Using transmission electron microscopy and 3View® to determine collagen fibril size and three-dimensional organization

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Running title: Size and organization of collagen fibrils *in vivo*

Collagen fibrils are the major tensile element in vertebrate tissues where they occur as ordered bundles in the extracellular matrix. Abnormal fibril assembly and organization results in scarring, fibrosis, poor wound healing and connective tissue diseases. Transmission electron microscopy (TEM) is used to assess formation of the fibrils, predominantly by measuring fibril diameter. Here we describe an enhanced protocol for measuring fibril diameter as well as fibril-volume-fraction, mean fibril length, fibril cross-sectional shape, and fibril 3D organization that are also major determinants of tissue function. Serial section TEM (ssTEM) has been used to visualize fibril 3D-organization *in vivo*. However, serial block face-scanning electron microscopy (SBF-SEM) has emerged as a time-efficient alternative to ssTEM. The protocol described below is suitable for preparing tissues for TEM and SBF-SEM (by 3View®). We demonstrate the power of 3View® for studying collagen fibril organization *in vivo* and show how to find and track individual fibrils. Time scale: ~8 days from isolating the tissue to having a 3D image stack.

[Introduction]

Collagens are a superfamily of triple helical proteins that are essential for a broad range of tissue functions including scaffolding, force transmission, cell adhesion, cell migration, morphogenesis and repair (for an overview see [1]). Of the ~30 different types of collagen and collagen-related proteins in vertebrates, collagen types I, II, III, V and XI assemble into *D*-periodic (where $D \sim 67$ nm (for review see[2])) cross-striated fibrils in the extracellular matrix (ECM) of tissues. The fibrils vary in diameter from ~12 to ~500 nm as a function of tissue and age[3], and are the primary tensile element of tissues such as tendons, ligaments, skin, cartilage, bone, fascia, joint capsule, blood vessels and other hollow organs. Much is known about the structure of fibrillar collagen genes but fundamental questions remain unanswered about the assembly and organization of the fibrils. TEM is a popular technique for studying collagen fibrils in which the diameter is the most commonly measured parameter. However, cell and tissue functions depend not only on collagen fibril diameter distributions but also on collagen fibril number, 3D organization, fibril volume fraction (FVF), and fibril length. Consequently, changes to any of these parameters can lead to altered tissue mechanics as is observed in diverse collagen diseases including fibrosis, scarring, adhesion formation, and

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heritable disorders of connective tissues such as osteogenesis imperfecta and the Ehlers-Danlos syndromes.

Electron microscopy of individual collagen fibrils

Individual collagen fibrils in embryonic tissues are ~12 to ~50 nm in diameter, close packed in curvilinear bundles, and are, therefore, not readily detectable by light microscopy. Fibrils in older tissues can be ~500 nm in diameter but even these are at the limit of resolution of most light microscopes because the fibrils are crowded into bundles and sheets. Combination of tissue cryosectioning with non-destructive atomic force microscopy has successfully visualized macromolecular structures of unstained and unfixed fibrillar collagens[4]. However, surface imaging techniques have limitations in measuring fibril length, FVF, fibril 3D organization and tracking the long-range course of individual fibrils.

Measurement of 3D connective tissue architecture

The varied 3D organizations of collagen fibrils in different tissues was first revealed by ssTEM, which showed parallel bundles in tendon and ligament, orthogonal lattices in cornea, and basket-weaves in skin and bone. Using ssTEM, Trelstad and Hayashi[5], and later Birk and Trelstad[6], were among the first to show that the collagen fibrils in embryonic tendon and cornea originate in slender plasma membrane invaginations. Subsequent studies using a combination of ssTEM and electron tomography showed that the slender invaginations and finger-like projections (called fibripositors, which contain collagen fibrils) were co-aligned with the long axis of the cell[7] and could be disassembled by cytochalasin B[8]. An advantage of serial sections over single sections was the ability to track individual collagen fibrils. However, despite these advantages, the technical challenges associated with obtaining undistorted sections by ssTEM and ensuring precise alignment between sections have been significant hurdles to many research groups.

