

How To Analyze Filaments: The Principles Of Helical Reconstruction

David DeRosier

A. Imaging helical structures

1. Sample preparation
2. Microscope set up

B. Image selection

1. Looking at micrographs
2. Optical diffraction
3. Criteria for selection
 - a. Not too curved
 - b. Nothing crossing over particle
 - c. Surface texture visible or layer lines visible

C. Densitometry

1. Raster step needed
2. Length of particle needed
3. Number of particles needed

D. Calculating the FFT

1. Straighten and make vertical
2. Determine width, width= $2r_0$
3. Box
 - a. Fairly tightly but better too generous than too tight
 - b. Box should be symmetric
4. Float and apodize
5. Select transform size
 - a. Reciprocal space sampling
 - b. Display of transform

E. Indexing

1. Rules governing n and $l(z^*)$
2. Finding layer lines in the transform: determining $l(z^*)$
3. Drawing lattice on transform
4. Determination of n
 - a. Measure R_0 for $G=\max$
 - b. Calculate approximate value $n \sim (2\pi R_0 r_0) - 2$
 - c. Determine if n is odd or even from phases
 - d. Concentrate on small values of n
5. Determine hand
 - a. Shadowed preparation
 - b. Tilt axis out of plane
6. Draw helical $n, l(z^*)$ plot
7. Calculate rotation and rise per subunit

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1. Collect layer line data
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G. Data averaging and 3-D map

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- a. Selection of asymmetric unit to search
- b. Radial scaling
- c. Polarity
- 2. Averaging and error analysis
- 3. Calculating the map
 - a. Correcting values of I to obey helical symmetry strictly
 - b. Surface views: choosing the right contour
 - c. z-sections
 - d. Cylindrical sections

H. Interpreting the map

- 1. Resolution achieved
- 2. Stain vs. ice
- 3. Seeing domains
- 4. Seeing secondary structure

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How To Index The Diffraction Pattern Of A Helix

David DeRosier

1. Assumptions.

In the electron microscope, long helical structures on a grid have their long axes perpendicular to the electron beam and are seen as 2-D projections along the direction of the beam. In the following, helical structures are assumed to be viewed in this manner unless stated otherwise. Further we orient the long axis vertically. We also use the term particle to indicate one individual helical structure in a population.

2. Appearance of the images.

The domains of the subunits in helical structures generate sets of helical channels and grooves on the surface and in the interior of the particle. These morphological features often give the particles' images a rough texture. The grooves and channels occur in sets each of which can be associated with a low order layer line in the diffraction pattern. For example, the twin stranded feature of actin which has crossovers every 360Å gives rise to the $1/360\text{Å}=0.00278\text{Å}^{-1}$ layer line seen in diffraction patterns.

3. The appearance of the diffraction pattern.

A horizontal line through the center of the diffraction pattern is called the equator. and a vertical line through the center, the meridian. Usually the strongest intensities lie along the equator in the form of a streak. The rest of the pattern consists of a set of streaks or layer lines which run parallel to the equator. Most layer lines have a region of weak intensity at the meridian. On either side the intensity builds up to a maximum with weaker maxima extending away from the meridian. (The strongest maxima needn't always be the first pair, however. If a structure rich in internal detail, the secondary peaks can be stronger than the primary or first peaks, although usually not on all layer lines.) It is possible for layer lines to have a strong maximum exactly on the meridian. These are special layer lines having order, $n=0$. (More below). The meridian is generally quite noisy and it can be hard at first to decide if a peak on it belongs to a layer line or not. The reproducibility of a feature is an important guide whether it belongs to the layer line or is just noise. The distance from each layer line to the equator is the height, Z , of that layer line.

