Molecular Tomography - Imaging Molecular Conformation using EM Tilt Series

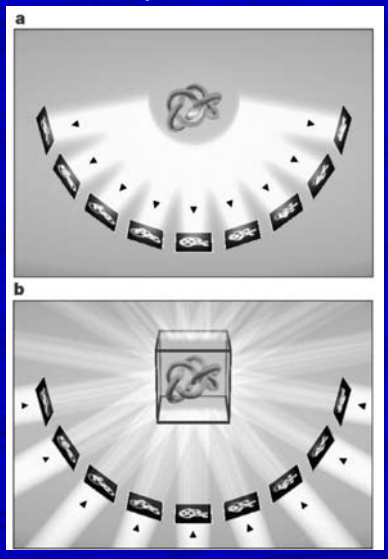
Outline

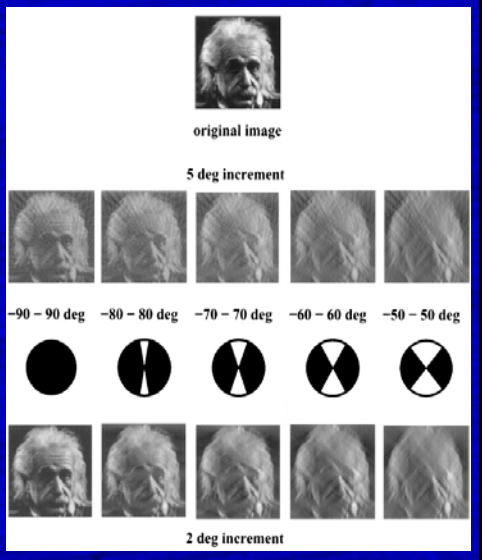
1. Why?
2. Methods
   a. Marker free alignment
   b. Saxton Rule (cosine rule) angles
   c. Multivariate Statistical Analysis
   d. Defocus gradient compensation
3. 3 specimens
   a. Insect Flight Muscle
   b. HMM decorated actin
   c. Actin-villin rafts
   d. 2-D arrays of myosin V

Why Use Electron Tomography to Determine the Structure of a Macromolecule?

- Your molecule is ***big***, ***bad*** & ***ugly*** & there is not much of it (there may only be one of it)
- You know the structure of your molecule in one context (e.g. in a crystal, detergent) & want to verify its structure in another (e.g. in situ, in a membrane)
- Your molecule is conformationally/ compositionally variable as part of its function (e.g. components bind weakly)
- You need to visualize your molecule in a difficult to trap state, i.e. under tension

Principle of Electron Tomography

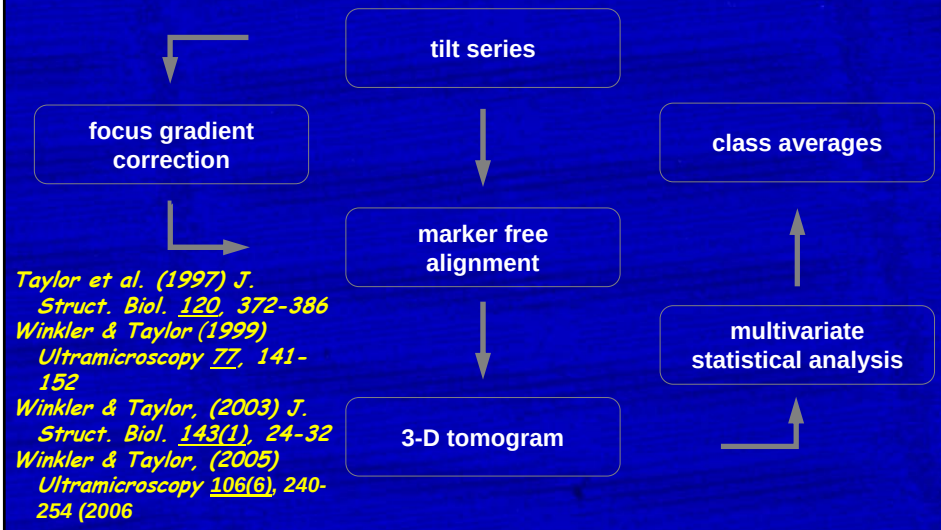




Sali A. et al. Nature (2003)

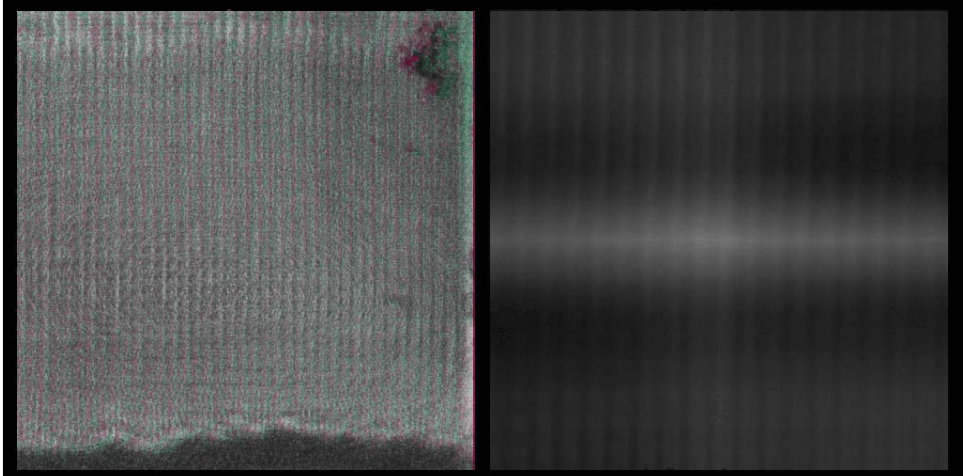
Koster B. et al. JSB (1997

Tomography Flowchart



Taylor et al. (1997) J. Struct. Biol. 120, 372-386
Winkler & Taylor (1999) Ultramicroscopy 77, 141-152
Winkler & Taylor, (2003) J. Struct. Biol. 143(1), 24-32
Winkler & Taylor, (2005) Ultramicroscopy 106(6), 240-254 (2006

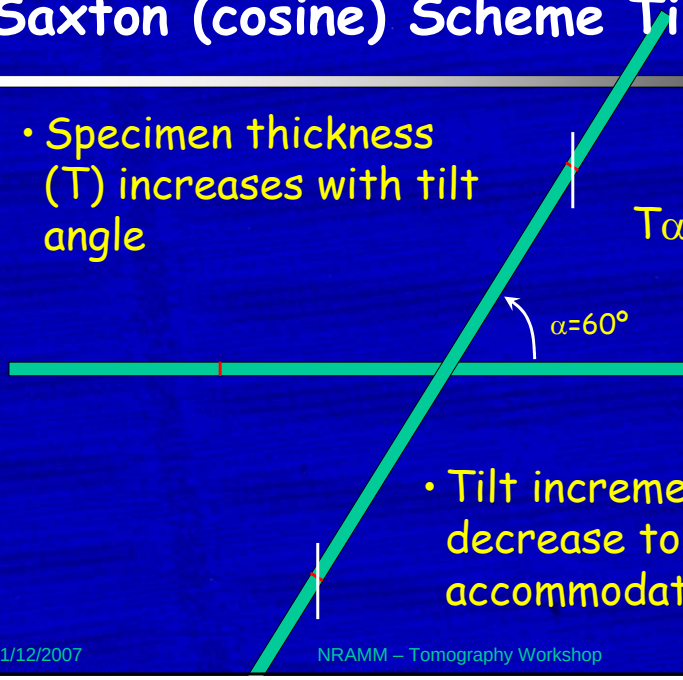
Marker Free Alignment



Saxton (cosine) Scheme Tilt Angles

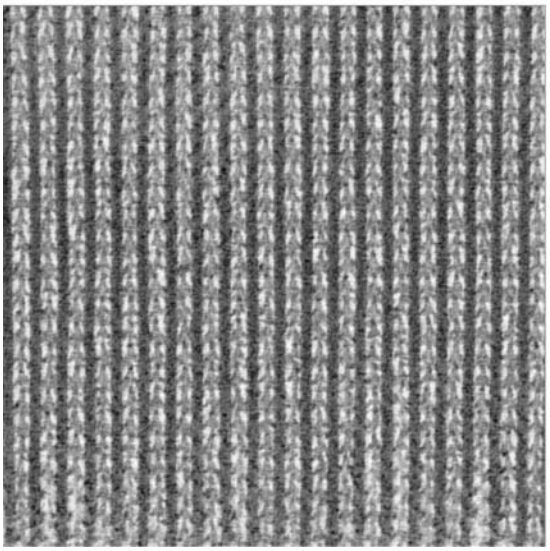
- Specimen thickness (T) increases with tilt angle

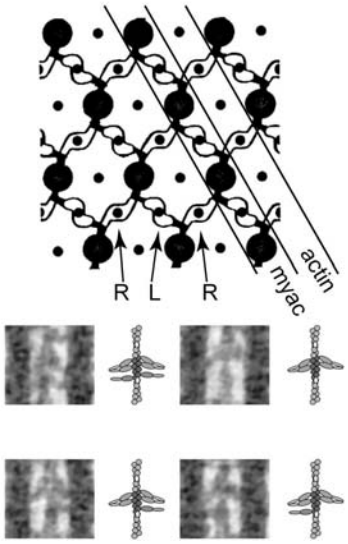
$T\alpha = T0/\cos\alpha$



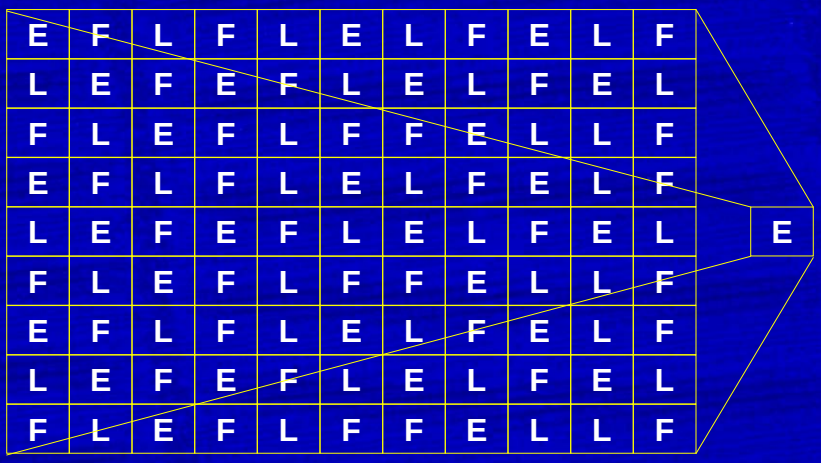
- Tilt increment must decrease to accommodate it

