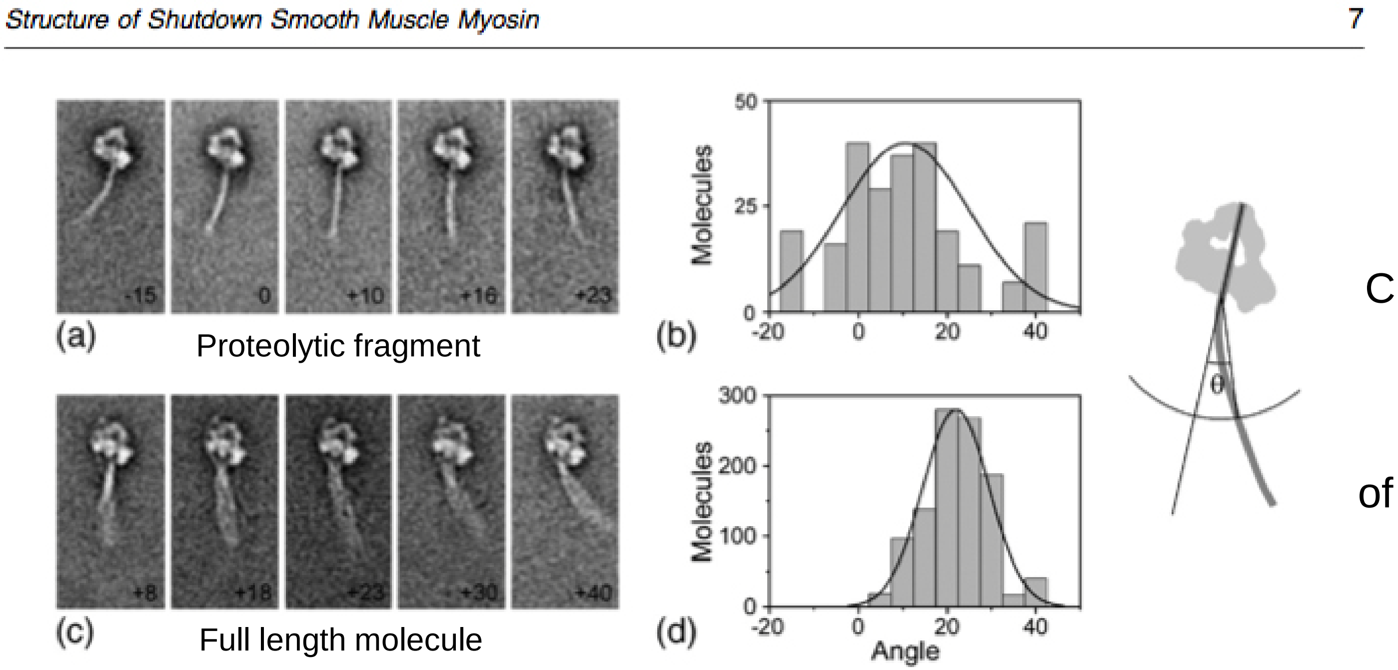
Head

tail

Detail in tail seen
Distal end

Structures of smooth muscle myosin and heavy meromyosin in the folded, shutdown state
Burgess, S.A., Yu, S., Walker, M.L., Hawkins, R.J., Chalovich, J.M. and Knight, P.J. (2007)
J. Mol. Biol. 372, 1165-1178.

Head aligned
(tail flexing)



Structure of Shutdown Smooth Muscle Myosin 7

Proteolytic fragment

Full length molecule

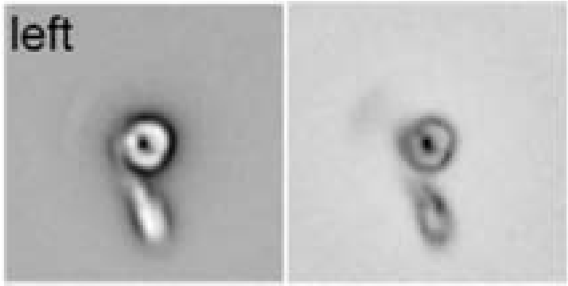
Change in
extent
and
mode
of flexibility

Another example:
Smooth muscle myosin molecule (also has Head and Tail domains)
Coiled-coil folded back on itself twice (3 coiled coil bundle)
Coiled coil bundle ~50nm long
Whole molecule alignment shows detail in tail
Head alignment and tail classification shows flexibility
(Compare whole molecule to proteolytic fragment)

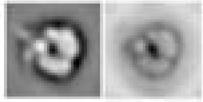
Structures of smooth muscle myosin and heavy meromyosin in the folded, shutdown state
Burgess, S.A., Yu, S., Walker, M.L., Hawkins, R.J., Chalovich, J.M. and Knight, P.J. (2007)
J. Mol. Biol. 372, 1165-1178.

Describing the structure/flexibility of a domain
2
Fixing the orientation of one domain to examine flexibility of the other domain

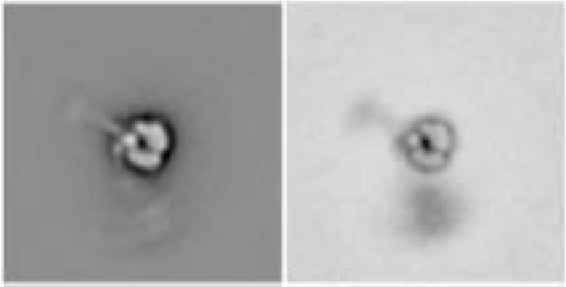
Whole molecule alignment



Average Variance



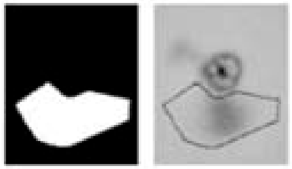
Head only



Average Variance

Flexibility between head and tail
Whole molecule alignment means neither all heads nor all tails aligned
Detail in each is lost (or distributed between many classes)

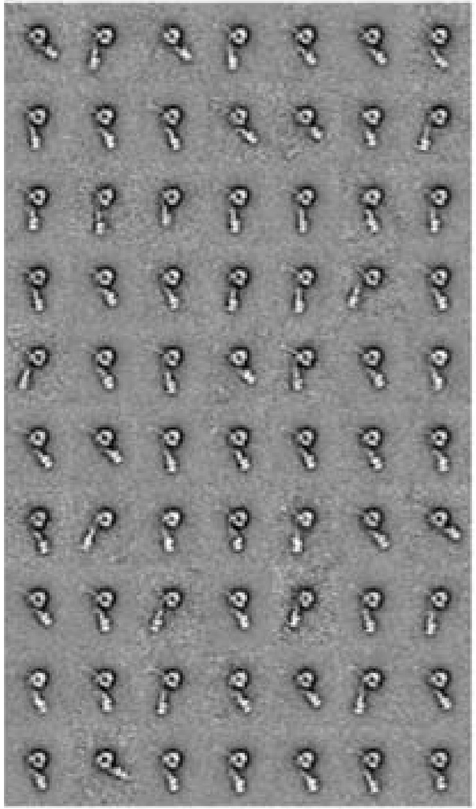
Fix one domain (by alignment) and examine distribution/position of other