4. The rules governing layer line heights, Z .

All layer line heights can be related to each other using only two independent parameters. Take for example the diffraction pattern of actin. The pattern has strong layer lines at $1/360\text{Å}=0.00278\text{Å}^{-1}$ and $1/59\text{Å}=0.0169\text{Å}^{-1}$. These two are known as the first and sixth layerlines respectively because the latter is about 6 times the former. All other layer lines are derived from the sums and differences of integral multiples of these two layer lines: $Z_n=hZ_1+kZ_6$ where h and k are integers. For example, the seventh layer line ($h=1$ $k=1$) is found at $1*0.0169+1*0.00278=0.0197\text{Å}^{-1}$. This is the $1/51\text{Å}$ layer line. Sometimes, different linear combinations (e.g., $h=5$ $k=0$ and $h=-1$ $k=1$) generate identical or almost identical layer line positions: $5*0.00278=0.0139$ and $1*0.0169-1*0.00278=0.0141$. We expect to find two layerlines close to or on top of one another. These two layer lines will have different orders and in the event only one of the two is visible, the reciprocal lattice helps tell us which one we are seeing. The layer line positions should strictly obey the above rule which acts as a guide to identifying which streaks in a diffraction pattern are noise and which are true layer lines.

5. The construction of the reciprocal lattice.

The first step in analyzing a helical structure is to divide the diffraction pattern into two mirror symmetric parts. The transform of a helical lattice (seen in projection) is very similar to the transform of a two dimensional lattice which has been mirror symmetrized about a vertical line through the origin. The reason is that one side of the helix (say the side nearest you) corresponds to one lattice (call it the solid lattice) and the other side (the far side) to its mirror lattice (call it the dashed lattice). Just as one can draw a reciprocal lattice through the transform of a two dimensional lattice, one can draw a pair of mirror related lattices through the diffraction pattern of a helical lattice. For an ideal helical structure the two lattices will not pass exactly through all the maxima. The failure is due to the curvature of the helix which tends to push the maxima out to higher radius in the diffraction pattern.

Let us begin by assuming one has no idea about the reciprocal lattice and let us try to construct one. To begin, pick the strongest layer line that is not a higher order of a lower order layer line. In the diffraction pattern of actin it is OK to choose the first layer line but not the second since the latter is the second order of the first. On the first layer line, assign the peak on the right hand side of the meridian to the solid lattice. (The left side peak then belongs to the dashed lattice.) Draw a solid lattice line from the origin of the diffraction pattern to this first (solid) point on layer line 1. (If the layer line by chance has a meridional peak (i.e., $n=0$), the spot belongs to both lattices.) Now move to the next strongest layer line that is not a second order. On the actin diffraction pattern, let us choose the sixth layer line. Does the right side peak belong to the solid or the dashed lattice? The only way to find out is to try one choice-let's say the right side peak belongs to the solid lattice. Draw a second solid lattice line from the origin to the right side peak on the sixth layer line. Now we can complete the unit cell. Draw a third solid lattice line through first solid peak making it parallel to the second solid line. Draw a fourth line through (solid peak) on layer line 6 and parallel to the line between the origin and solid peak on layer line 1. These four lines should define a parallelogram which is the unit cell.

Continue the lattice by drawing additional lattice lines on the diffraction pattern in a perfect grid. If the second point is correctly chosen then the lattice will go through the strong peaks on the remaining layer lines. Of course it will go through only one peak on each layer line. (The dashed lattice will go through the other. Remember that if the peak to one side goes with the solid lattice the mirror symmetric peak across the meridian goes with the dashed lattice.) Also the weaker subsidiary maxima on a layer line are generally all associated with the primary peak and the lattice will not go through these peaks but should go through the primary peaks on either the left or right side of the meridian.

In our example, we chose wrongly. The lattice doesn't go through the peaks on the layer lines. If the lattice misses layer lines, then we try the other choice to see if it works better which in this case it does. As a general rule the radial positions of the peaks may not lie exactly at lattice points, but the axial position for the layer line must fall exactly on the lattice point.