Muscle is Structurally Heterogeneous



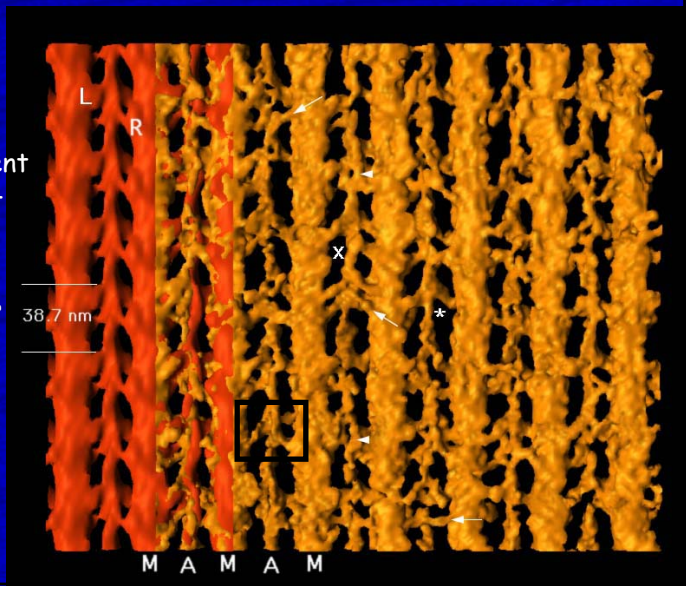


Spatial Averaging



Rigor IFM Tomogram

- orange = spatial average
- gold = tomogram
- M = myosin filament
- A = actin filament
- L = averaged lead chevron
- R = averaged rear chevron
- * = lead chevron
- X = missing rear bridge
- --> = rear bridges



"Classified" Averaging

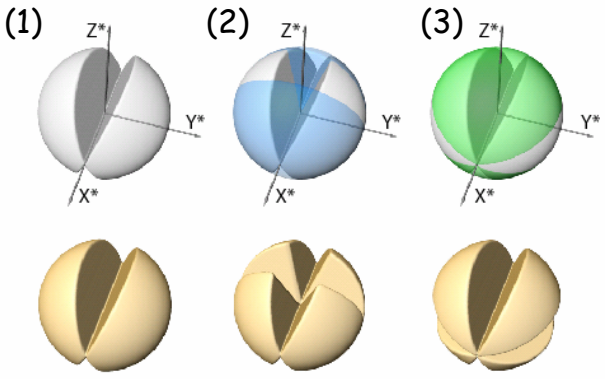
E	F	L	F	L	E	L	F	E	L	F
L	E	F	E	F	L	E	L	F	E	L
F	L	E	F	L	F	F	E	L	L	F
E	F	L	F	L	E	L	F	E	L	F
L	E	F	E	F	L	E	L	F	E	L
F	L	E	F	L	F	F	E	L	L	F
E	F	L	F	L	E	L	F	E	L	F
L	E	F	E	F	L	E	L	F	E	L
F	L	E	F	L	F	F	E	L	L	F

F

E

L

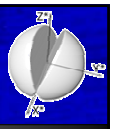
The Missing Wedge – 3 Conditions



1. Repeats (subvolumes) have similar orientation w.r.t missing wedge
2. Repeats differ mainly by rotation about z
3. Repeats differ in rotation about all 3 Euler angles

Our Model System: Insect Flight Muscle of *Lethocerus sp.*

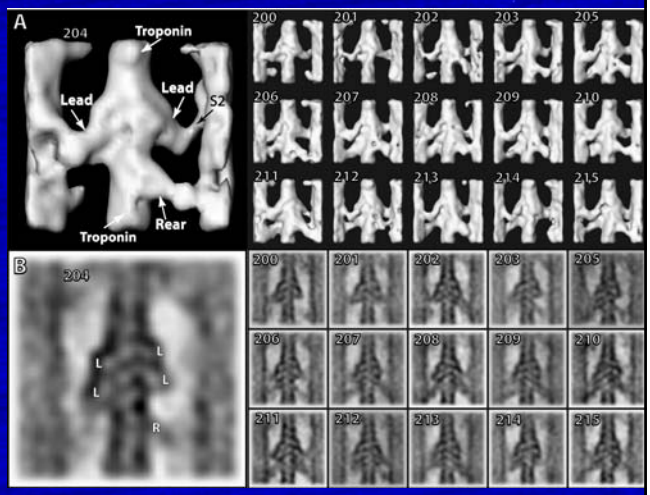
20 Class Averages from IFM Rigor



- Dual axis tomogram (on film)
- Hierarchical Ascendant Classification (HAC)
- Multireference alignment
- Not all classes average the same number of repeats
- Resolution ~7 nm

Stretch Rigor Fiber - Class Averages

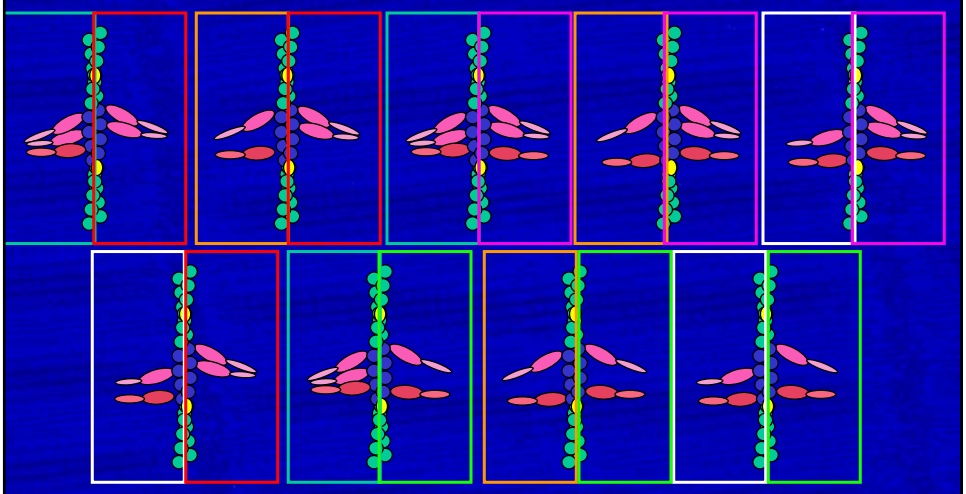
- Single reference alignment - each repeat aligned on a thin filament reference
- Classify with a mask that includes crossbridges,& no thin filament



Alignment & Classification Scheme

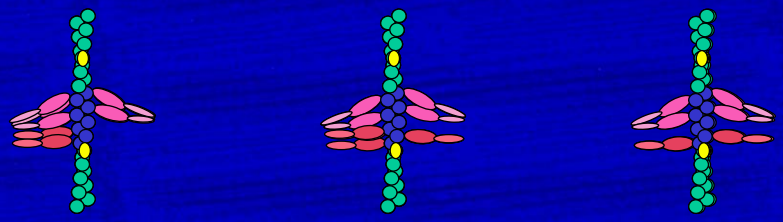
I. Align each of N repeats (38.5 nm actin periods) on the actin filament
II. Classify the repeats based on the crossbridges
  A. How many classes?
III. 2 classification models
  A. Create $N^{1/2}$ class averages of complete repeats
  B. Classify separately left & right sides of actin filaments
    1. $N^{1/2}$ left side class averages
    2. $N^{1/2}$ right side class averages
  C. Signal to noise improvement is $N^{1/4}$ for both models
IV. Then reassemble the tomogram by replacing the raw repeats with the class averages
  A. $N^{1/2}$ class averages of complete repeats
  B. $N^{1/2} * N^{1/2} = N$ class averages reconstituted from left & right sides

Classifying Heterogeneous Structures



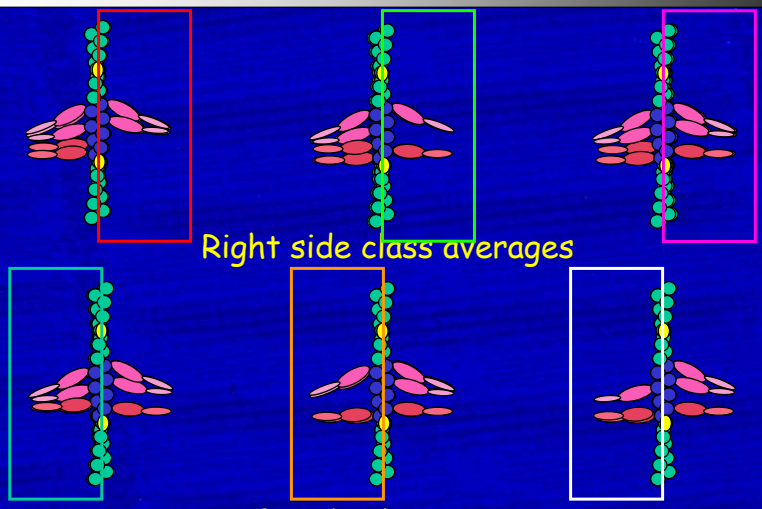
Heterogeneous set of motifs with no 2 alike

Classifying Heterogeneous Structures

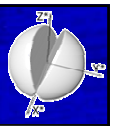


Left + right side class averages

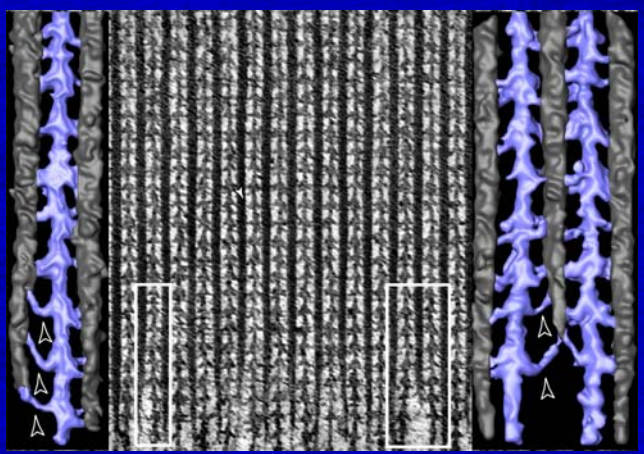
Classifying Heterogeneous Structures



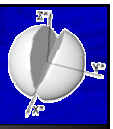
Swollen Rigor IFM



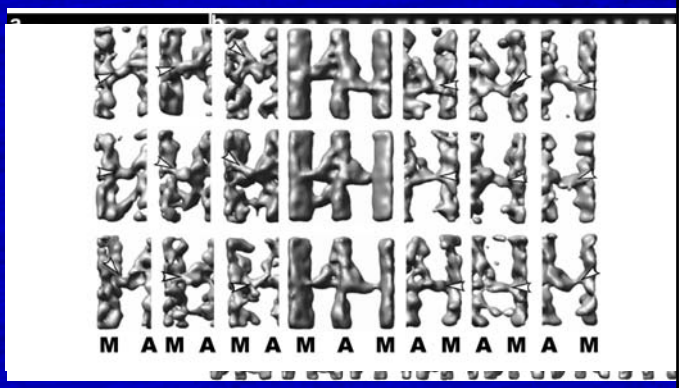
- Single axis tomogram (on film)
- Single reference alignment - each repeat aligned on a thin filament reference
- Classify with a mask that includes crossbridges,& no thin filament