Serial block face-scanning electron microscopy (SBF-SEM)[9] provides an opportunity to automate serial section electron microscopy and, as we show here, can replace ssTEM for some studies of collagen fibrils. A detailed review of SBF-SEM is outside the scope of this article but examples of its use include studies of neuronal tissue organization[10-12], chromatin architecture[13], and elastin organization[14]. These studies have shown that SBF-SEM can add to the information obtained by ssTEM. A working SBF-SEM system was developed by Winfried Denk[15] and has been commercialized by Gatan Inc. and called 3View®. Here, images of the resin block face are formed by collecting the back-scattered electrons before an in-chamber ultramicrotome removes a section. The images are acquired (following sectioning) by Gatan DigitalMicrograph™ software. These images can be converted into other image formats as required. The protocol described below shows how 3View® can be used to image the 3D organization of connective tissues including the shape and dimensions of cells and collagen fibrils.

With conventional TEM sectioning using an ultramicrotome, section thickness is estimated by the interference color of the section floating on water. The precise thickness of the section can be determined by labeling the surfaces of the section with nanogold beads and taking images of tilt pairs. These methods are not applicable to 3View®. The instrumental settings provide an average section thickness after the manufacturer's calibration. The actual section thickness should be determined experimentally, for example, by having internal standards such as polystyrene latex beads of defined diameter.

As discussed by Briggman and Bock[12], there is no best 3D EM imaging method; the challenge for 3D TEM is to acquire datasets of sufficient z-depth, x-y resolution, and completeness so that the tortuosity of cellular protrusions, cellular organelles (and in our case collagen fibrils) can be identified. For collagen fibrils this problem equates to having sufficient contrast and resolution to measure fibril diameters of individual fibrils, to find the ends of fibrils, to track narrow (~30 nm diameter) fibrils that are crowded by other collagen fibrils, and to estimate lengths of fibrils in the range 1 µm to several millimeters. The superior resolution of TEM over SBF-SEM makes it the better choice for measurement of fibril diameter, FVF and fibril profile. However, the convenience of serial sectioning using 3View® makes it the technique of choice for tracking individual collagen fibrils for studies of connective tissue 3D architecture. A downside of ssTEM is that cutting sections before imaging destroys the continuity between sections, and sections need to be aligned (rotationally and translationally, and occasionally flipped) and corrected for physical distortion. Although the issue of alignment

for ssTEM is being improved by novel software tools (for example see[16-19]), SBF-SEM is a powerful 3D EM approach that is worthy of consideration for studies of collagen fibril architecture. A distinct advantage of SBF-TEM is its ability to generate large numbers (hundreds to low thousands) of serial images in a workable time frame of a few days.

The ideal situation is to have the opportunity to perform both TEM and 3View® on the same specimen block. Therefore, ultrathin sections can be prepared for measurements of fibril diameter, FVF and circularity by TEM, whilst 3View® can be used to study 3D organization, fibril assembly, and fibril length. We compared a number of published EM preparation procedures and settled on a reduced osmium procedure that is similar to one published by Ellisman and co-workers[20]. Figure 1 shows a comparison of TEM and 3View® using the same specimen block. The sample was embryonic tendon prepared using the reduced osmium staining protocol, as described below. The fibrils are ~30 nm in diameter tapering to finite tips and are close packed into bundles at the cell surface.

As shown in Supplemental Video 1 (3DMOD-movie.mov), 3View® images of samples prepared using the reduced osmium protocol have sufficient contrast to identify individual collagen fibrils and to obtain quantitative information on cell number, cell shape, cell-cell interactions, collagen fibril number, and to identify collagen fibrils in cell surface fibripositors. Does 3View® replace ssTEM for studies of collagen fibril organization in vivo? The answer has to be 'Probably yes', because the resolution is sufficient to identify the fine tapering tips of fibrils. The answer to the question, 'Does 3View® supersede TEM for all studies of collagen fibrils?' is most probably 'No'. This is because TEM has superior resolution, which is especially useful for diameter measurements. A further consideration is that the cut depth (section thickness) in SBF-SEM can be greater that the volume of interaction i.e. the depth of information collected from the surface of the block face[15]. Therefore information is lost as parts of the sample that have not contributed to the image are cut away. 3View® can cut sections as thin as 50 nm (even less on ideal samples) but we have found that for studies of collagen fibrils that are at least several microns in length, there is no advantage of generating sections thinner than 100 nm. For 3View® studies of collagen fibrils in mature tissues (in which tips of fibrils are extremely rare) it is likely that the section thickness can be increased.