This process of drawing in the lattice is important for breaking the layer lines into their near and far sides. One can't tell whether the solid side is the near or far side until one determines the hand of the helical structure.

The lengths of the lines between points in the lattice should be approximately equal to the inverse of the subunit dimensions. The approximate dimension d of a subunit of molecular mass m is given by the following

$$d = 1.34(m)^{1/3}$$

where d is in Å and m is in daltons. For actin $d \sim 50$ Å. One clue about selecting peaks as belonging to the same (solid) lattice is not to pick two peaks that are too close to one another because this would be at odds with respect to the subunit dimensions. For example, suppose one had decided to choose layer lines six and seven rather than 1 and six to begin drawing the reciprocal lattice. One of course will get the same lattice whatever suitable pair one starts with. Let us choose the left side peak on layer line 6 as the solid point and then ask which peak on layer line 7 is the solid peak. If we pick the left side peak, we generate a unit cell with a side approximately equal to 360 Å. This is too big and hence we would reject that as a good beginning pair of points. We would try the right side peak which you can see is indeed the right one.

We now have located all the layer lines and have divided them into two halves: the solid lattice and the dashed lattice. Each layer line has both a Z and an order n . The next task is to determine the order.

6. Measurement of the radius of the particle from the image.

Believe it or not this is a hard quantity to measure accurately. Most people underestimate the diameter of a particle, especially if the particle is in negative stain. The temptation is to measure from the darkest part of one edge to the darkest part of the other. Generally, the true edge of the particle is farther from the axis and if one looks at the structural features along the edge, one sees that the periodic part extends beyond. It is better to be a little to the high side than to the low side. Some particles, like actin, show a variation in apparent width along their length. You want to measure it at its widest point. Take the diameter and halve it to find the radius, r .

Flattening of the particle introduces errors. If the particle is a thin tube and is totally flattened, then the width is half the circumference rather than the diameter. One way to estimate the amount of flattening is by tilting and observing the change in particle width. For a discussion of flattening, see Moody (1990). For our example, we will use the value of 48Å as the outer radius of actin.

7. Measurement of the radial position of the first maximum on each layer line.

This is easy if the first peak on every layer line is the strongest. Measure the horizontal distance, R , from the meridian to the first peak on either side of each layer line. There are two measurements per layer line. In a perfect particle, they are the same. Often they are not in reality. Partial, one sided flattening or differential staining, can cause the two peaks to lie at slightly different radii. One can keep two sets of R a solid set and a dashed set for each layer line according to the lattice scheme you have. For our example using actin, we construct the following table. Since the peaks are streaky, we might enter the minimum value of R and also the maximum reflecting the spread in the peak:

layer line	R min	R max
1	.0099	.0155
2	.0183	.0239
3	.0233	.0289
4	.0250	.0290
5	.0120	.0144
6	.0050	.0110
7	.006	.0120

8. Estimation of n from the values for r and R .

An approximate value for $|\mathbf{n}|$ for each layer line is given by the following formula:

$$2\pi Rr \approx |\mathbf{n}| + 2$$

This formula is approximate and works best for large values of \mathbf{n} . The table below gives the correct values for the positions of the peaks.

order	$2\pi Rr$
0	00.0
1	01.8
2	03.1
3	04.2
4	05.4
5	06.4
6	07.5
7	08.6
8	09.6
9	10.7
10	11.8
11	12.8
12	13.9
13	14.9
14	16.0
15	17.0
16	18.1

The values of \mathbf{n} are approximate because the features which give rise to a particular layer line may not be at a radius corresponding to the outside of the particle. If the radius r used is truly from the outside then the expression $2\pi Rr$ is a maximum and hence represents an upper bound on \mathbf{n} . Generally one can estimate a range of values for \mathbf{n} . The smallest range of possible values for \mathbf{n} are those corresponding to small values of \mathbf{n} since the possible sources of error make less of a change.