Swollen Rigor IFM



- Single axis tomogram (on film)
- Single reference alignment
- 1-sided left & right classification
- Combine classes



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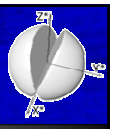

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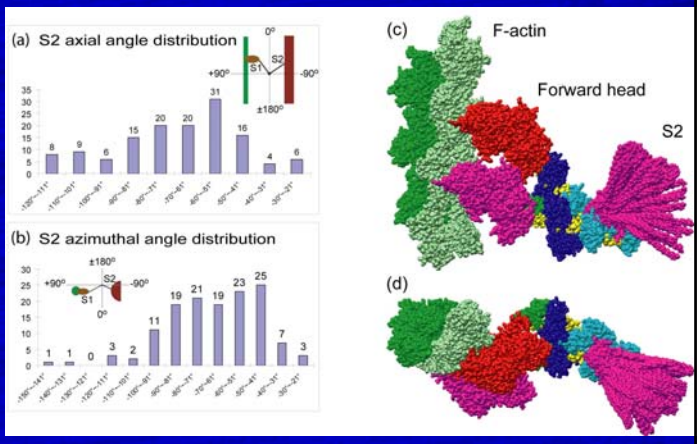

Visualizing Active Muscle Crossbridges

Multivariate Data Analysis of Subvolumes from Tomograms of Quick-frozen, Ca^{2+}-activated Isometrically Contracting Insect Flight Muscle Reveals Unconventional Cross-bridge Conformations

Visualization of Actin Filament Decorated with Smooth Muscle HMM by Cryo Electron Tomography

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Kathy Trybus[2] and Kenneth A. Taylor[1]

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Structure of the Villin-Actin Cross-link using Single-Particle Analysis of 3D Volumes

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Taylor Lab
Institute of Molecular Biophysics
Florida State University

3D Structure of Myosin-V in Inhibited State

Pushing the resolution in Cryo-Electron Tomography

Jun Liu

The Institute of Molecular Biophysics
Florida State University

Taming "The Big, the Bad & the Ugly"

1. Efficient subvolume alignment & averaging techniques absolutely essential
2. Multivariate Statistical Analysis (classification) is an important component for extracting molecular conformations from subvolumes
3. ~20Å possible from tomograms of ice embedded specimens

Visualizing Active Muscle Crossbridges

Multivariate Data Analysis of Subvolumes from Tomograms of Quick-frozen, Ca^{2+}-activated Isometrically Contracting Insect Flight Muscle Reveals Unconventional Cross-bridge Conformations

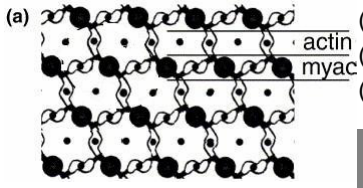
1

Acknowledgement

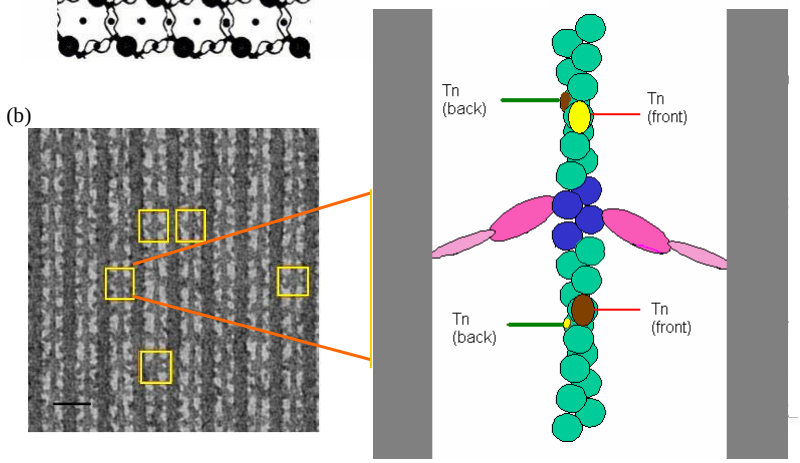
- Jun Liu, Mary C. Reedy, Richard T. Tregear, Hanspeter Winkler, Clara Franzini-Armstrong, Hiroyuki Sasaki, Carmen Lucaveche, Yale E. Goldman, Michael K. Reedy, and Kenneth A. Taylor
- Funding from NIGMS

2

Insect flight muscle (IFM)



(a) A cross section of *Lethotherus* IFM
(b) Projection of the tomogram of a myac layer
(c) Myosin shelf origins (Sh) and actin target zone (Ta)



3

HST state--the experimental model

- The fully active isometric state in IFM
- High static tension (HST) state: it can reach tension levels comparable to stretch activation.
- Active cross-bridges show a wide range of attachment angles, from pre-stroke to rigor-like end-stroke.

4

Bridges outside of actin target-zone

- Unique properties of IFM thin filament and myosin head:

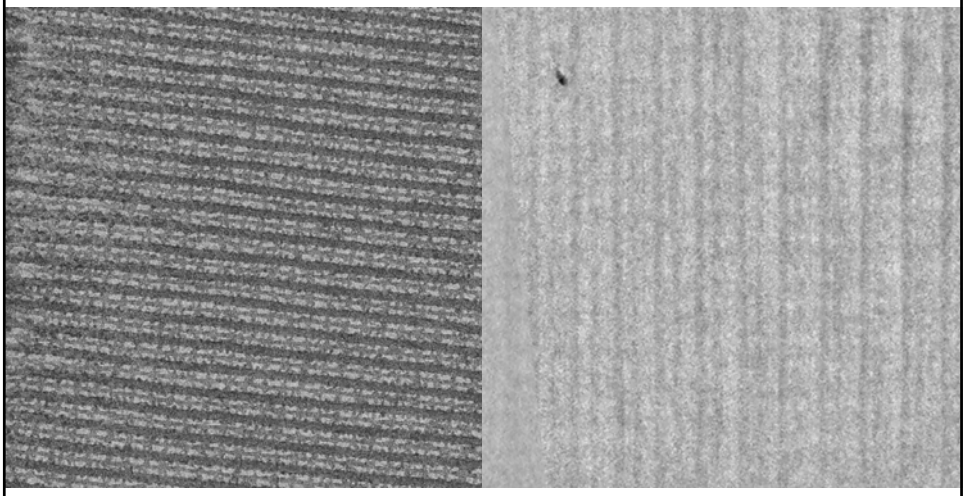
  --High molecular weight tropomyosin isoforms (historically called “heavy troponin”, TnH)

  --Tn isoforms with different Calcium affinity (F1, F2)

  --Myosin regulatory light chain N-terminal extension

Links between thick and thin filaments other than the predominant target zone bridges.

5



- (a) collect 2D images that are projections of a 3D object at various orientations
- (b) a 3D image of the object is reconstructed by projecting all 2D images back into a common volume

3D image (repeat sub-volume) processing

- 1. Sub-volume extraction
- 2. Sub-volume alignment
- 3. Masks for multivariate data analysis (MDA)
- 4. MDA and classification
- 5. Repeat reassembly

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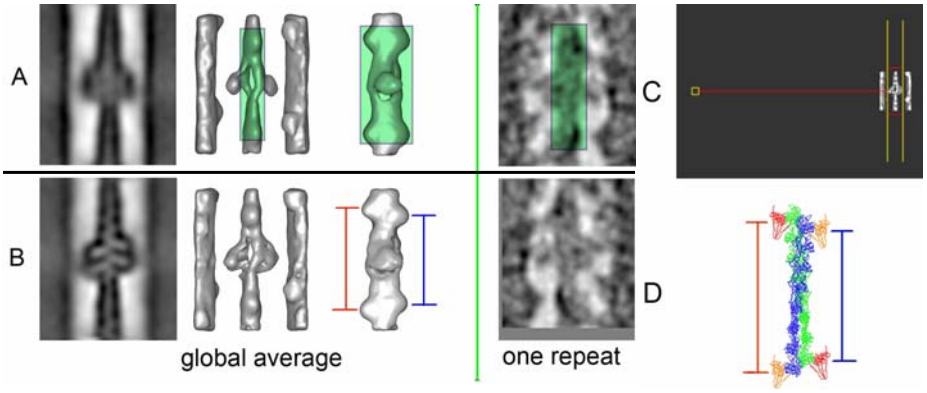
Repeat Sub-volume Alignment

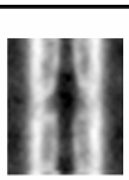


- **A**: before alignment; **B**: after alignment.
- Alignment involves x, y, & z translation and 180° rotation along the thin filament axis.