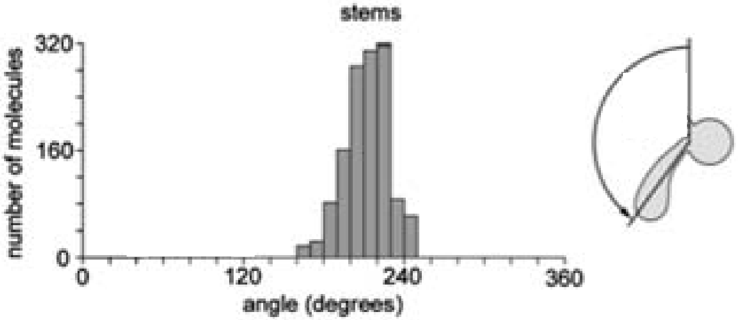
Tail mask

Heads alignned
Tails classified

F

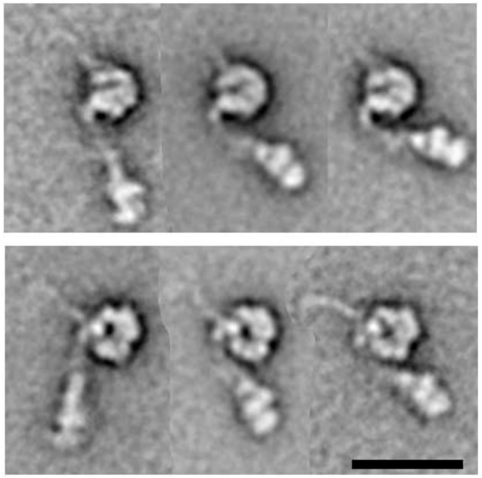


Class averages



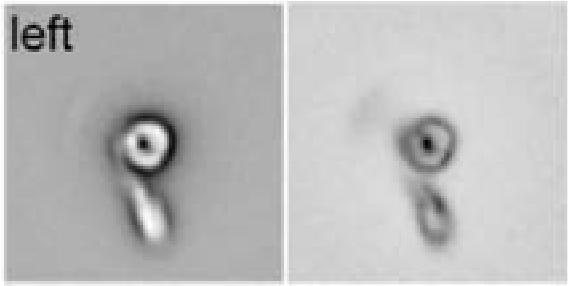
Measure angle of tails
in each class

Tail Flexibility
(left views)
Length unaffected by nucleotide condition

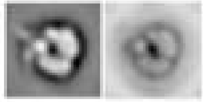


Assemble class averages
Into movie sequence
According to tail angle

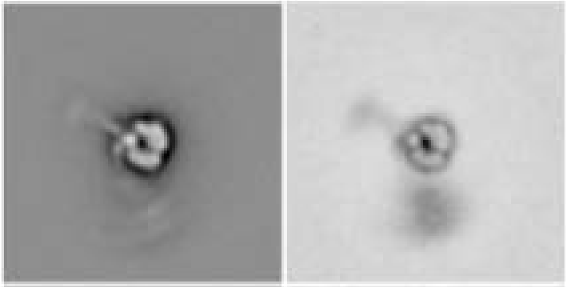
Whole molecule alignment



Average Variance



Head only



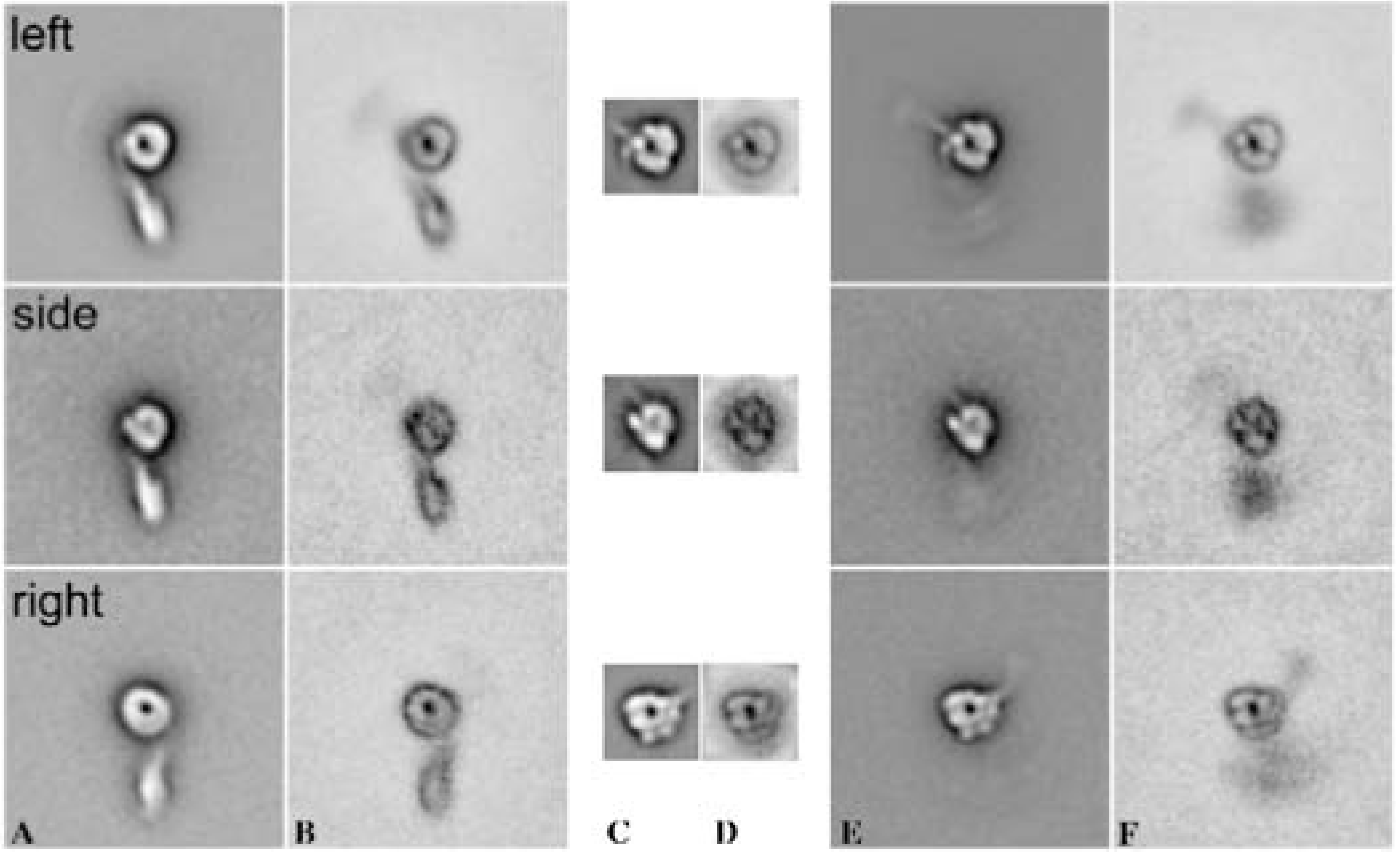
Average Variance

Flexibility between head and tail
Whole molecule alignment means neither all heads nor all tails aligned
Detail in each is lost (or distributed between many classes)

Fix one domain (by alignment) and examine distribution/position of other

Whole molecule alignment

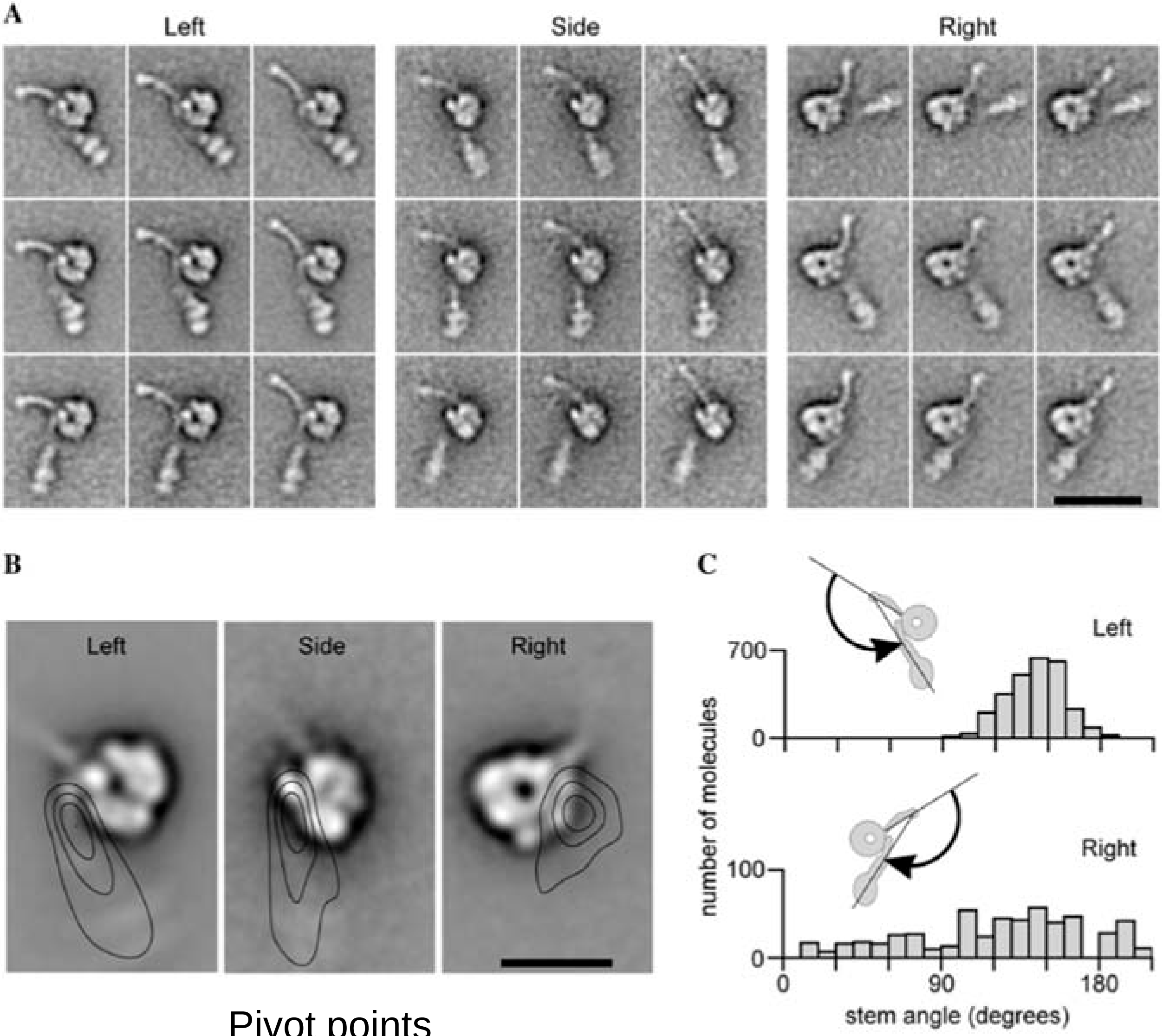
Head only



Average Variance

Average Variance

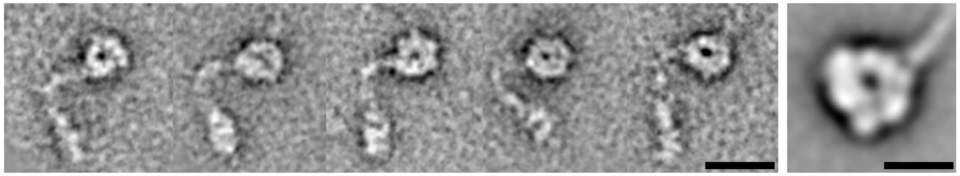
Tail flexibility analysis- fit with straight lines (manually), measure angle & pivot