Our table for the range of values of \mathbf{n} for actin is as follows:

layer line	$2\pi Rr$	range of $ \mathbf{n} $
1	2.98 - 4.67	1 to 3
2	5.52 - 7.21	4 to 5
3	7.17 - 8.71	5 to 7
4	7.40 - 8.74	5 to 7
5	3.61 - 4.34	2 to 3
6	1.50 - 3.32	1 to 2
7	1.80 - 3.62	1 to 2

9. Determination whether \mathbf{n} is odd or even.

We can narrow down the uncertainty by determining if \mathbf{n} is odd or even. If \mathbf{n} is even, then the phases of the pair of mirror symmetric peaks across the meridian will be the same. If odd they will differ by 180° . What we find from our computed diffraction patterns (taken from actinicen.big and actinicef.big) is that the phase differences on layer lines 1 and 2 are about 0° and hence \mathbf{n} must be even. On layer lines 4, 5, 6 and 7, the phase differences are about 180° and values of \mathbf{n} must be odd.

layer line no.	phase left peak	phase right peak	phase difference
1	164°	163°	1°
2	-61	-62	1
3	not in data set		
4	50	-128	178
5	25	-152	177
6	140	-40	180
7	-41	139	180

10. The hand of the particle.

In the diffraction pattern of a helical particle, each layer line has a hand. Some correspond to left handed helical grooves on the particle and some to right-handed grooves. The hand of the layer lines can be determined by shadowing so one is looking only at one side say the near side. The diffraction pattern of the near side of a right handed helical groove will give rise to peaks in the upper left and lower right quadrants. This method was used in determining the handedness of the flagellar filaments of *Rhizobium* (Trachtenberg et al. *J. Mol. Biol.* **195**, 603-620 1987).

Another method of hand determination is to take images of the structure with its helical axis tilted out of the plane perpendicular to the direction of the electron beam. The effect of tilting a helix is to produce a cycloid pattern for each set of helical grooves in the particle. One side of the image will have a gentle scalloping and the other side will be serrated. Assume the top of the helix is tilted away from you. A right-handed helix will be serrated on the right side whereas a left handed helix will be serrated on the left. In principle, one needs only to look at the image, detect the serration for each layer line and assign hand. In practice, it is difficult to see the serration. There is a trick using diffraction patterns that seems to work very well.

If one diffracts from the right hand half of a tilted structure (the top is tilted away from the viewer), one will obtain the transform of the serrated half of a right-handed helical groove in the image. This is a stronger feature and will give rise to a strong intensity on the layer line corresponding to that right-handed helix. If one diffracts from the left hand side of the image, that same layer line will be weaker. Thus what one does in practice is to obtain a diffraction pattern of the right-hand side of the particle and one of the left-hand side. Those layer lines that are stronger on the diffraction pattern of the right hand side compared to that of the left-hand side are right handed and vice versa. In the diffraction pattern of tilted actin, layer line 6 is left handed while layer line 7 is right handed. This is reflected in the reciprocal lattice because the two corresponding lattice points (say the solid points) are on opposite sides of the meridian. Thus once one has assigned an absolute hand to one layer line, the hands of all other layer lines are fixed by the reciprocal lattice. This then determines which of the two lattices (solid or dashed) corresponds to the near side of the particle. Since the hand of the first layer line of actin is right handed, its peak will be in the upper left quadrant. Hence the dashed lattice is the near side and the solid the far side.

All right-handed layer lines are assigned values of $n > 0$ and those that are left handed $n < 0$.