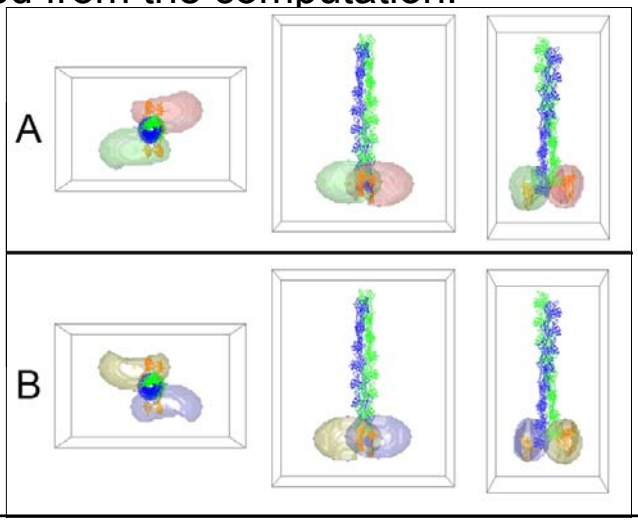
8

Masks for MDA

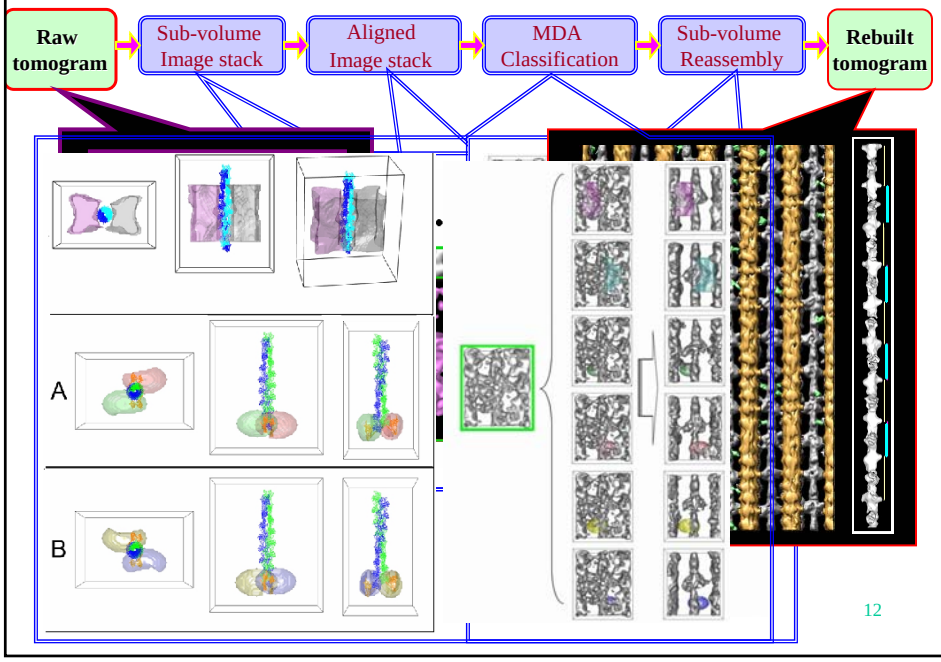
- Only those pixels within the mask are subjected to MDA. Pixels outside the mask are excluded from the computation.

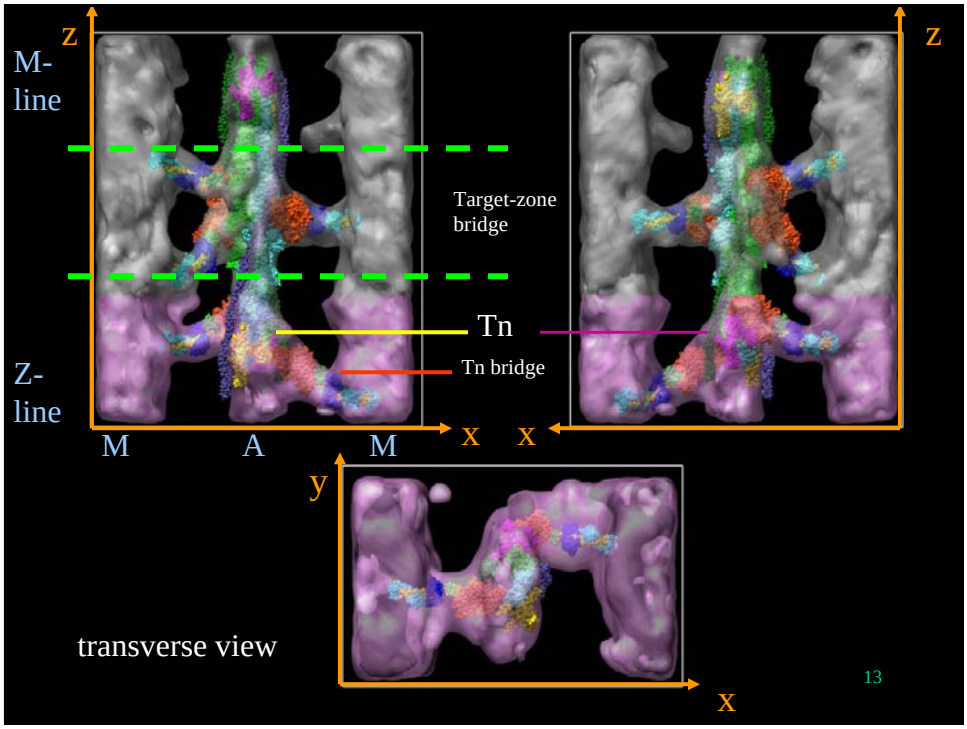


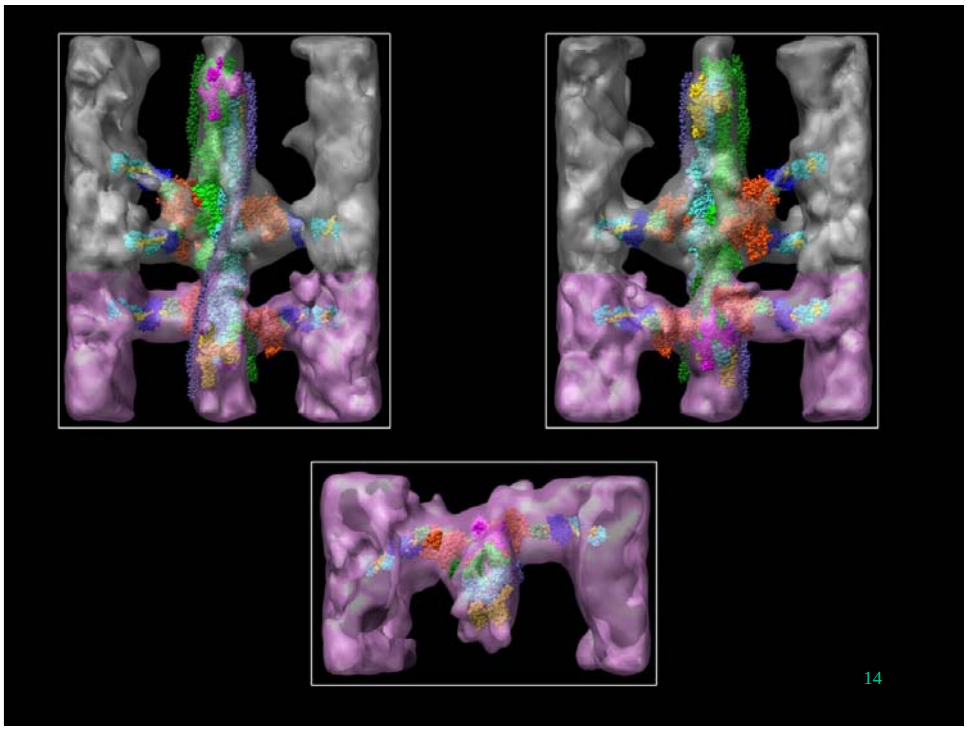
A projection image of the global variance map



Journey of a cross-bridge repeat sub-volume




z
M-
line
Target-zone
bridge
Tn
Tn bridge
Z-
line
M
A
M
x
x
z
y
x
transverse view
13

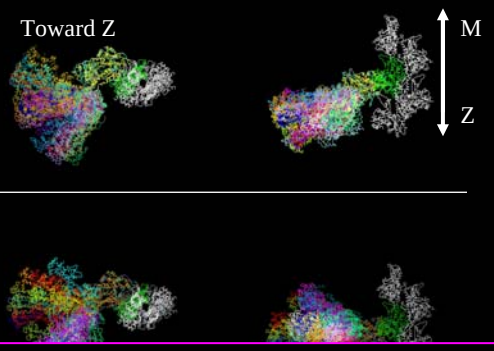

14

Evaluation and analysis

- Model fitting (fit atomic structures into class averages)

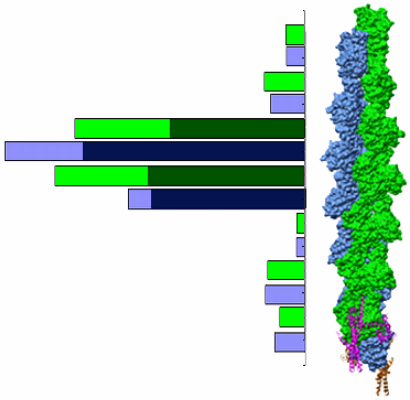
Motor domain in strong binding configuration.

Starting model--
Upper: Rigor (Holmes KC 2003)
Bottom: transition scallop (Houdusse A 2000)



Motor domain deviated from the strong binding position

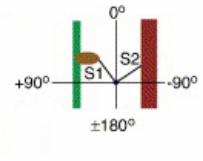
Actin occupancy by myosin heads

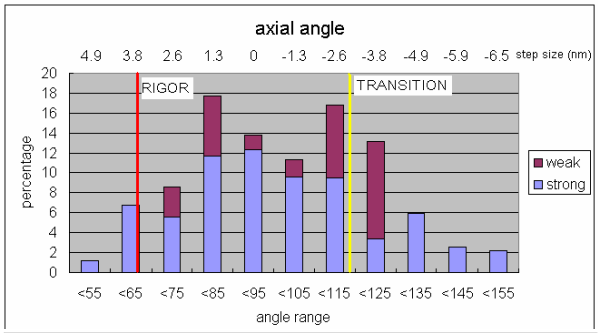


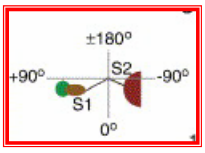
- Target-zone: actin H, I, J, K
- ~71% of target-zone bridges are at strong binding configuration

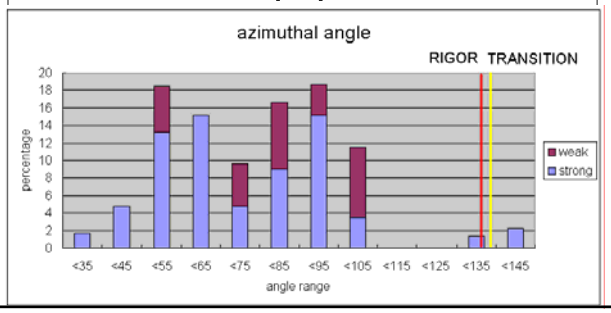
16

2 - lever arm









SUMMARY

- The revised image processing technique reveals many unconventional cross-bridge conformations
  -- a broad distribution of lever arm angles of strong binding bridges
  -- weak binding bridges on target-zone
  -- bridges formed outside of target-zone, e.g. Tn region
- Model fitting provides information beyond the resolution of density maps and helps the characterization of different bridges
- MDA and classification as data reduction tools, facilitate the statistics (e.g. actin occupancy, angle distribution)