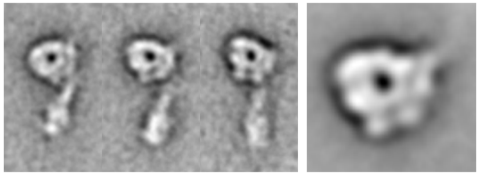
Pivot points

Small numbers of molecules with extreme flexibility hard to align and classify (n~150)

~8 molecules per class



~40 molecules per class



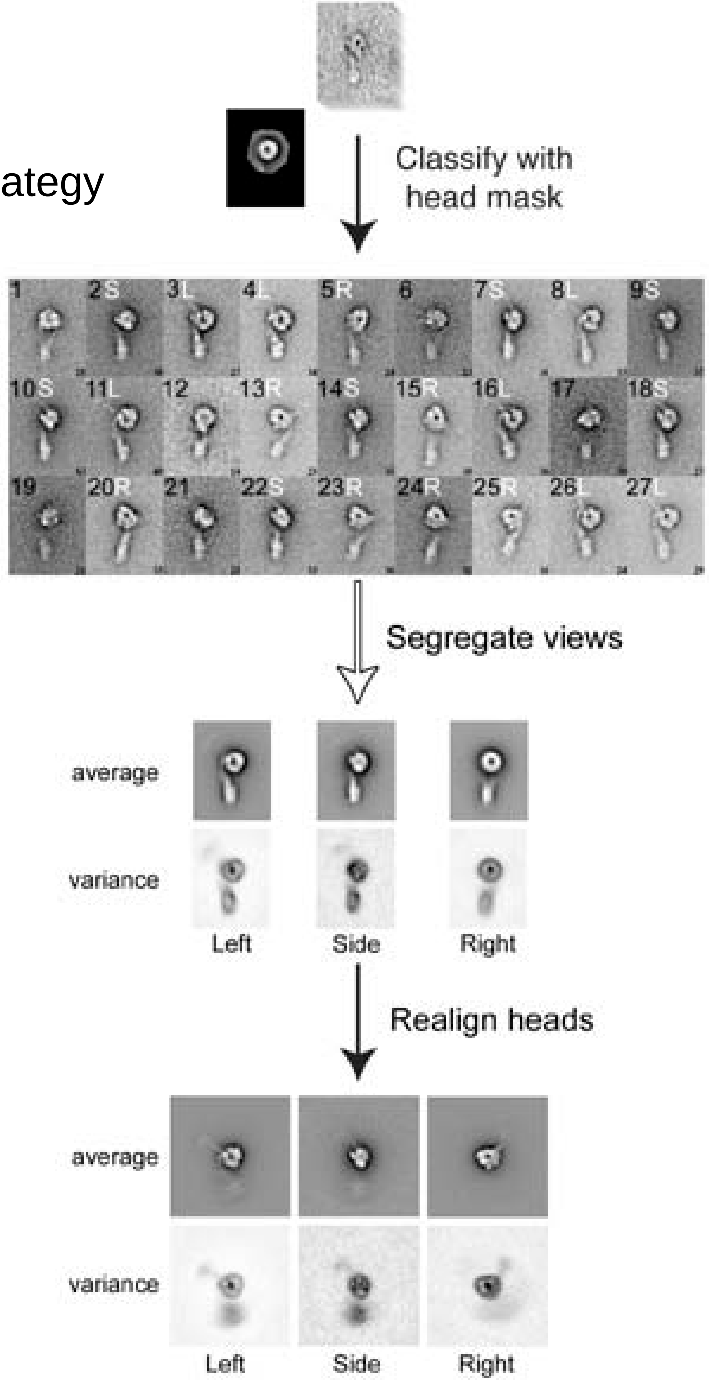
Nevertheless,
Movie sequence can be made by obtaining coordinates of distal tail in individual (head aligned)molecules

Movie demo

Single molecules
(hence noise)

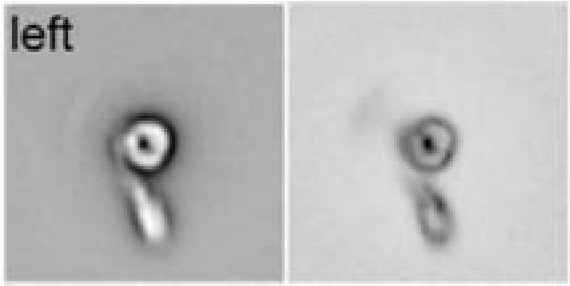
RECAP

Summary of alignment and classification strategy

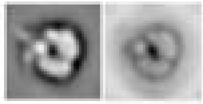


Describing the structure/flexibility of multiple domains

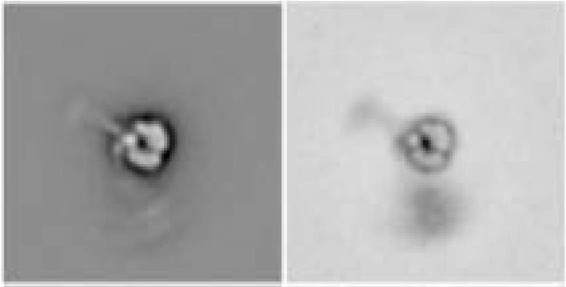
Whole molecule alignment



Average Variance

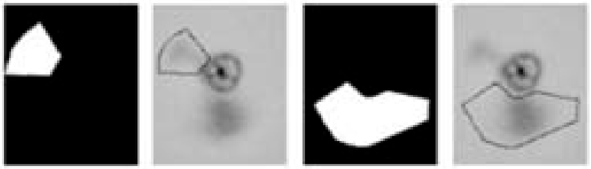


Head only

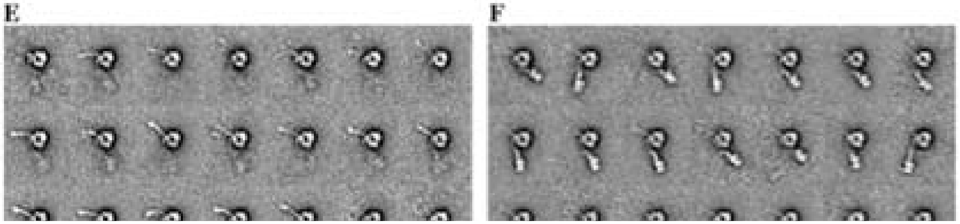


Average Variance

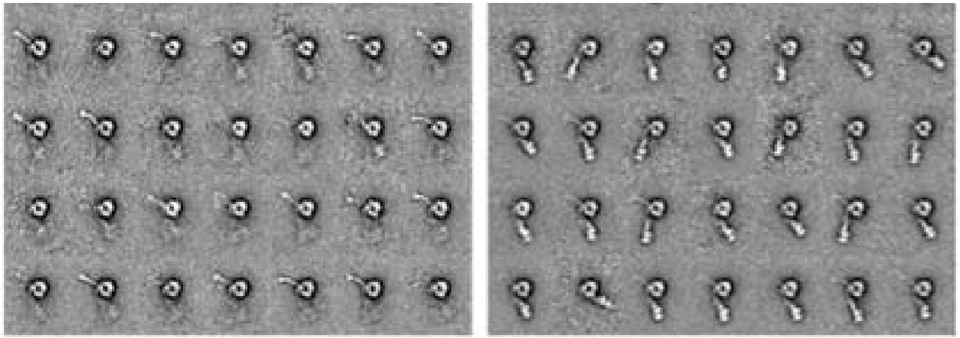
Flexibility between head and tail
ALSO
Flexibility between head and stalk