11. Finishing up the indexing.

The layer lines obey simple rules similar to those of a lattice. Let us use our two vectors from the origin to our first two chosen layer lines as our "basis set". Let the values for these layer lines be n_1, Z_1 and n_2, Z_2 . Now pick a third layer line. Let's choose the one at $Z_3 = h * Z_1 + k * Z_2$. Then $n_3 = h * n_1 + k * n_2$. Using this rule we can now decide which values of n are correct by trial and error. One should check various combinations of n within the range of

values. Remember that the value of n is an upper bound. Smaller values are allowed but not on every layer line. Suppose that everything fit except one layer line for which the range was say 14 to 16 and where you got a value based on the other layer lines of 10. This may be OK because $n=16$ is a max. It would be a problem however, if the value obtained from the other layer lines was 20 which to be true would come from a feature that was at a radius larger than the outermost radius of the structure. If one is lucky, there will be only one solution that fits all layerlines. For our actin example this is the case. We know the first layer line value of n lies between +1 and +3 (it is right handed) and we know it is even. Hence $n=+2$. Likewise, on the sixth layer line n must be between -1 and -2 and must be odd. Hence $n=-1$. The rest of the values can be assigned using the reciprocal lattice drawing. We have chosen layer line 1 to lie at the $h=1$ $k=0$ point of our lattice and the sixth to lie at the $h=0$ $k=1$ point. The other layer lines are derived from these. For example the fifth layer line is at the $h=-1$ $k=1$ point. Hence its value of n is $-1*(+2) + 1*(-1)=-3$ and Z is $-1*(0.00278) + 1*(0.0169) = 0.0141\text{\AA}^{-1}$ or $1/71\text{\AA}$.

l	h	k	n	Z
1	1	0	+2	0.00278
2	2	0	+4	0.00556
3	3	0	+6	0.00834
5	-1	1	-3	0.0141
6	0	1	-1	0.0169
7	1	1	+1	0.0197

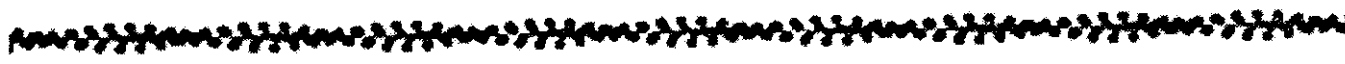
12. Drawing an n,Z plot.

When the solution is found one can sum up the results by plotting the pairs of points, n,Z . These will form a perfect 2D lattice that describes the helical lattice. Z is plotted vertically and n horizontally. One can also produce a layer line selection rule of the form $l=tn + um$ where l is the layer line number, n is order of the layer line. If the structure has rotational symmetry about its helix axis, then the values of n that are allowed are multiples of the rotational symmetry. My opinion is that the layer line selection rule is a less useful form of the information than the n,Z plot. For example, for actin the rule is: $l=-6n + 13m$. The first layer line has values $l=1$, $n=2$ and $m=1$ and the fifth has $l=5$, $n=-3$ and $m=-1$. Can you figure out what value(s) of n are allowed on the 12 layer line?

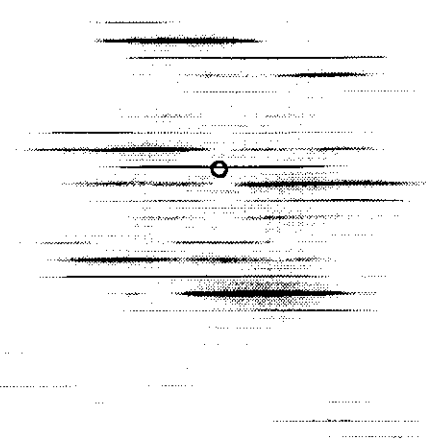
Figure caption

- Figure 1 model actin filament and its diffraction pattern
- Figure 2 front half of the filament and its diffraction pattern
back half of filament and its diffraction pattern
- Figure 3 wrong reciprocal lattice drawn on diffraction pattern of filament
right reciprocal lattice (both solid and dashed)
- Figure 4 tilted filament
left half and its diffraction pattern
right half of tilted filament and its diffraction pattern
- Figure 5 n,Z plot for actin