Measuring collagen fibril diameter *in situ* by TEM

Collagen fibrils are conspicuous in TEM longitudinal section (LS, side-on) by their characteristic *D*-periodic staining pattern (Fig. 2a,b). The *D*-periodicity is explained by the *D*-stagger of collagen molecules that are 4.4 x *D* in length; the molecular length of fibrillar collagen molecules is ~300 nm, which is ~4.4 times the *D*-spacing (discussed in [2]). In transverse section (TS, end-on) the fibrils typically have a near-circular profile (Fig. 2c,d).

Measurement of diameter from LS views – If fibrils are removed from tissue (e.g. by ultra sonication and homogenization[21]) and imaged on EM grids, fibrils will flatten on the support surface[22], which will lead to an over-estimate of fibril diameter. If fibrils are imaged within LS sections, the entire width of the fibril might not be included in the section and therefore the distribution will be skewed to smaller diameters.

Measurement of diameter from TS views – If the images have sufficient contrast and signal-to-noise then they can be segmented into binary images using a thresholding algorithm followed by application of the Watershed segmentation step to separate fibrils in contact with each other. Various size and shape parameters can then be computed in ImageJ (Fig. 3). Fitting of fibril sections to ellipses indicates the presence of local tilting of fibrils. The best measure of fibril diameter is to use the minor diameter of the fitted ellipse in accord with the standard method of manual measurement of fibril diameter where the minimal diameter is selected by eye.

Fibril-volume-fraction (FVF)

FVF is the proportion of the ECM occupied by collagen fibrils and depends on fibril volume and number [23]. In the case of aligned tissues (e.g. tendons and ligaments), the FVF is equal to the fibril-area-fraction (FAF) calculated from transverse sections. The calculation of FAF is shown by Eq. 1:

Equation 1,

$$FAF = \sum \frac{A_i}{A}$$

where A_i is the transverse area of a single fibril in the image and A is the total image area. The sum is for all fibrils appearing in the image rectangle (whole and part fibrils). In tissues in which fibrils are not parallel to the tissue long axis, FVF can be estimated from FAF taking into account the thickness of the sections and the tilt of the fibrils with respect to the sectioned axis. Quantitative measurements of fibril diameter and FVF can be interpreted in terms of a simple fibril growth mechanism [24].

Fibril profile shape – measurement of circularity

Collagen fibril profiles can deviate from circular and exhibit bulges and surface irregularities, even in healthy tissues. Large deviations from circular are common in diseases caused by mutations in genes that encode fibrillar collagens [25-29], enzymes involved in fibrillar procollagen processing[30], and in animal models of small proteoglycan deficiencies[31] (for review see [32]). Cross-sectional shape can be quantified by the circularity parameter, defined in Eq. 2, which has a maximum value of 1, for circles[33]

Equation 2,

$$\frac{4\pi A}{P^2} = circularity\ parameter$$

where P is the perimeter, A is the transverse area of the fibril.

Fibril length (L) and mean fibril length (L_m) by TEM

Collagen fibrils need to exceed a critical length (l_c) to achieve sufficient shear stress at the fibril-matrix interface and thereby ensure tissue mechanical integrity (discussed by [34]). Evidence suggests that l_c is approximately twice the length of the cell [34]. Therefore, knowledge of fibril and cell lengths provides a valuable insight into the mechanical status of a tissue. Two approaches have been developed to estimate fibril length: by fibril fragmentation and by serial section electron microscopy.

Estimation of mean fibril length (L_m) by fibril fragmentation – L_m can be estimated by fragmenting collagen fibrils *in situ*, adhering the fibril fragments to TEM grids, and measuring the total contour length of the fragments (L_{sum}) and the number of tapered tips (N_{tip}). Assuming that each fibril has two tips the mean fibril length can then be calculated using Eq. 3.