19

Visualization of Actin Filament Decorated with Smooth Muscle HMM by Cryo Electron Tomography

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Kathy Trybus[#] and Kenneth A. Taylor[§]

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[#]Dept of Molecular Physiology & Biophysics, Univ. of Vermont College of Medicine, Burlington, VT 05405

Background – Acto-HMM

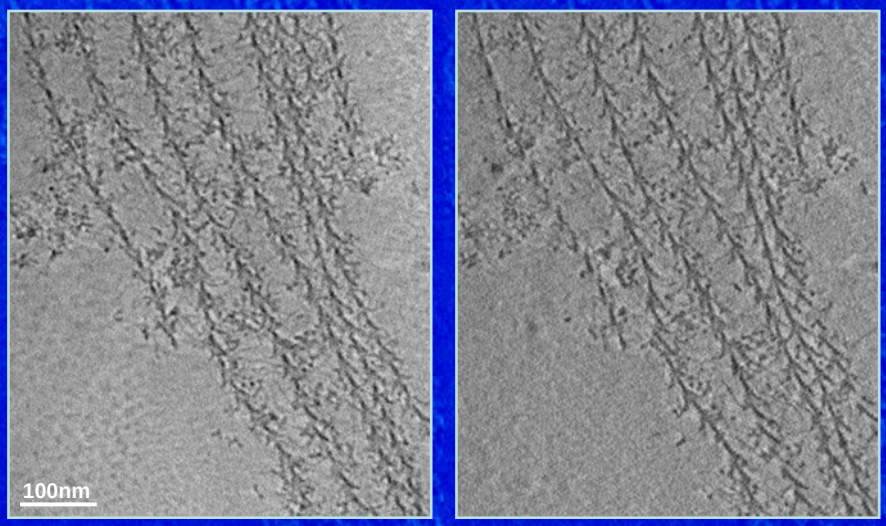
- Helical reconstruction of negatively stained specimens.
  - Katayama & Wakabayashi. (1991) The cardiac ... Chem. Analysis. (2002) Molecular modeling of average structure of actin-hea... Biochem. 90, 703-714
  - However, Acto-HMM is not a...
  - Liu et al., (2004) Electron tomography of fast frozen, stretched rigor fibers reveals elastic distortions in the myosin crossbridges. J. Struct. Biol. 147, 268–282.
  - Limited resolution

Tomograms of fixed, embedded & sectioned rigor insect flight muscle.

Conditions

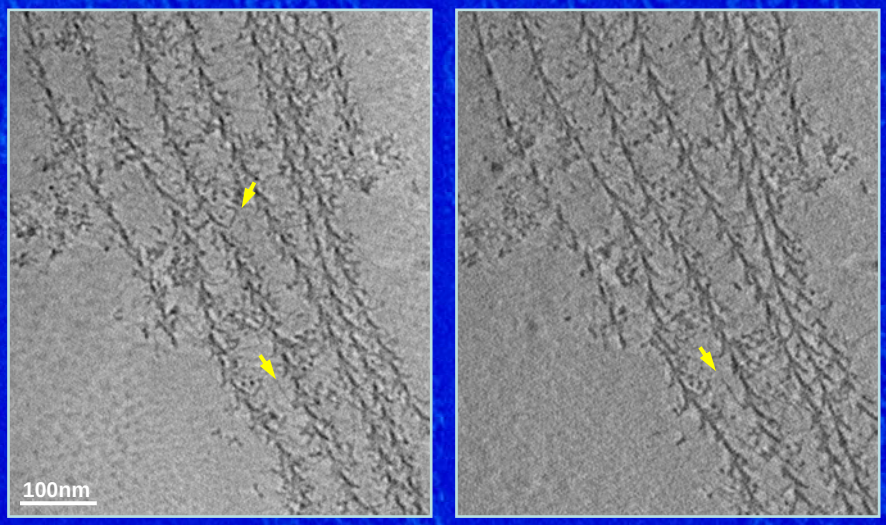
- Cryoelectron tomography to obtain 3-D image
- Acto-HMM rafts formed on a lipid monolayer by polymerizing actin in the presence of HMM
- Conditions –
  - 1 mM ADP, 20 mM Pi, 2 mM Mg^{2+}, 80 mM KCl
- A novel approach developed to obtain the 3-D averaged structure of acto-HMM