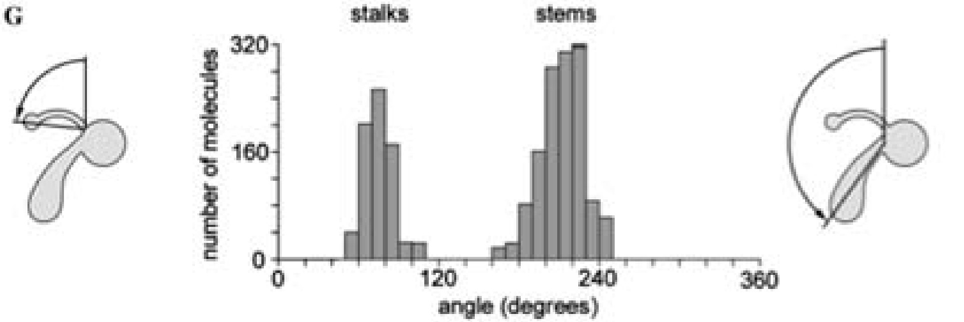


Perform a
SECOND classification
of the SAME set
of head-aligned
molecules



Determine position (x,y) in class averages of
tip of tail
tip of stalk
Measure angle of these (arbitrary axis)





Stalk conformation is nucleotide dependent

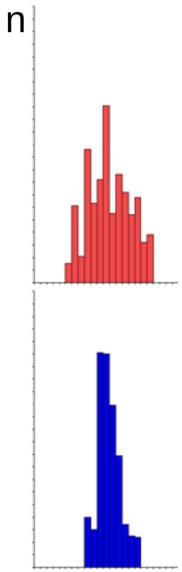
Burgess *et al* (2003) Nature 421, 715-718

ADP.Vi

Movie demo

Apo

Movie demo

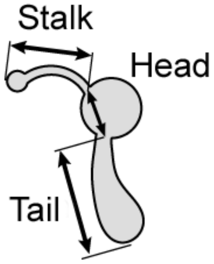


stalk angle
relative to head

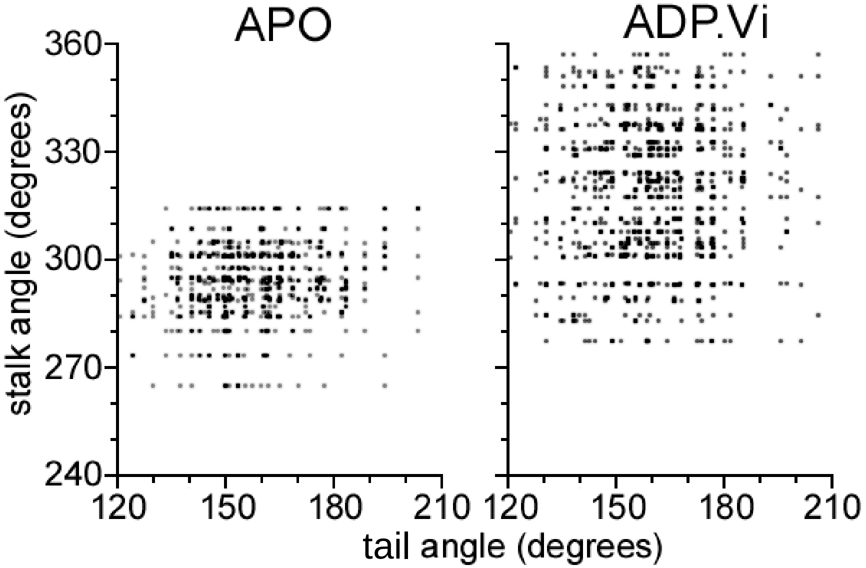
ADP.Vi-dynein stalk is curved along its length
Apo-dynein stalk is rigid with a kink and less ‘flexible’

What is the mechanism, sliding ??? YES

For those molecules where tail AND stalk angles obtained- scatter plot



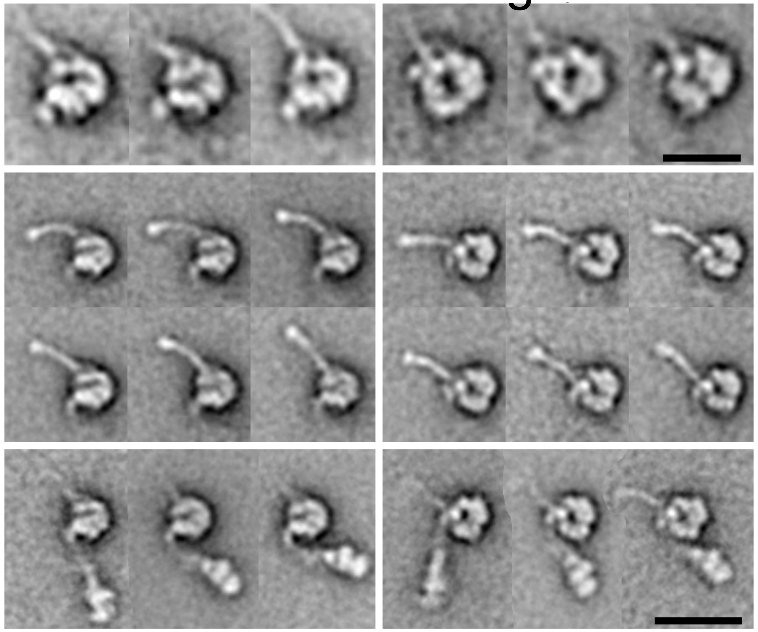
Left views

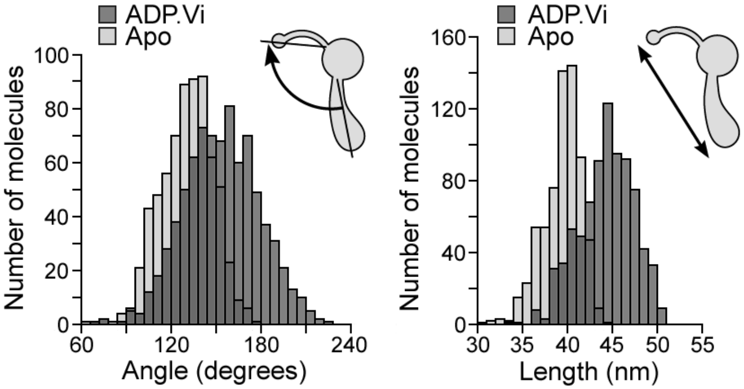


Angle of tail and stalk measured relative to the head

Their movements are not coupled

For those molecules where tail AND stalk angles obtained
Measure angle BETWEEN two domains
End-end lengths





So far seen molecules with head+tail OR head+stalk but not BOTH
How to combine to show WHOLE MOLECULE in its entirety?

Realign the ‘reconstituted’ molecules according to tail

Either

use class averages to perform alignment

Or

determine coordinates of tails in original micrographs and realign from scratch

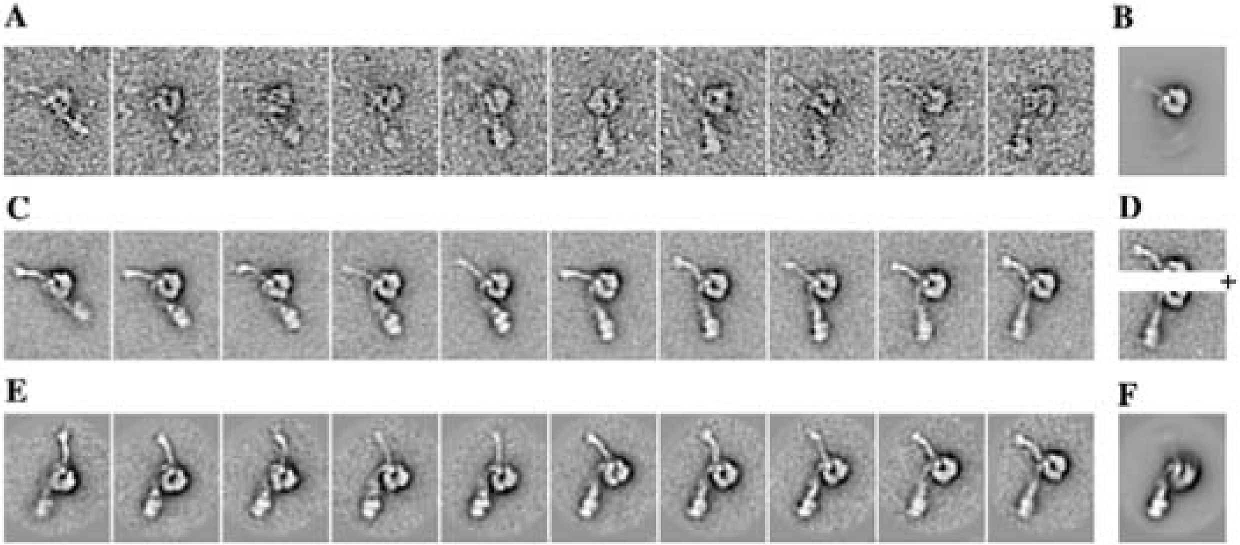
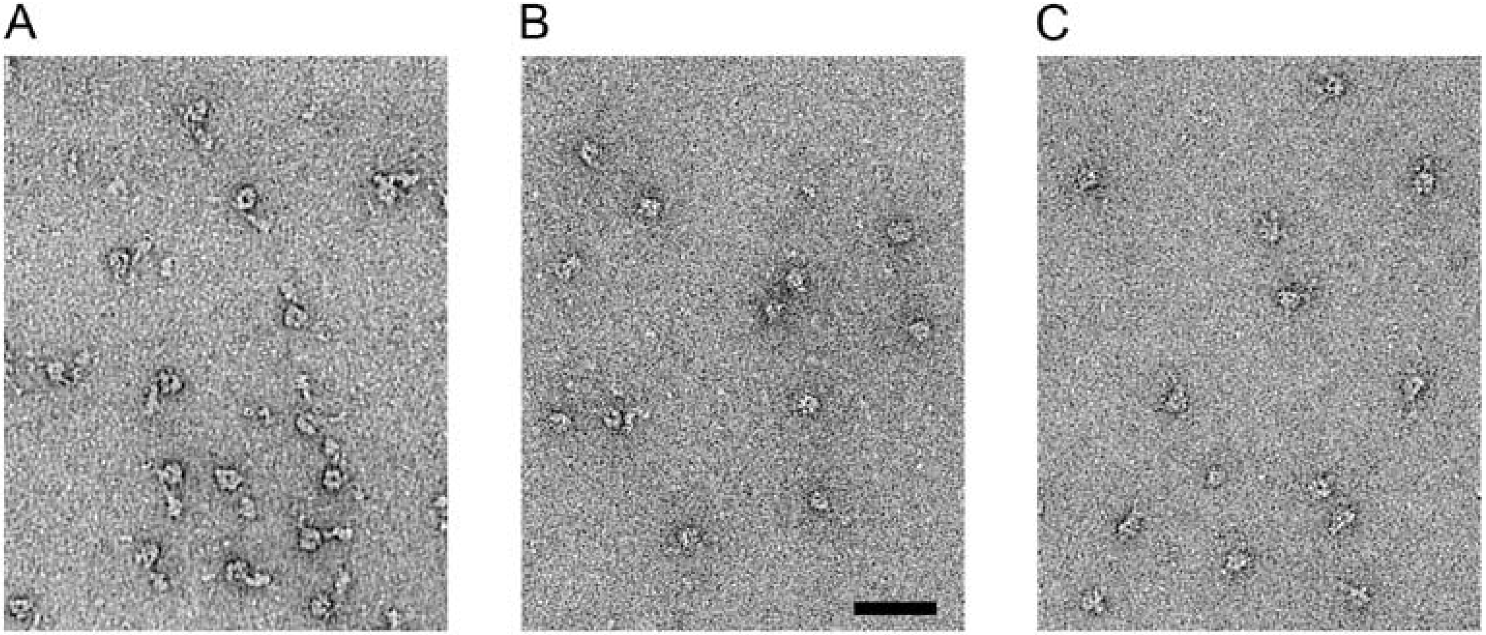