Equation 3,

$$Lm = \frac{2.L_{sum}}{N_{tip}}$$

It should be noted that this approach provides no information on the length distribution. Furthermore, free tips of collagen fibrils in the extracellular matrix can fuse with the shaft of a neighboring fibril *in vivo* to generate Y-shaped structures [35]. If Y-junctions are observed in the fibril fragments, the value of N_{tip} should be increased accordingly.

Measurement of collagen fibril lengths in invertebrates: a special case – Intact collagen fibrils have been isolated from the dermis of the sea cucumber (class *Holothuroidea*) by treatment with EDTA. The fibrils were imaged by negative staining TEM and unstained by dark-field scanning transmission electron microscopy (STEM)[36]. The ability to release intact fibrils means that a precise length distribution can be obtained. The lengths of the long flexible fibrils have been traced and shown to be in the range 14-444 µm. STEM images were analyzed to provide axial mass distributions from fibrils of different lengths. Examination of the staining pattern showed that the fibrils were exclusively N, N-bipolar i.e. the fibrils had two N-tips. Here, the collagen molecules are oriented with their amino-termini pointing to the tip and the carboxyl-termini oriented towards the middle of the fibril. Staining of the fibrils identified an 8-*D*-period region (the polarity transition region, PTR) precisely mid-distance between the tips where the collagen molecules flipped through 180°. Therefore, the distance from a tip to the PTR equates to half the length of the fibril. In contrast, the tissues of all vertebrates that have been examined to date have both N, N-bipolar and unipolar fibrils [37]. Unipolar fibrils have a C-tip and an N-tip with all collagen molecules oriented in one direction

with no PTR. The C-ends contain binding sites for tip-to-tip fusion with either another C-tip or an N-tip, thereby facilitating increase in fibril length [21]. The ability of a C-tip to fuse with either another C-tip (to produce a PTR) or an N-tip of either a bipolar or unipolar fibril, produces bipolar fibrils in which the PTR can be located at any position along the fibril length. Therefore, in vertebrates, the distance from the tip to the PTR is not a meaningful indicator of fibril length.

Estimation of fibril length by ssTEM and 3View® – Craig, Parry and co-workers were amongst the first to use ssTEM to estimate the mean fibril length in rat tail tendon as a function of age [34]. These researchers cut thin sections (~80 nm) transverse to the tendon long axis and tracked the course of 1368 collagen fibrils across 17 sections (z-depth ~1.4 µm) and made estimates of the mean length of tendon fibroblasts, the fraction of tendon occupied by cells (the cell-volume-fraction), L_m and l_c. Their interpretation of the data suggested that L_m ranged from 25-45 µm (in 5 day postnatal rat tail tendon) to 6400-12,700 µm (at 6 months of age).

The ability of 3View® to generate volumetric data with z-depths much larger than 1.4 µm, means that more fibril tips can be counted leading to greater confidence in the estimate of mean fibril length (L_m). Thus, by generating a 3D reconstruction by 3View® and counting the number of fibril tips and the total length of fibrils, L_m can be estimated according to Eq. 4.

Equation 4,

$$L_m = \frac{2 \times \Sigma(\text{total length of fibrils in a bundle})}{(number\ of\ fibril\ tips\ in\ a\ bundle)}$$

Eq. 4 can be shown to be accurate, to a close approximation, according to the following analysis.

Suppose that all fibrils are aligned parallel to the z-axis, and have length L. Suppose that the field of view along z runs from z=0 to z=f. If L>f, then we will only see one end of any fibril, the other being out of the view. A random fibril of length L will only be visible in the field of view (FOV) if its bottom tip starts in the range z=-L to z=f (a region of width f+L). The bottom tip itself will only be visible if it is in the range z=0 to z=f. Thus if a fibril is visible in the FOV, the probability that the bottom tip is visible is p = f/(f+L).

Suppose a total of N fibrils are visible in our FOV. Assuming we do not know the value of N explicitly, so can estimate it.

We have an estimate of the total length of fibrils visible, T.

If we knew p and N, we could estimate T as:

> T = (Total visible length of fibrils without visible ends) + (Total length of fibrils with an end visible)

> = (1-p)Nf + 0.5pNf; [since average visible length of fibril with an end visible is 0.5f]

> = Nf – 0.5pNf

We can count the number of bottom tips visible, n, which we expect to be n = pN.