Myosin HMM Decorated Actin Filament

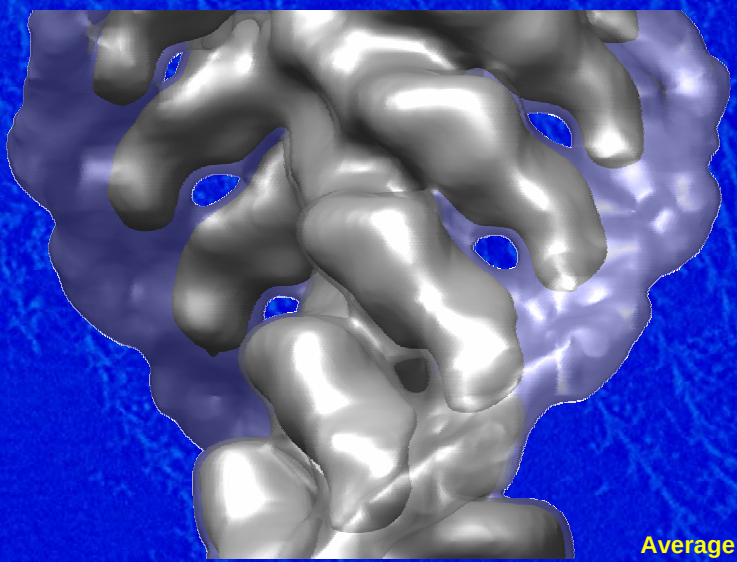


projection one Z-section

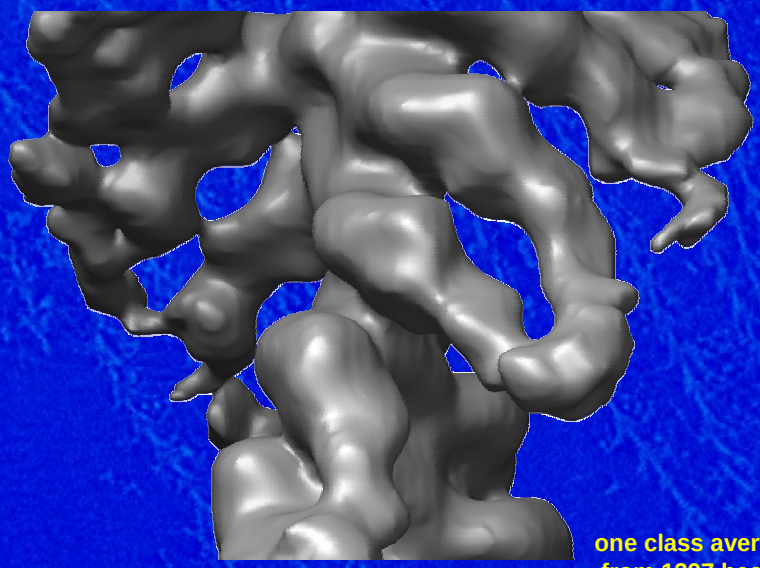
Cryo Tomgram Reveals S2



HMM Decorated Actin Filament

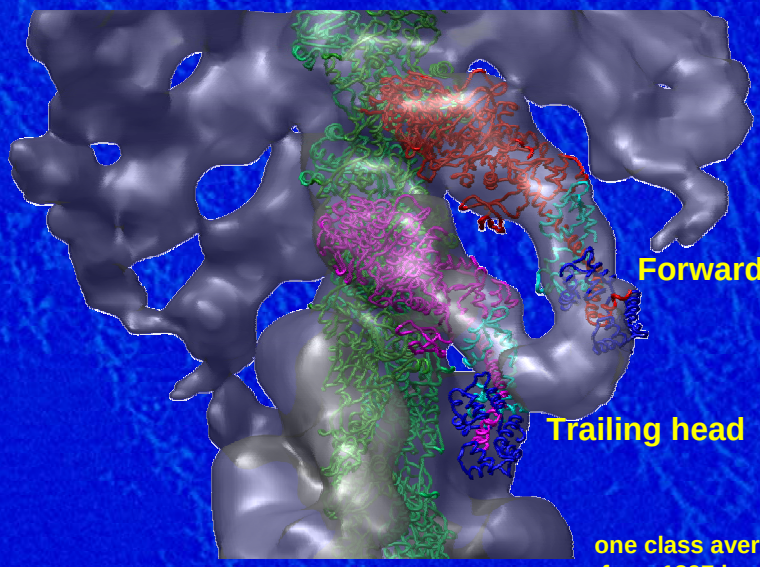


HMM Decorated Actin Filament



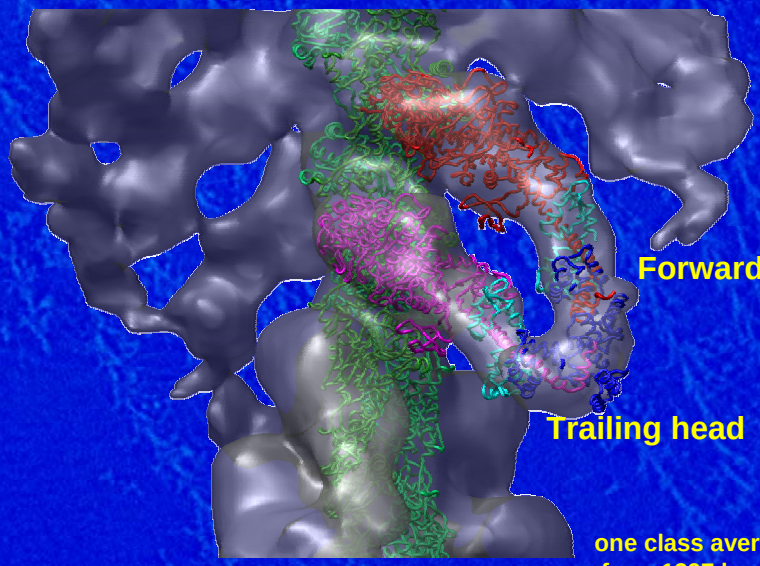
one class average
from 1397 head pairs

HMM Decorated Actin Filament



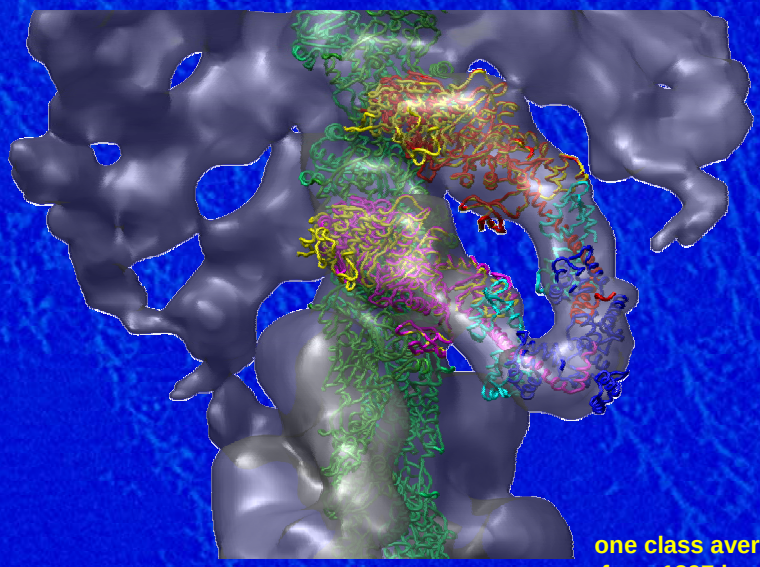
one class average
from 1397 head pairs

HMM Decorated Actin Filament



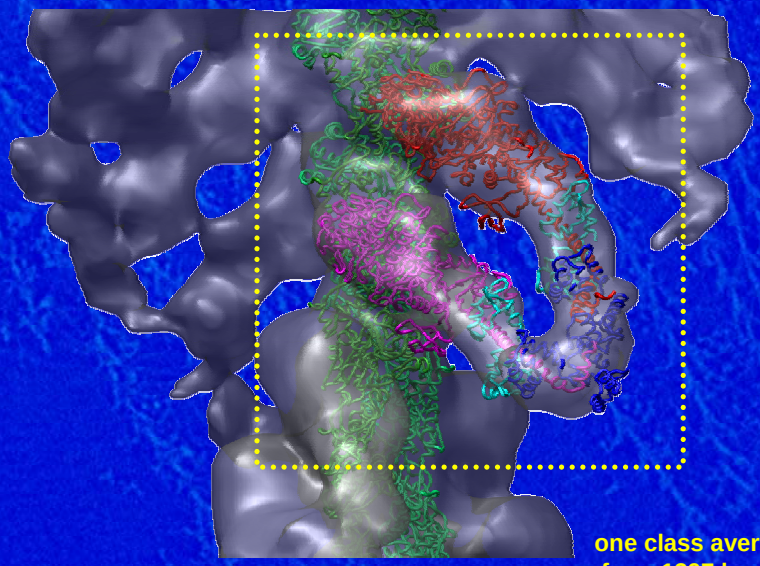
one class average
from 1397 head pairs

HMM Decorated Actin Filament



one class average
from 1397 head pairs

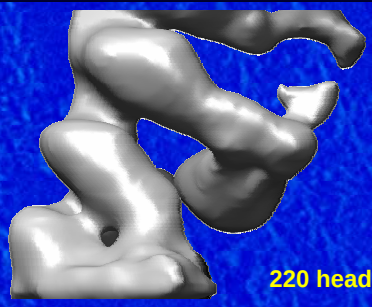
HMM Decorated Actin Filament



one class average
from 1397 head pairs

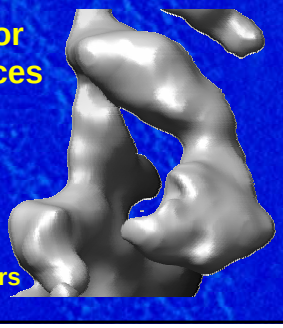
Parking is always a problem for someone

HMM – consumer
of parking spaces

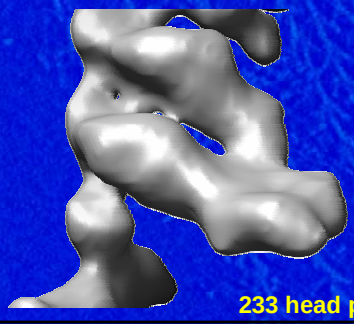


220 head pairs

Actin – purveyor
of parking spaces



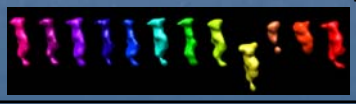
91 head pairs



233 head pairs

Structure of the Villin-Actin Cross-link using Single-Particle Analysis of 3D Volumes

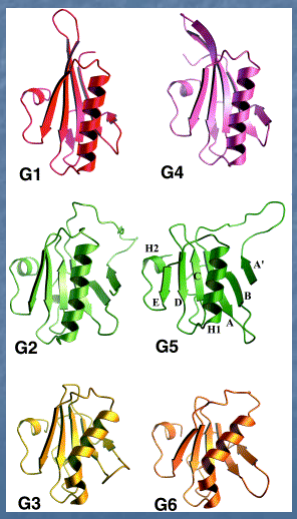
Cheri Hampton
Taylor Lab
Institute of Molecular Biophysics
Florida State University

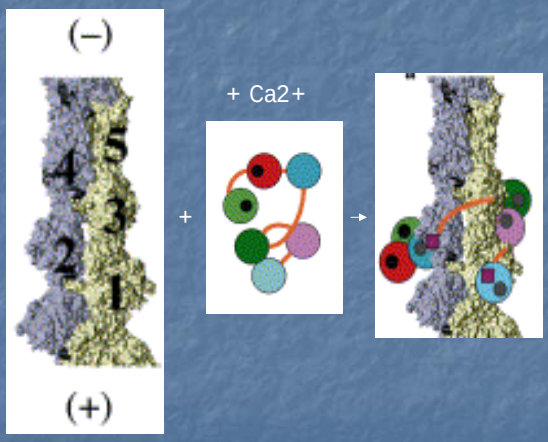


Introduction to Villin

- An 95 kD actin nucleating, cross-linking, severing and capping protein found in microvilli along with fimbrin
- Cross-links w/o calcium, requires high calcium or phosphorylation to activate severing and capping
- 45% sequence identity to gelsolin
- Has a C-term "head-piece" domain that confers cross-linking ability
- There is one crystal structure for the 1st gelsolin-like domain and an NMR structure for "head-piece" and HP + 6th gelsolin-like domain
- Assumed to bind/ sever F-actin similar to gelsolin

Gelsolin Structure and Function

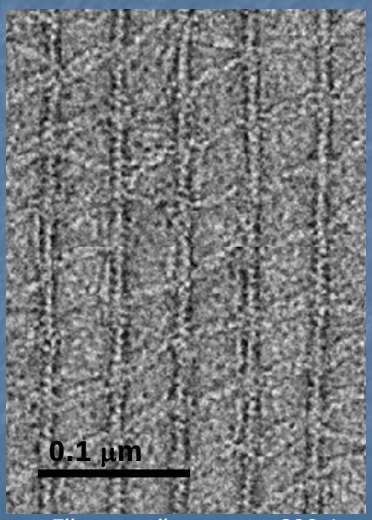




Gelsolin severing and capping model adapted from McGough, et. al. 2003. *FEBS Letters*, 552(2-3):75-81.

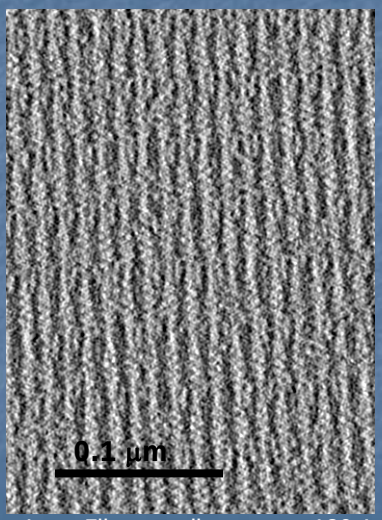
Villin Forms a Tight Bundle

α-Actinin-F-actin Rafts



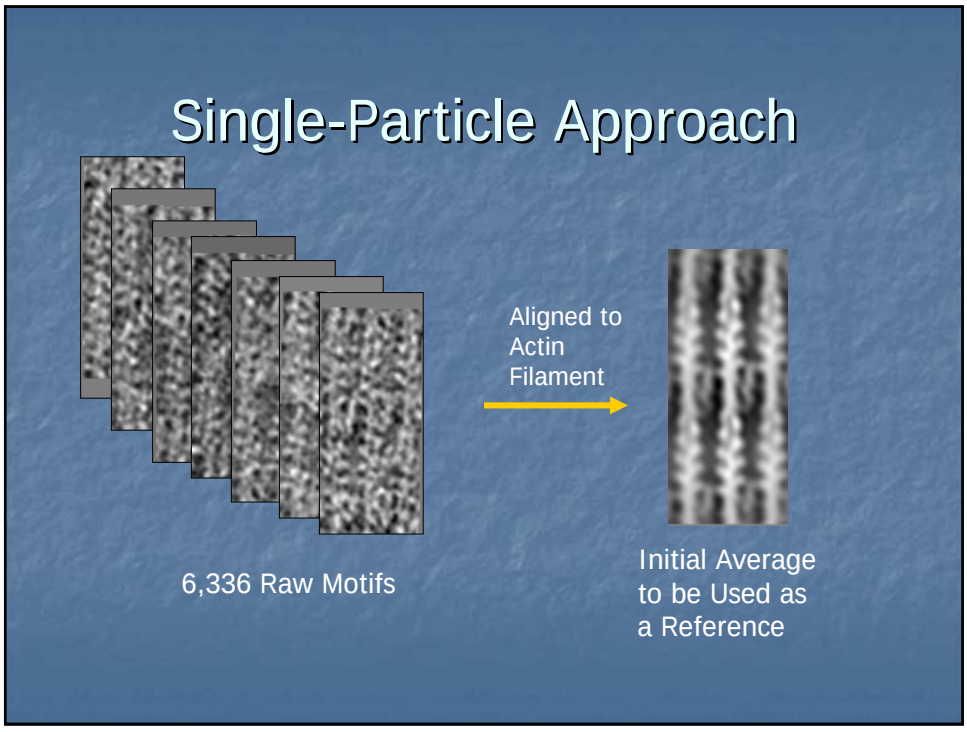
Inter-Filament distance= ~390 A

Villin-F-actin Rafts



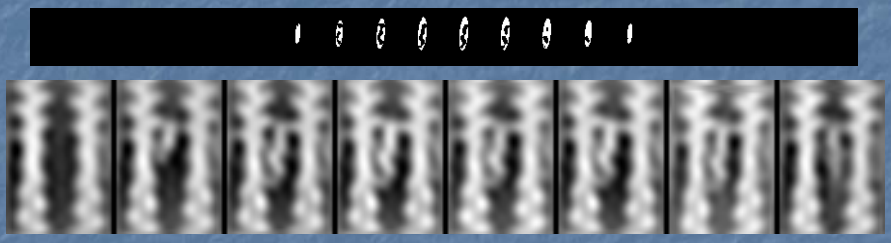
Inter-Filament distance= ~126 A

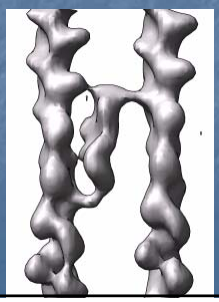
Factin-Villin
Tilt Series
Movie
Courtesy of
Dr Jun Liu