Fig. 7. Whole molecule averages of dynein. (A) Examples of individual head-aligned dynein molecules and (B) corresponding global average from this alignment. (C) Whole-molecule averages of molecules shown in (A) created by splicing together their corresponding stalk and stem class averages, illustrated for the last panel in (D). (E) Whole-molecule averages shown in (C) after alignment of their stems. (F) Global average of stem-aligned images.

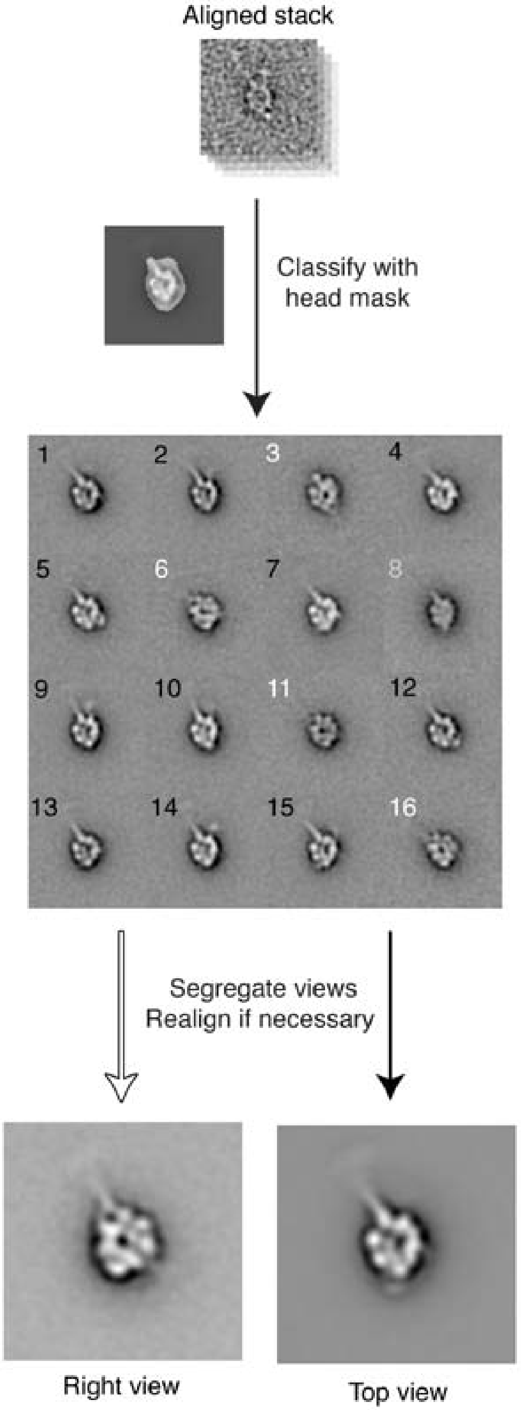
Using GFP based tags to map polypeptide path within macromolecules