Therefore

> T = Nf – 0.5nf

and we can estimate the number of fibrils as N = (T+0.5nf)/f

This allows us to estimate p = n/N = nf/(T+0.5nf)

Since p = f/(f+L), re-arranging gives L = f(1-p)/p

Thus an approximation of the length is given by:

> L = f(1-p)/p, where p = nf/(T+0.5nf)

[n = number of bottom tips, f = width of field, T = total length of fibrils visible]

Further simplification: If T is much larger than nf (number of ends x FOV width), then p is ~ nf/T

Then L = f (1-p)/p, which is approximately f x 1 x T/nf = T/n
Note that n is the number of one end (tip), which is half of the total number of ends.

So in this case, L = 2T/(number of ends) (*Eq. 4*).

This calculation makes several assumptions:
- The fibrils have the same length.

- Fibril tips occur randomly along the z-axis.

- Sufficient numbers of tips are identified that p is reasonable.

- The fibrils have two tips.

Collagen fibrils are almost certainly of different lengths in embryonic tissues and during wound repair. Therefore, according to Assumption 1, L_m is likely to have a large range (as previously suggested [34]). Evidence suggests that Assumption 2 is satisfied; the location of the base of fibripositors (plasma membrane invaginations that contains one end of the fibril) appears to be randomly positioned along the z-axis; furthermore, the location of the (second) tip in the ECM appears to be random. Assumption 3 is achievable for embryonic tissues using 3D reconstructions with z-depth of 10 μm. For late embryonic and postnatal tissues in which very long fibrils occur, a larger FOV is required to increase the probability of observing fibril tips; the 3View® protocol can readily provide volumetric datasets of z-depths of 100 μm.

Experimental Design

Choosing the 3View® microscope settings

When choosing imaging conditions it is necessary to find a balance between image quality, acquisition speed and sample damage. For the highest resolution it is desirable to use the lowest accelerating-voltage to reduce the beam's volume of interaction. One downside with this is that the detector efficiency decreases with the lower beam energy and so image noise becomes a problem. Lower accelerating voltage does have the advantage that charging is reduced, so it is possible to work closer to, or in, high vacuum conditions, which reduces beam spread and helps with collection efficiency. It is possible to counter some of the reduced detector efficiency by increasing the dwell time at each pixel, but this increases the time per image. The most important determinant of imaging conditions is the sample itself. More stain increases the back scattering potential and helps conduct some of the primary beam away from the sample surface. Harder resins allow a higher total dose to be used before cutting artifacts start to appear. When testing a new stain protocol, or resin, we have found that it is helpful to collect a secondary electron image along with the back-scattered image during sectioning. The secondary electron image shows more topographical information, which allows you to see damage accumulating on the block face earlier than you would notice it in the back-scattered image. In this way it is possible to identify what maximal imaging conditions (dose and vacuum level) a particular sample can cope with. Suggested settings for users examining collagen fibril-rich tissues are shown below:
- **3.8 kV operating voltage and a spot size of 3.5 (FEI Quanta setting)** – a good balance between speed of image acquisition and quality of image. To balance charging, a vacuum setting of 0.3-0.4 Torr was used.

- **100 nm cut thickness** – the shortest collagen fibril identified by fragmentation were in the range 1-5 μm (ref. [21]). A cut thickness of 0.1 μm would be ~1/10th the length of the shortest identified fibril and thus provide sufficient z-axis resolution to identify tapering tips of entire fibrils.

- **1000 sections** – A z-depth of 100 μm is ample to identify entire early fibrils forming at the plasma membrane and sufficient to enclose entire fibripositors, of which the longest recorded was ~18 μm (ref. [7]).

- **4900 x indicated magnification collecting 3000-x pixels, 3000-y pixels at 10 μs/spot dwell time** – These settings provide a pixel resolution of 10 nm, which is sufficient resolution to clearly identify fibripositors and individual collagen fibrils. (Collagen fibrils in embryonic tendon, skin and healing

wounds are ~ 30 nm in diameter.) The scan time is approximately numX x numY x dwell time, where numX and numY are the number of pixels used to make up the images, and the dwell time here is 10 µs. Therefore each scan takes ~1.5 min. The cut time is ~0.313 min, therefore, collection of 1000 sections is achieved in ~30 hours. Therefore, the 3View® can be set up in the morning, run through the day, overnight and through the next day, and data collected in the evening of the second day. The 3View is then ready to be set up for another data collection on day 3.