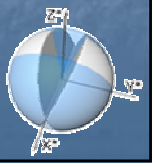

Single-Particle Approach
Aligned to
Actin
Filament
6,336 Raw Motifs
Initial Average
to be Used as
a Reference

Villin Cross-Links are Homogeneous

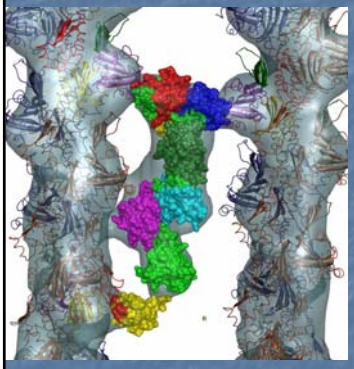
6,356 total repeats were sorted into classes based on variance



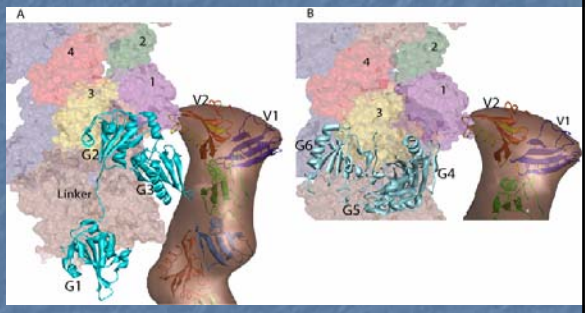




Non-quantitative positioning of gelsolin domains plus HP



Villin and gelsolin binding sites do not overlap.

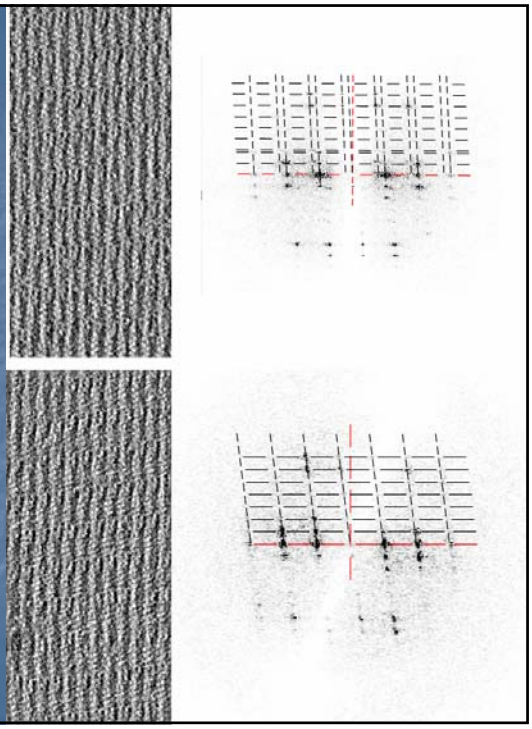


Gelsolin N-terminus (PDB 1RGI) docked onto F-actin. Note that G1 is capping the filament end.

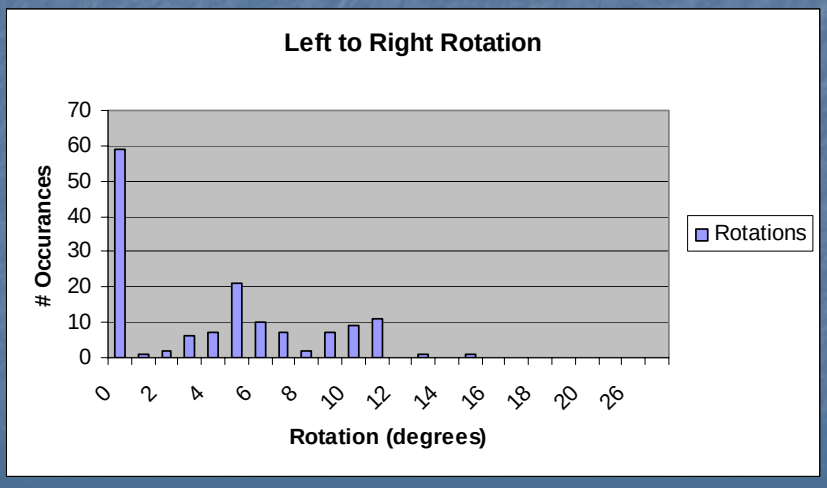
Gelsolin C-terminus (PDB 1H1V) docked onto F-actin.

Raft Analysis

- Actin symmetry varies: 13/6, 80/37, 186/86
- Interfilament spacing ~126A
- Axial shift of 17A from left to right filament
- Axial rotation of the right filament relative to left is 0, 5, 10 degrees
- Diffraction spots out to 28A resolution



Filament Rotations from Class Averages

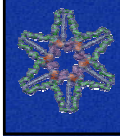


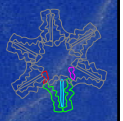
3D Structure of Myosin-V in Inhibited State

Pushing the resolution in Cryo-Electron Tomography

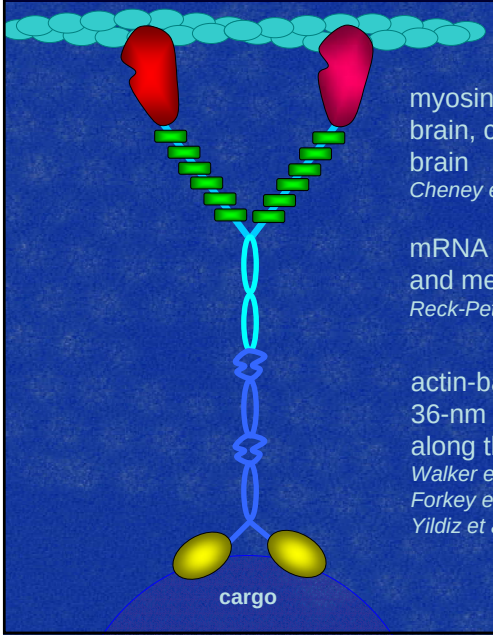
Jun Liu

The Institute of Molecular Biophysics
Florida State University





Functions of Myosin-V



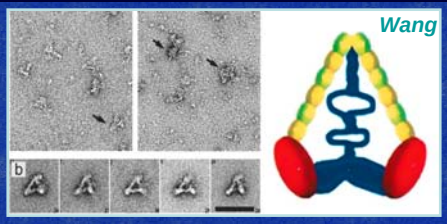
myosin-Va expressed at high levels in brain, correspond to 0.2% of total protein in brain
Cheney et al. Cell. 1993;75:13-23

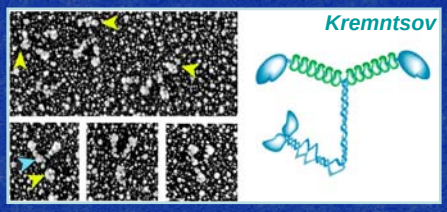
mRNA transport, cell polarity and membrane trafficking
Reck-Peterson et al. BBA 2000; 1496:36

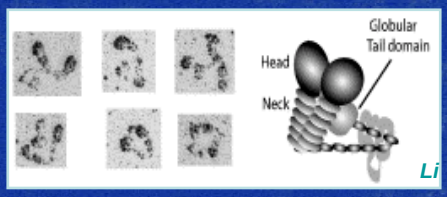
actin-based processive motor that takes large, 36-nm steps in a hand-over-hand manner along the actin filament.
Walker et al. Nature. 2000;405:804.
Forkey et al. Nature 2003; 422:399
Yildiz et al. Science 2003; 300:2061

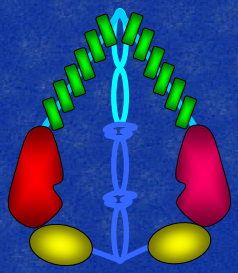
The Muscle Group, Leeds

3 Models of Myosin-V in the Inhibited State









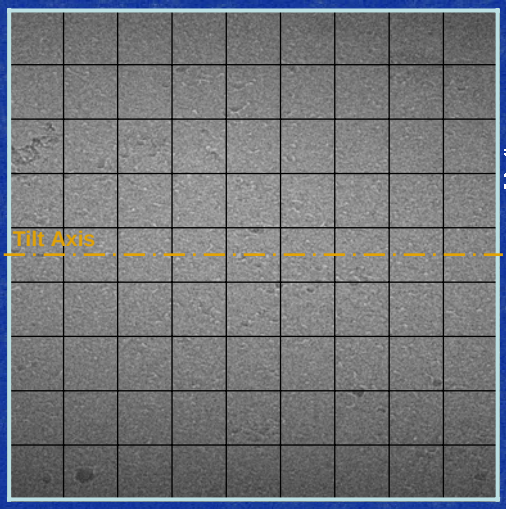
Wang et al. model

Kremntsov et al. JCB 2004; 164:877
Wang et al. JBC 2004; 279:2333
Li et al. BBRC 2004; 315:538

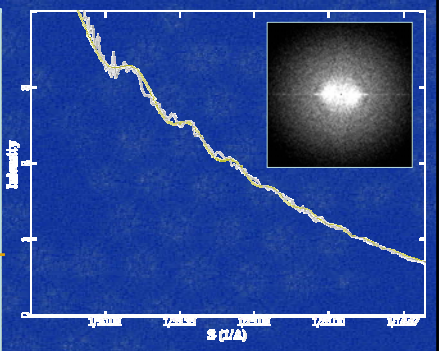
Cryo Tomogram of Myosin-V 2-D Arrays

- 60 images
- ±70˚ tilt range
- Cosine rule
- Focus gradient corr.
- Low dose (~30e/Å^2)
- -172˚C
- Magnification 43,200x
- CM300FEG at 300kV
- 2k x 2k CCD camera
- Defocus at ~8-10µm