Axonemal dynein isoform c

Cytoplasmic dynein motor

Cytoplasmic dynein GN-motor-B2



Aligned stack

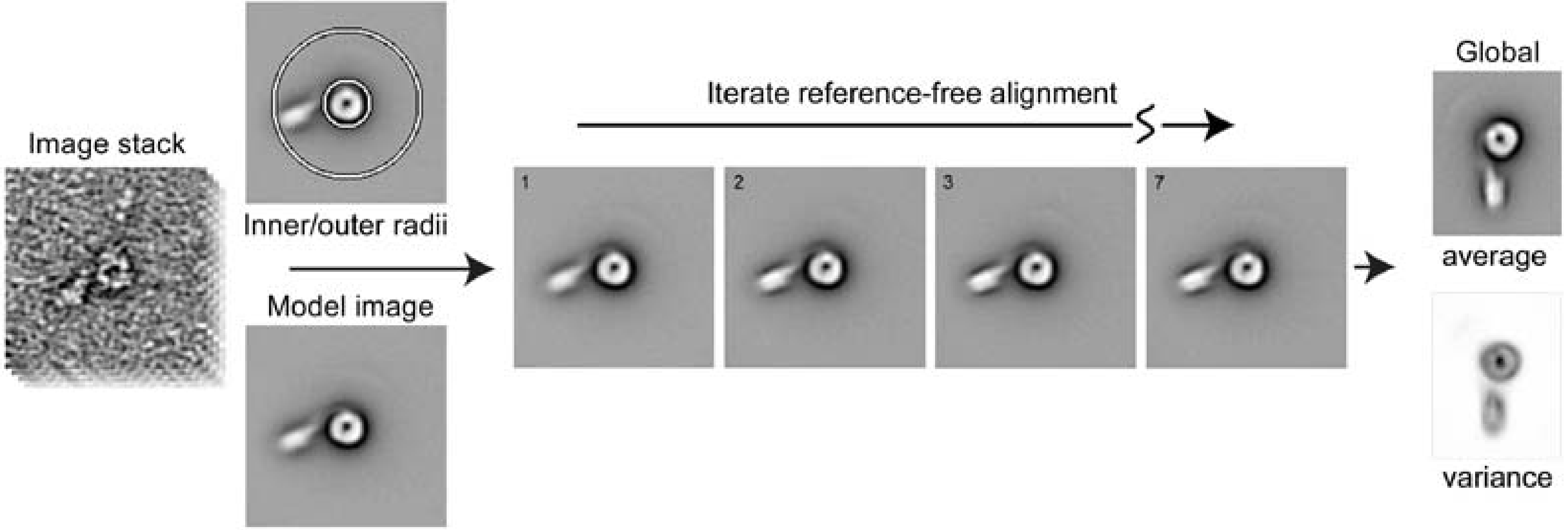
Classify with head mask

Segregate views
Realign if necessary

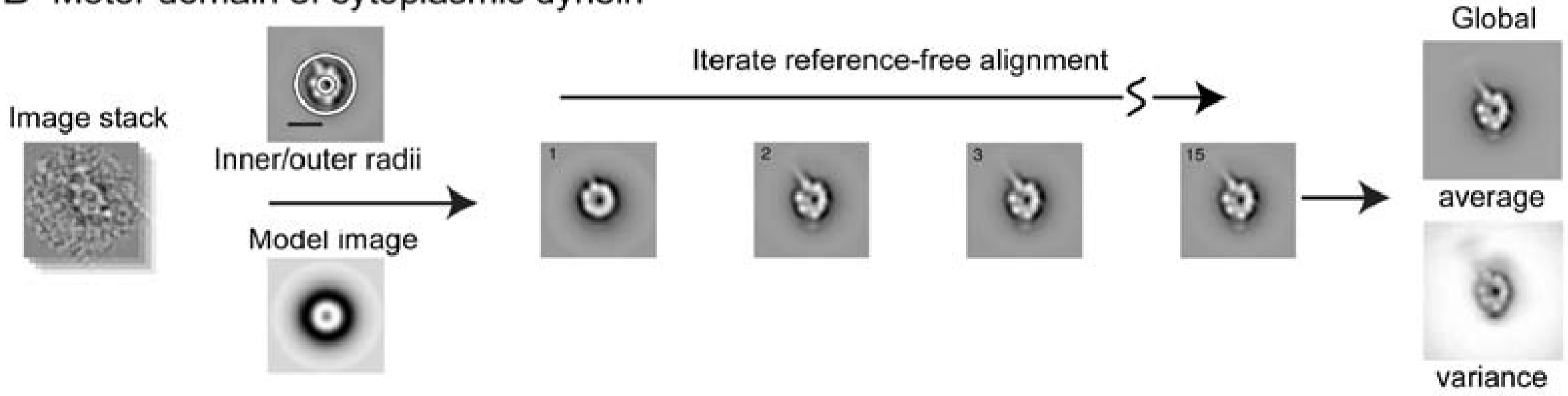
Right view

Top view

A Full-length flagellar dynein-c



B Motor domain of cytoplasmic dynein

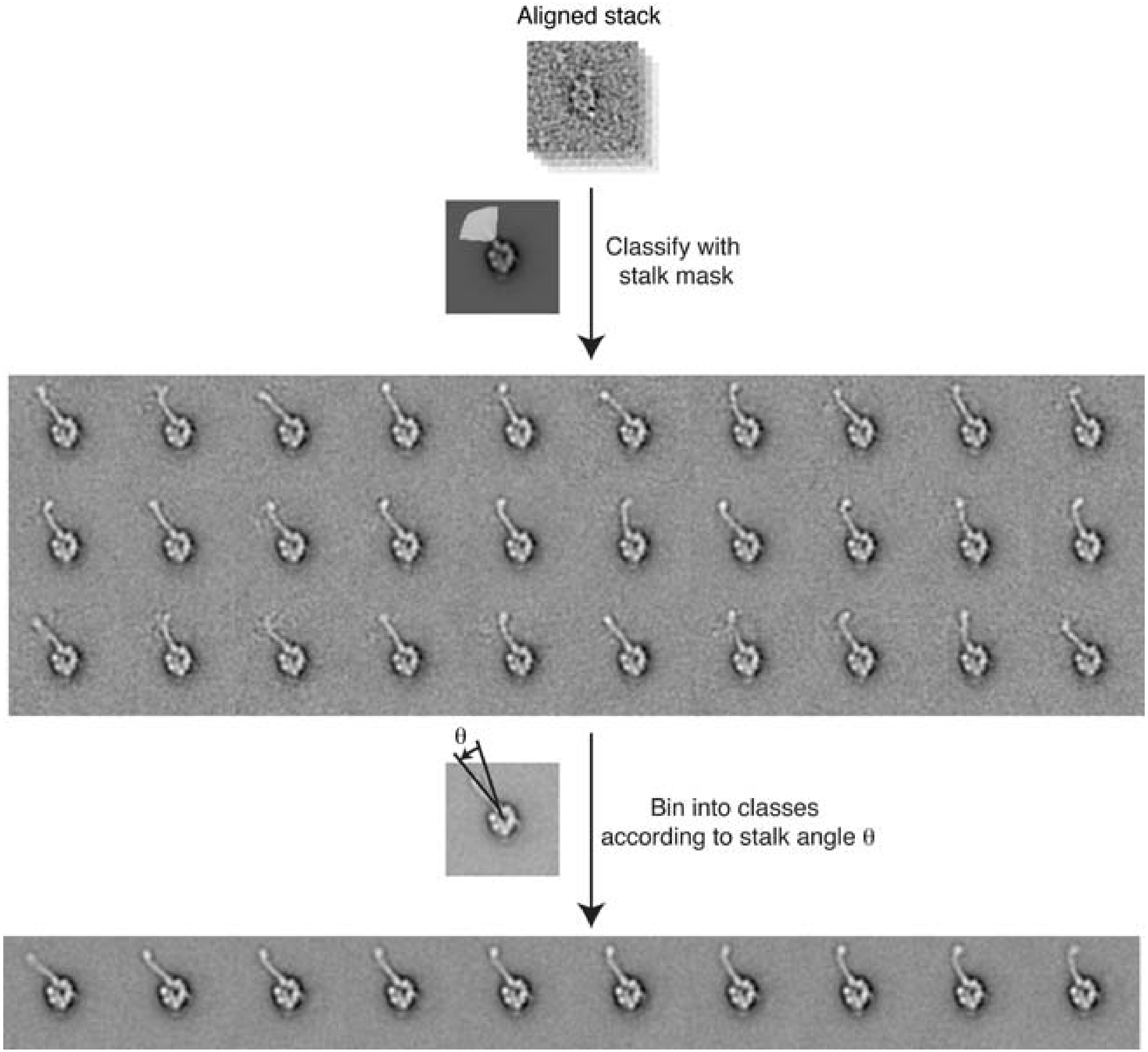


Classifying a flexible domain

Domain is not aligned
Classification mask must encompass all positions/conformations of flexible domain
Mask typically much larger than flexible domain
Classification includes considerable amount of background -leads to poor classes
How to get around this problem?

Two solutions

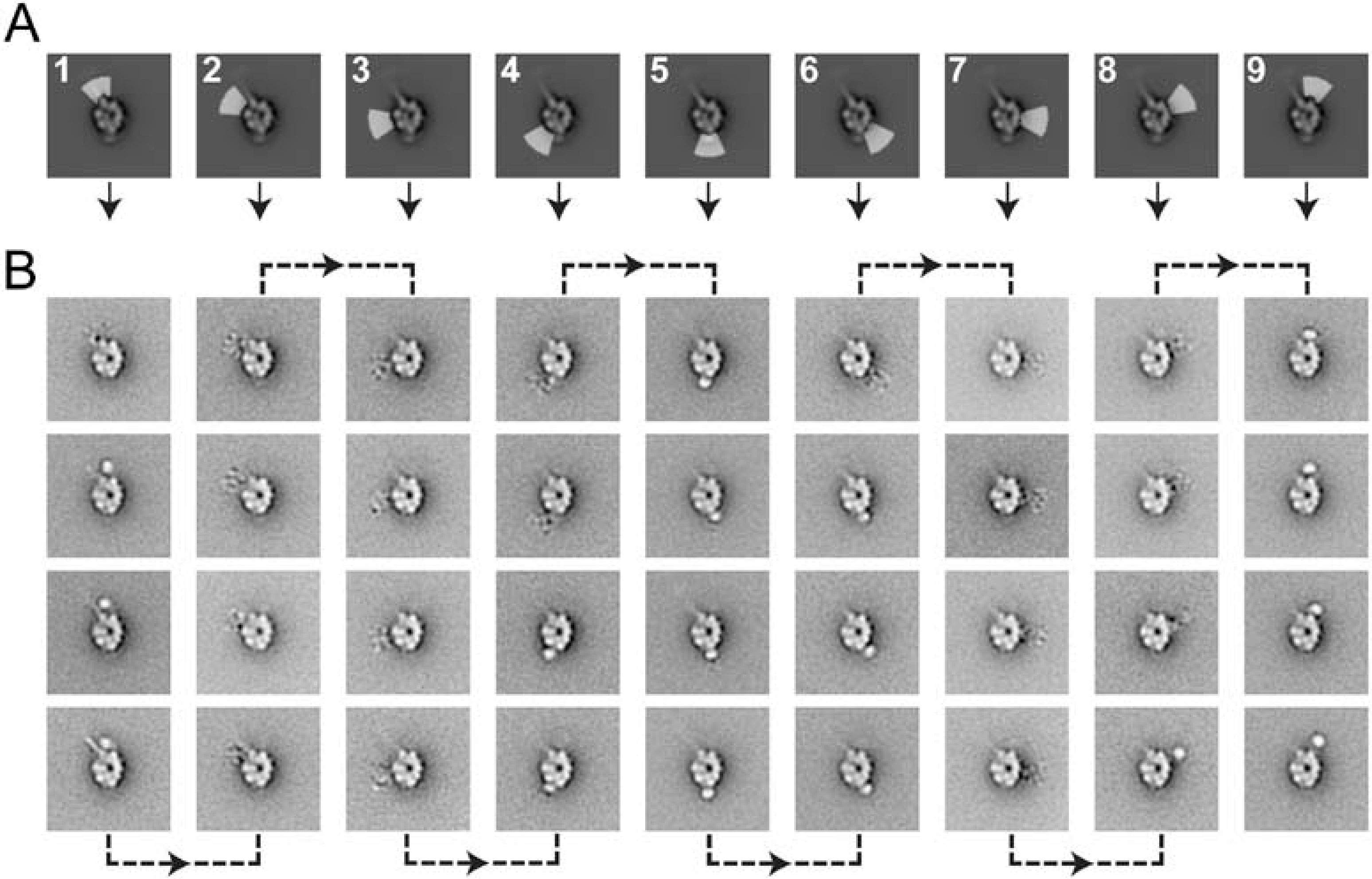
1) Classify according to mask then identify position of domain and reclassify based on coordinates
2) Classify only a small portion of potential flexible domain area and repeat