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Front



Back

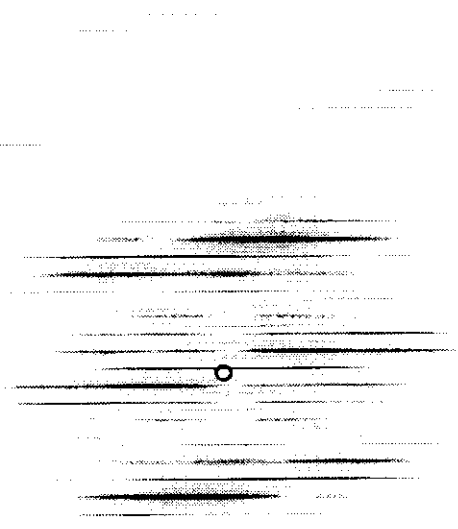
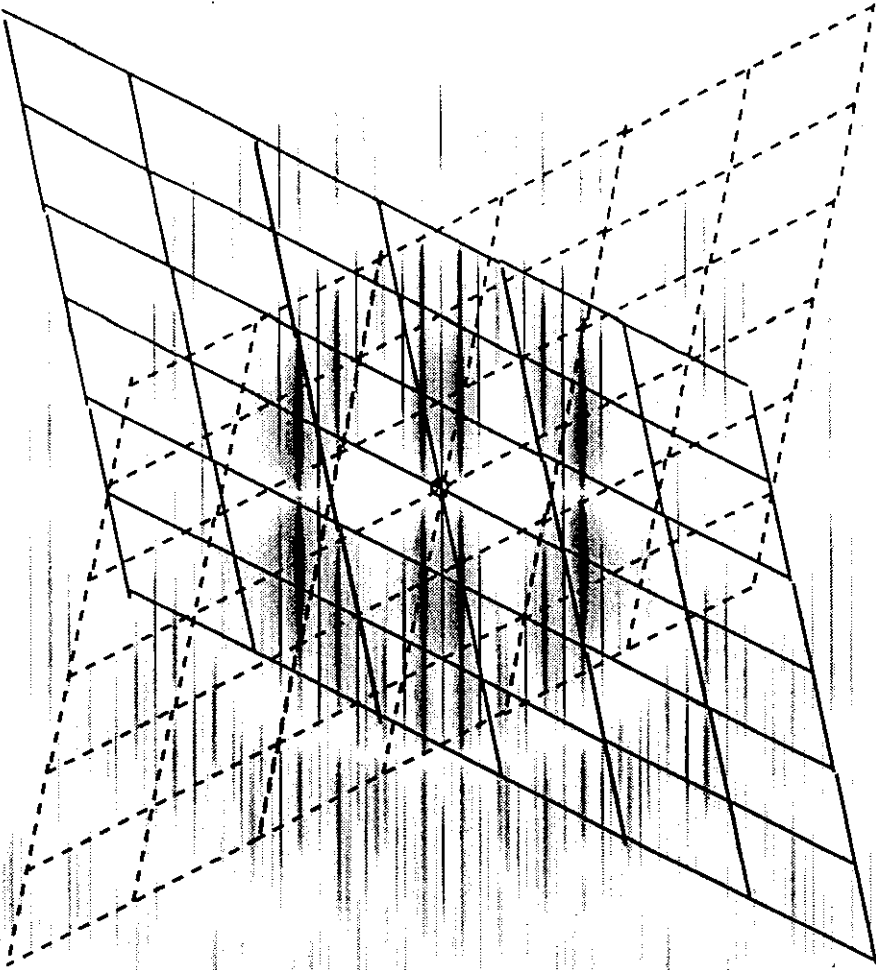
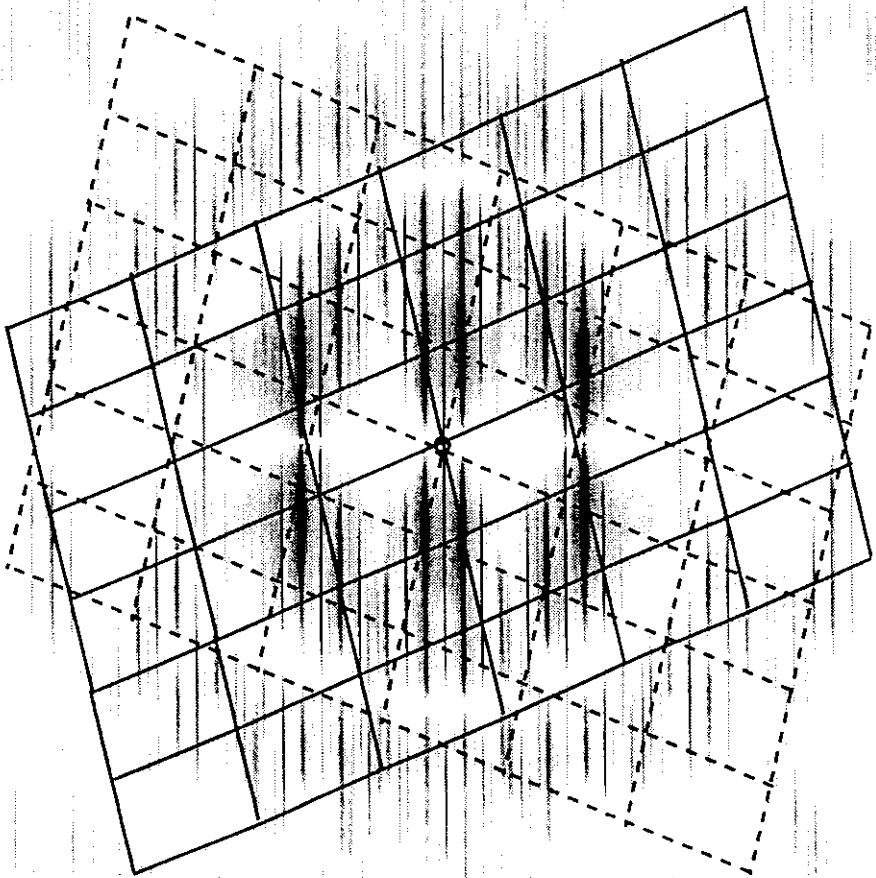