Choice of image-processing software

Measurements of fibril morphometric parameters and the generation of 3D reconstructions from serial electron microscope images are best performed using image-processing software. A wide variety of packages are available, some of which will have features better suited to some applications than others. In the examples shown below we describe the use of ImageJ[33], Fiji[38], SigmaPlot, Gatan DigitalMicrograph™ and IMOD[39] although the use of the protocol is not restricted to these programs. ImageJ is a public domain, image processing and analysis programs. Fiji is ImageJ, bundled with a host of useful plugins, including TrakEM2, which is plugin for morphological analysis and 3D analysis. SigmaPlot is a data analysis and graphing program that we use to display the diameter and FVF data. IMOD is a suite of image processing modeling and display programs for tomographic and 3D reconstruction from serial section EM. IMOD was developed by David Mastronade and co-workers at the Boulder Laboratory for 3-D Electron Microscopy, who provide expert advice on all aspects of the IMOD suite. IMOD is available for Linux, Windows and Mac OS X and is supported by an online User's Guide. IMOD can be downloaded from http://bio3d.colorado.edu/imod/, which also provides detailed descriptions of how to install and use the programs. Gatan DigitalMicrograph™ is an image acquisition and processing program that is available from Gatan and accompanies 3View®. The Gatan DigitalMicrograph™ images from 3View® can be converted to MRC format and inputted into IMOD for computational analysis.

Future perspectives

Future developments will include automation of collagen fibril measurements by TEM and single fibril tracking by SBF-SEM (3View®). For the automation of collagen fibril measurements, it should be possible to perform real-time analysis on images collected from a TEM fitted with a digital camera. With the microscope suitably calibrated for magnification, information would be obtained on fibril diameter distribution, fibril-volume-fraction and fibril profile circularity.

Automation of single fibril tracking would provide a major step-up in our understanding of fibril growth and length distribution. As shown in the examples below (Anticipated Results), it is possible to identify early fibrils at the cell surface because one tip of the fibril is contained within a recess in the plasma-membrane. However, the identification of entire fibrils in the ECM (e.g. contained with bundles of fibrils) is much more challenging and requires manual searching for fibril tips. Particle-picking routines would need to be developed that track fibrils and identify tapering tips, count the number of collagen fibrils in consecutive sections, and track the curvilinear path of individual fibrils within a bundle of fibrils.

MATERIALS

REAGENTS

Samples for analysis

This protocol has been validated on a wide range of collagen-rich tissues including:

- 3D cell culture tendon-like constructs and fibroblast populated collagen gels.
- demineralized bone
- embryonic and postnatal mouse and rat tendons,
- embryonic chick tendon,
- human Achilles tendon,
- human skin,
- mouse heart,
- mouse kidney,
- rabbit patella tendon
- rat diaphragm,

  ! CAUTION All animal and human tissue samples should be collected in accordance with the relevant ethics regulations, Animals Act and animal licences.

Tissue preparation and glutaraldehyde fixation

- Cyanoacrylate glue (no preferred supplier). **! Caution** Avoid contact with skin and eyes.
- Glutaraldehyde 25% (vol/vol) EM grade (Agar scientific, cat. no. R1011). **! Caution** Glutaraldehyde is harmful and may cause dermatitis or skin sensitization with prolonged skin contact. Wear gloves and safety goggles, and work in a fume cupboard. Avoid contact and inhalation.
- Hydrochloric acid (Sigma-Aldrich). **! Caution** HCl is poisonous, can be explosive, might be fatal by inhalation, and will cause burns. Open and use it inside a fume cupboard, wear gloves and safety goggles. Avoid skin contact and inhalation.