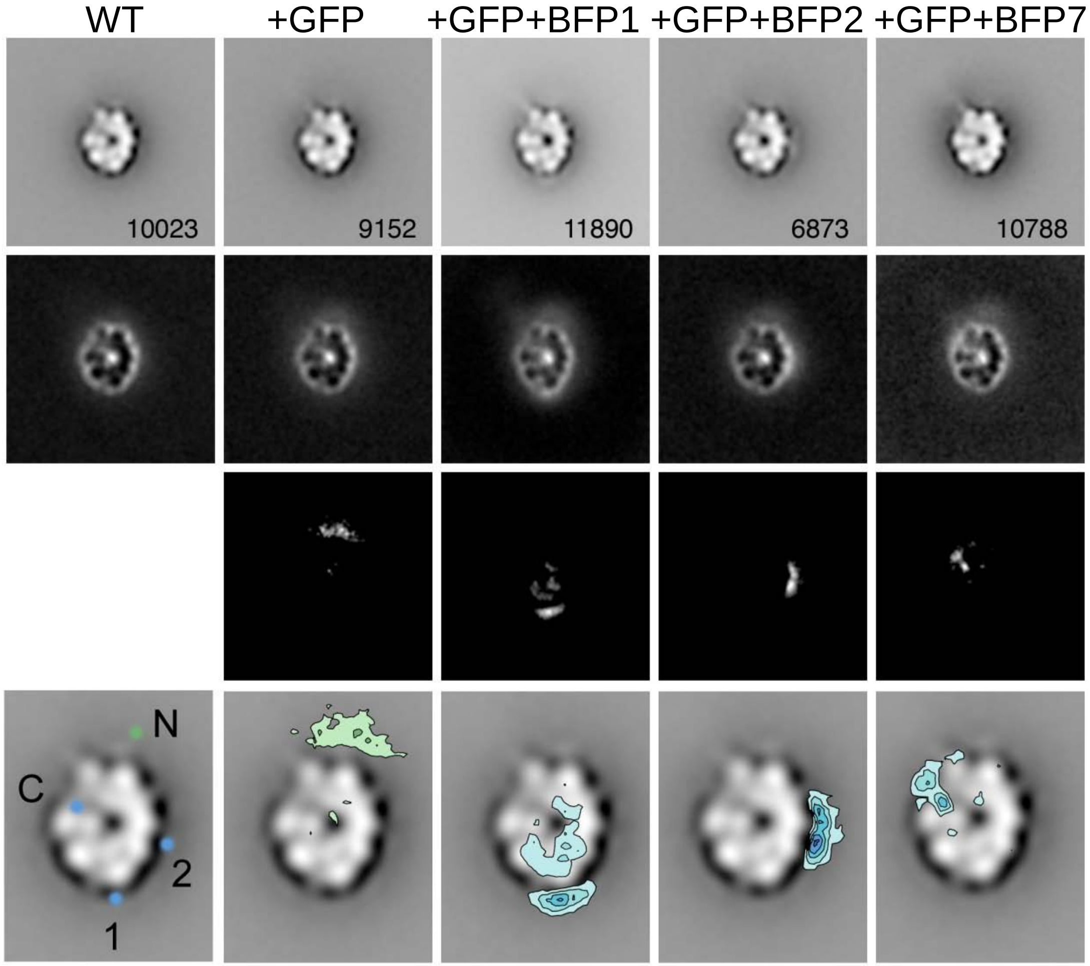
Aligned stack

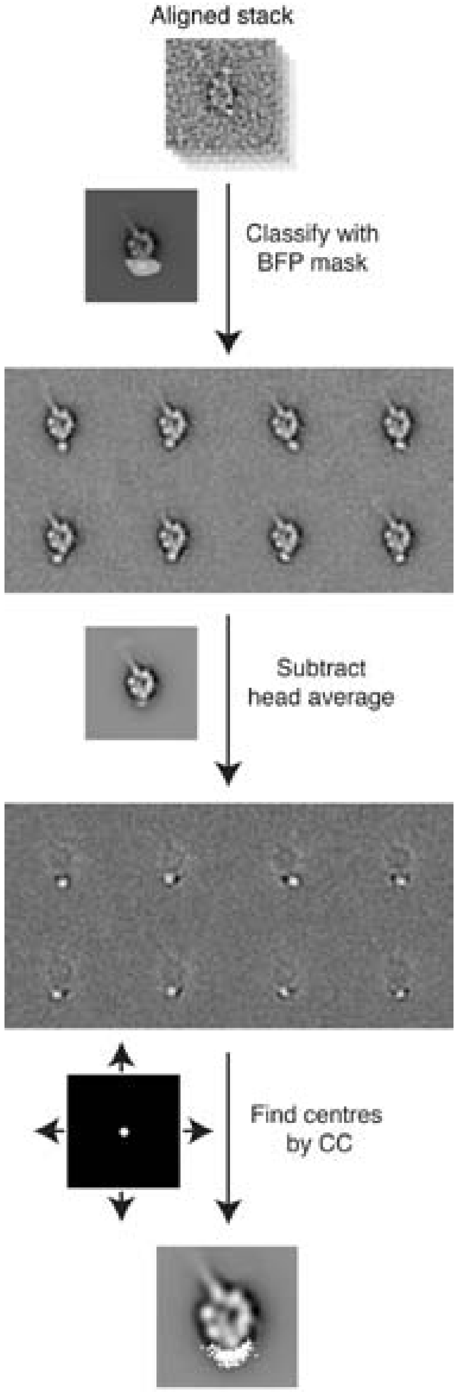
Classify with stalk mask

Bin into classes according to stalk angle θ


A
1
2
3
4
5
6
7
8
9
B

Difference mapping to summarize position of (unseen) flexible domain





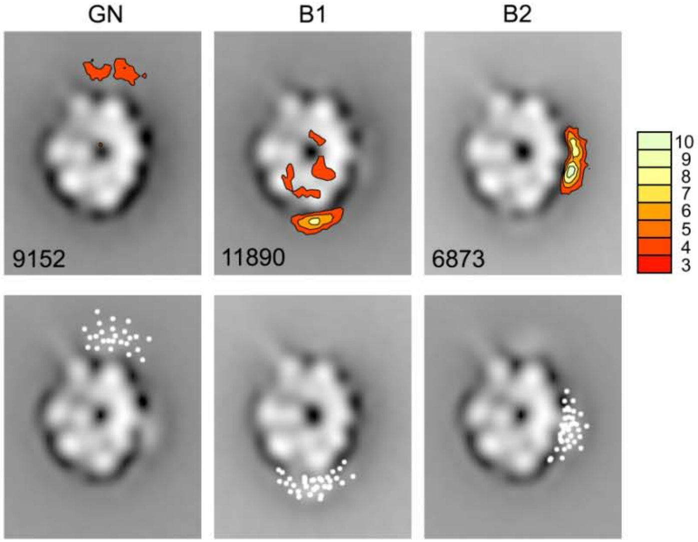
Aligned stack

Classify with
BFP mask

Subtract
head average

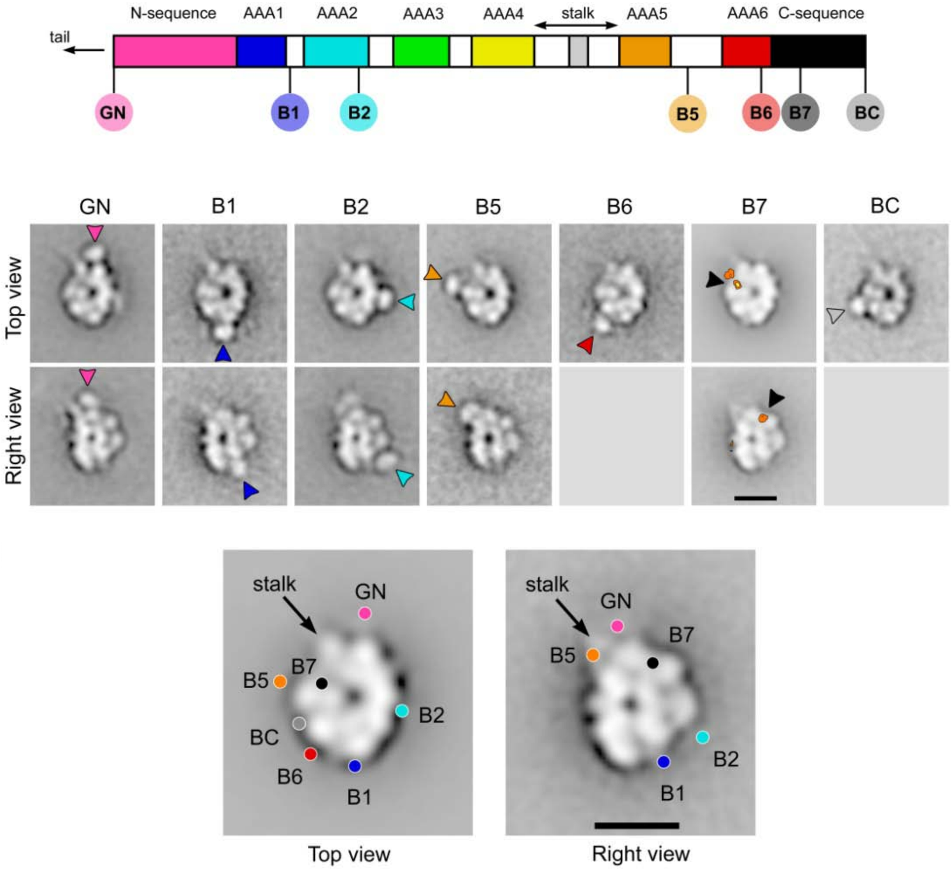
Find centres
by CC

Correspondence between difference mapping and auto-detection



Evidence for the linker in recombinant cytoplasmic dynein

Roberts *et al* (2009) Cell 136, 485-495

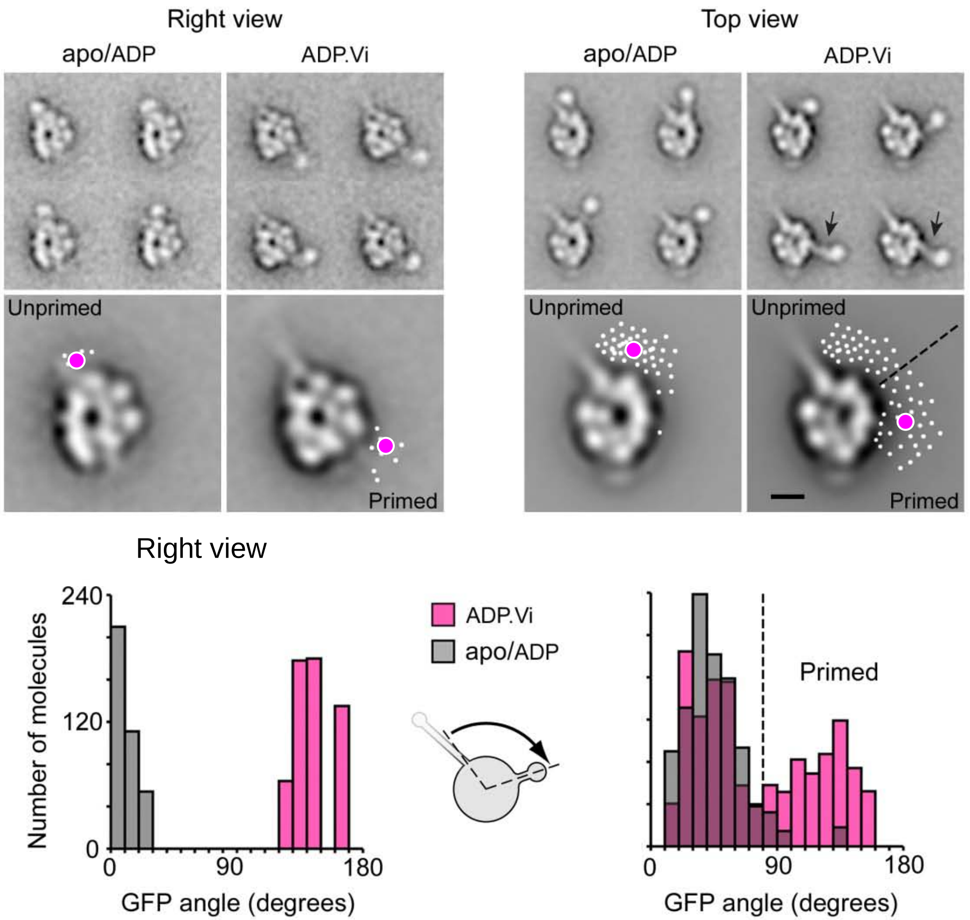


Two characteristic views- both rather asymmetric

GFP-based tags detected by negative stain EM

GN and B1 tags located at opposite sides of the head- intervening sequence must span the head

Structure of the motor in ADP.Vi (“primed” conformation)



In top view there is a broad distribution of linker positions in ADP.Vi

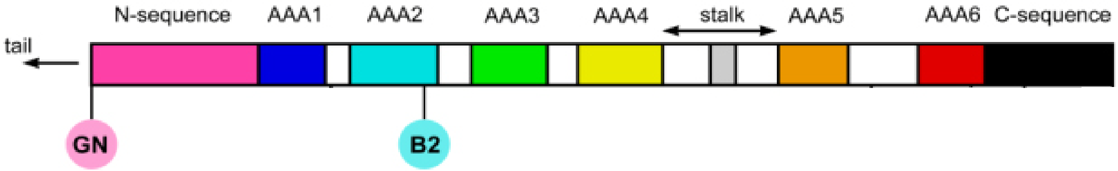
Two populations-
a ***mixture*** of
unprimed and primed
linker positions

N-terminal GFP
In unprimed motor is close to stalk base
In primed motor is near AAA2

Roberts *et al* (2009) Cell 136, 485-495

Evidence for the linker in recombinant cytoplasmic dynein

Roberts *et al* (2009) Cell 136, 485-495



Movie demo

N-terminal GFP (GN) moves from base of stalk towards AAA2 (B2) during priming stroke (apo/ADP to ADP.Vi)

Are any of these techniques useful for cryo-EM data?

QuickTime™ and a
TIFF (LZW) decompressor
are needed to see this picture.

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TIFF (LZW) decompressor
are needed to see this picture.

Negatively-stained molecules
Adsorbed to a carbon film
dried and embedded in heavy metal stain