Figure 3

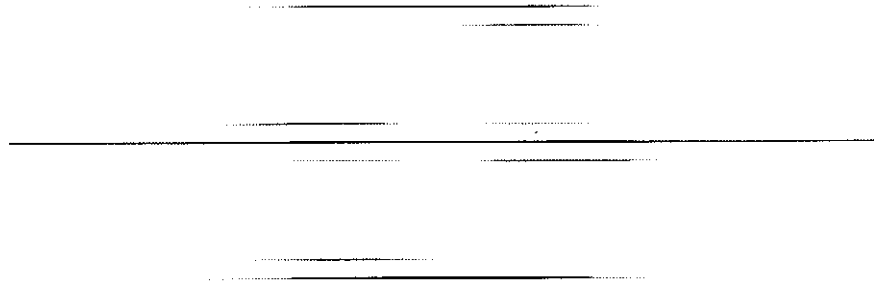
Wrong



Right



Left (15 degrees Tilt)



Right

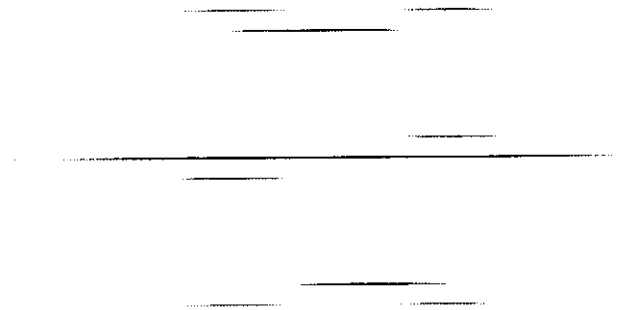


Figure 5

n,Z Plot for Actin