Osmium and tannic acid impregnation

- Osmium 4% (vol/vol) (Agar scientific, cat. no. R1024). **! Caution** Osmium tetroxide is highly toxic and may have reproductive effects. The vapour is an irritant and can cause burns to eyes and skin. It can cause dermatitis and ulceration. Open and use it inside a fume cupboard. Wear gloves and safety goggles.
- Potassium ferrocyanide (The Brtish Drug House Ltd, Laboratory Chemicals Division. cat. no. 214022). **! Caution** potassium ferrocyanide might cause irritation to eyes and might be harmful in contact with skin. May be harmful if inhaled. Might cause irritation. Open and use it inside a fume cupboard. Wear gloves and safety goggles. Avoid skin contact and inhalation.
- Sodium cacodylate (Agar scientific, cat. no. R1102). **! Caution** Sodium cacodylate is toxic and inhalation of small concentrations over a long period of time will cause poisoning as it contains arsenic. It may also be carcinogenic and mutagenic. Open and use inside a fume cupboard, wear gloves and safety goggles. Avoid contact and inhalation.
- Tannic Acid (TAAB scientific, cat. no. TO46)

Stains

- Uranyl acetate (Agar scientific, cat. no. R1100A). **! Caution** Uranyl acetate is toxic, radioactive and may be carcinogenic. There is a danger of cumulative effects.

Open and use it inside a fume cupboard. Wear gloves and safety goggles. Avoid skin contact and inhalation.

- Toluidine blue (Agar scientific, cat. no.R1727).

EM dehydration and infiltration

- Ethanol (Fisher scientific cat.no. e/0650DF/P17). **! Caution** Ethanol is highly flammable and intoxicating if inhaled or ingested. Wear gloves and safety goggles.
- Propylene oxide (Agar scientific, cat. no. R1080). **! Caution** Propylene oxide is toxic, flammable and carcinogenic. It is readily absorbed through the skin and causes blisters. It is irritating to skin, eyes, respiratory system and GI tract. Open and use it inside a fume cupboard, wear gloves and safety goggles, Avoid contact and inhalation. Use in glassware or polypropylene containers.
- Agar100 pre-mix kit, Hard Irritant (Agar scientific, cat. no. R1140). ! Caution Agar 100 resin is irritating to eyes and skin and may cause sensitization. The vapor may be harmful. Dodecenylsuccinic anhydride (DDSA) is irritating to eyes and skin. Methyl nadic anhydride (MNA) is irritating to eyes and skin and may cause sensitization. Dimethylbenzylamine (BDMA) is corrosive and can be absorbed through the skin. Worker must work in a fume cupboard. Wear gloves and safety goggles. Avoid contact and inhalation.

EQUIPMENT

Tissue handling, fixation and embedding

- 200 mesh copper EM grids (Agar Scientific, cat. no. G2700C)
- Compact light microscope (e.g. Leica DM LS)
- Fine forceps (Agar Scientific, cat. no. T5225)
- Fine forceps, curved tip (Agar Scientific, cat. no. T5131)
- Flat embedding molds (Agar Scientific, cat. no. G369)
- Glass vials (Agar Scientific, cat. no. B794)
- Glass slides (Agar Scientific, cat. no. L4339)
- Laboratory embedding oven capable of 60°C.
- Plastic Pasteur pipettes, 3 ml (Agar Scientific, cat. no. G3374)
- Polyethylene graduated containers, 100 ml (Agar Scientific, cat. no. G332)
- Razor blades. **! Caution** Sharp. Handle with care at all times.
- 1.5-ml tubes (Agar Scientific, cat. no. G3711)
- 15-ml tubes (Corning Incorporated cat. no. 430766)
- 50-ml tubes (Corning Incorporated cat. no. 430828)
- Single edge razor blades (Agar Scientific, cat. no. T585). **! Caution** sharp blades, handle with care at all the time. Dispose of used blades safely

Block trimming

- Glass knives (Agar Scientific)
- Diamond trimming knife (UltraTrim 45 Diatome)

Semi-thin and ultrathin sectioning and TEM

- Bright field light microscope (no manufacturer specified)
- Diamond knife (Ultra 45° Diatome)
- Dissecting microscope (OLYMPUS TL2)
- Hot plate (e.g. Kaiser 4141, temperature 20-45°C)

- Tecnai 12 Biotwin electron microscope (FEI Company) or similar.
- Ultramicrotome (Leica EM UC6 or equivalent)