Frozen-hydrated molecules
Adsorbed to thin carbon film or not
not dried, not embedded in stain

Tail is flexible also in cryo-EM
Variance images can be used to show its position

Negative stain

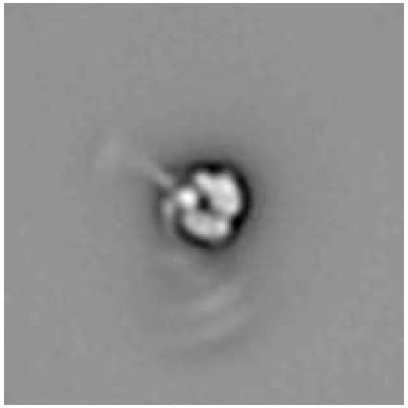

Image average

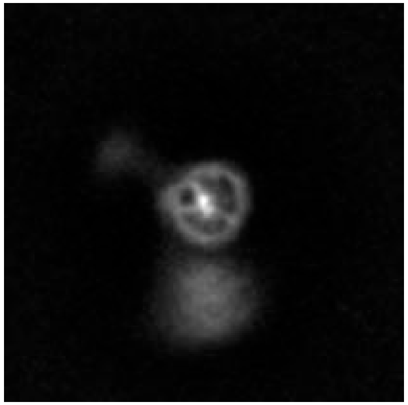

Image variance
(white =high)

Cryo-EM

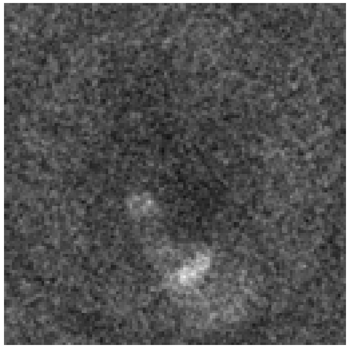

Image variance

Back-project 2D image variances of cryo data to create 3D variance map of tail position

Summary

- Conformational variability can be studied by EM
  - in negative stain easily and also in frozen-hydrated specimens
  - start with many molecules (> 10,000)
  - a wealth of biologically relevant information can be extracted, if done with care

- Crucial to obtain a robust reliable alignment of molecules (invariant part)
  - First and second rounds of alignment
  - Third round e.g. using class averages (to change which part is fixed)
  - Obtain coordinates from classes and rewindow from original micrographs
- Segregate views from first round of alignment
  - Improves subsequent classification
  - Subtract invariant part (global/class average) to reveal variable domain(s)
  - Automatic detection
  - Scanning classification
- Image variances are often very helpful in understanding heterogeneity
  - Locating variable domain lost in average
  - Difference mapping
  - Designing masks for classification
  - Back-projected to create 3D envelope
- SPIDER has many useful features for image processing and analysis
  - Scripting/automation HOUSEKEEPING essential
- Crucial to examine data carefully and critically at all steps in processing
  - (easy to make a mistake and produce a convincing untruth)
  - there is no substitute for a critical eye