Focus Gradient



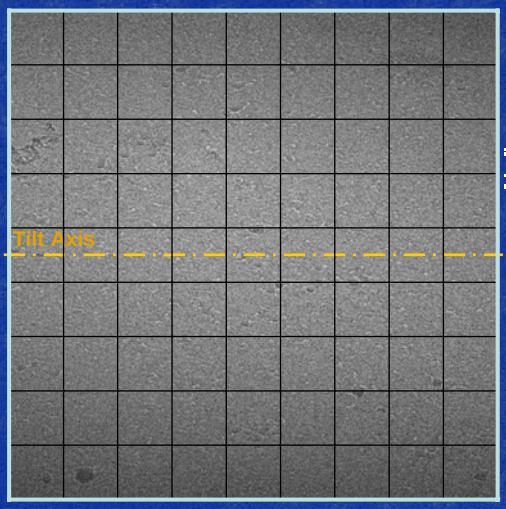
60° image



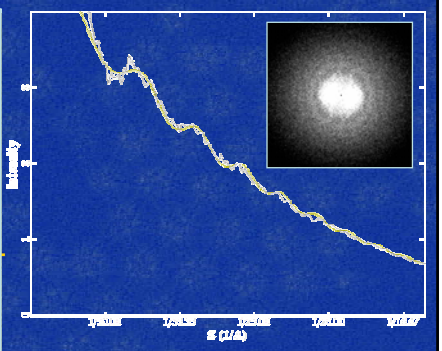
-12.5um → -11.8um → -10.9um

using CTFIT from EMAN

Focus Gradient



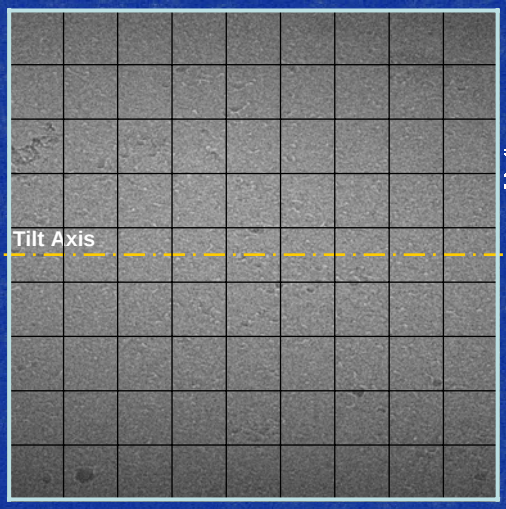
60° image



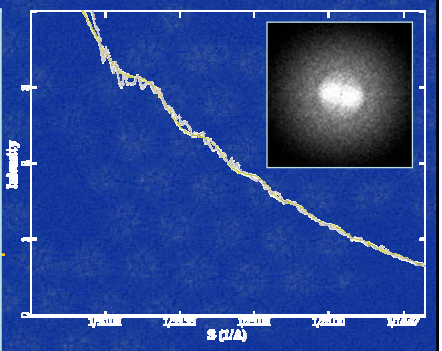
-12.5μm → -11.8μm → -10.9μm

using CTFIT from EMAN

Focus Gradient



60° image

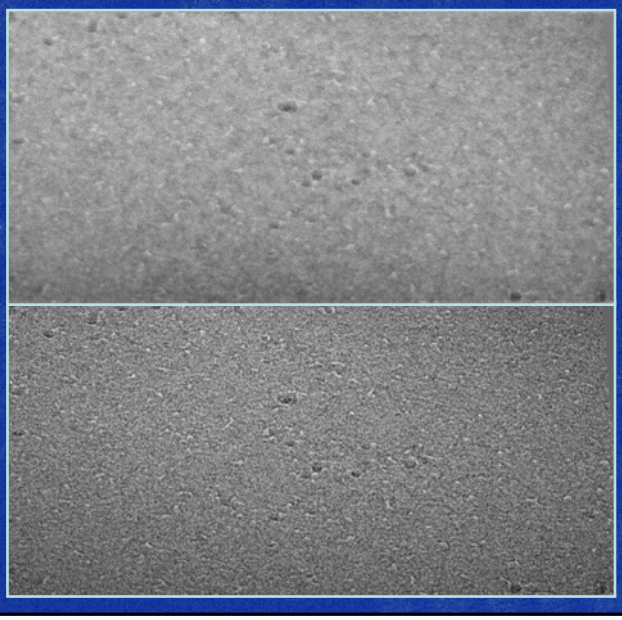


Defocus changes:

-12.5um → -11.8um → -10.9um

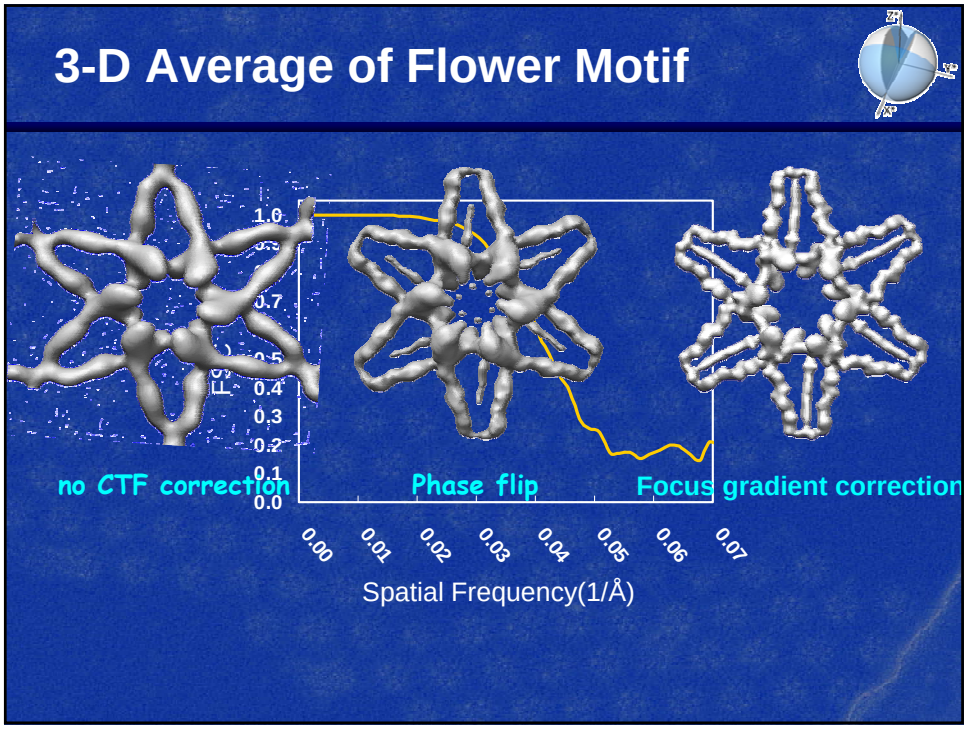
using CTFIT from EMAN

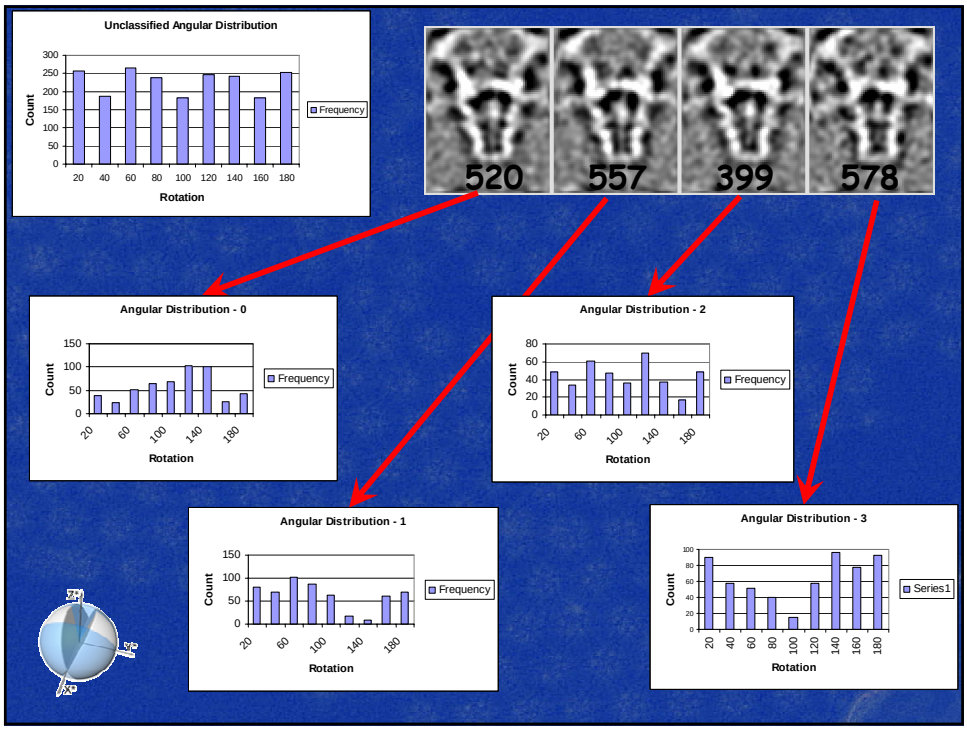
Focus Gradient Correction



Focus gradient correction applied to tilt series image data used in electron tomography

Winkler H. & Taylor KA, JSB. 2003, 43:24


3-D Average of Flower Motif
1.0
0.7
0.5
0.4
0.3
0.2
0.1
0.0
no CTF correction
Phase flip
Focus gradient correction
0.00
0.01
0.02
0.03
0.04
0.05
0.06
0.07
Spatial Frequency(1/Å)


Unclassified Angular Distribution
Count
Rotation
Frequency
520
557
399
578
Angular Distribution - 0
Angular Distribution - 2
Angular Distribution - 1
Angular Distribution - 3
Series1

Challenges

1. How many different structures you looking for?
2. Resolution & Signal/Noise Improvement
   a. Keep in mind that resolution ultimately depends on the tilt angle increment
   b. Once S/N improvement reaches the resolution limit, there is no further improvement
3. What is the resolution limit for biological tomography?
   a. Subvolume averaging
   b. CTF correction

U.S. Department of Health and Human Services

Cell Migration Consortium

Supported by the
National Institutes of Health

NIGMS & NIAMS