SBF-SEM by 3View®

- Aluminum cryo pin, bullseye (Electron Microscopy Sciences, USA, cat no 70446)
- Miniature hacksaw
- Minispin Eppendorf centrifuge
- QUANTA FEG 250 (FEI Company) equipped with a Gatan 3View® ultramicrotome

Computation and image processing

- Macintosh desktop or laptop computer running Mac OS X (preferred) or Linux or Windows PC with high performance graphics card.
- Wacom tablet (Cintiq) with interactive pen, 12 or 24-inch (Wacom Company)
- IMOD software
- SigmaPlot (Systat Software Inc.) – Optional.
- ImageJ

REAGENT SETUP

Glutaraldehyde, 2.5% (vol/vol) Prepare fresh for each experiment, 1 ml per sample. Combine 2.5% (vol/vol) glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2

Reduced osmium, **2% (vol/vol)** prepare fresh for each experiment, 0.6 ml per sample. Combine 2% (vol/vol) osmium tetroxide in 0.1 M sodium cacodylate buffer, pH 7.2 and 1.5% potassium ferrocyanide.

Osmium tetroxide, aqueous, **2% (vol/vol)** prepare fresh for each experiment, 0.6 ml per sample. 2% (vol/vol) osmium tetroxide in distilled water.

Potassium ferrocyanide stock solution, 3% (wt/vol). 3 g of potassium ferrocyanide in 100 ml of 0.2 M sodium cacodylate buffer, pH7.2. Can be stored at 4°C for 2 months.

Sodium cacodylate buffer stock solutions, 0.2 M (42 g of sodium cacodylate, 0.5 ml glacial HCl, make up to 1 litre of solution). Can be stored at 4°C for 3 months.

Tannic Acid (TAAB EM grade, MW 1701.23,cat. no. T046), **1% (wt/vol)** prepare fresh for each experiment, 1 ml per sample. 1% tannic acid in 0.1 M cacodylate buffer, pH 7.2.

Toluidine blue, add 1 g di-sodium tetraborate (borax) to 100 ml distilled water. Add 1g toluidine blue to the borax solution and allow to dissolve. Filter the solution and store in a brown bottle. Can be stored at RT (20-22°C) for 3 months.

Uranyl acetate aqueous 1% (wt/vol) prepare fresh for each experiment, 1 ml per sample. 1%(wt/vol) uranyl acetate in distilled water.

PROCEDURE

Tissue preparation and fixation ● TIMING 2.5 h

1 | Cut tissues into small pieces (ideally 0.5-1 mm cubes) to allow good penetration of fixative and stain.

! CAUTION All animal and human tissue samples should be collected in accordance with the relevant ethics regulations, Animals Act and animal licences.

∆ **CRITICAL STEP** Prevent tissues from drying.

2 | Transfer all samples to 2.5% glutaraldehyde made up in 100 mM cacodylate buffer (pH 7.2). Fix the specimens for 15 min at room temperature (RT, 20-22°C). Cut into smaller pieces (if required) and place into fresh fixative. Continue fixation for 2 h at 4□C after which wash the specimens in 100 mM cacodylate buffer (pH 7.2) for 5 x 3 min in RT.

? TROUBLESHOOTING

PAUSE POINT. Samples can be stored overnight or shipped to the EM laboratory without significant degradation of specimen quality. For shipping, transfer samples to fresh phosphate buffer (pH 7.4) in a sealed tube. Glutaraldehyde is highly effective at cross-linking collagen fibrils and introduces detectable mass leading to specimen stiffening, especially during extended reaction times [40]. Furthermore, from a health and safety viewpoint, it is preferable to transfer samples in phosphate buffer rather than glutaraldehyde. Label the tubes clearly with 'Sample ID', 'Date' and 'User ID'. The tubes should be filled with buffer and securely sealed with stretch film. The samples are safe to be shipped at RT (or cooler) for up to 5 days. If the samples are transferred to phosphate buffer the receiving laboratory must wash the samples (5 x 3 min) in fresh cacodylate buffer (100 mM, pH 7.2) before starting the next step

En bloc reduced osmium staining ● TIMING 1 h 15 min

3 | Incubate tissue in freshly-made 2% osmium tetroxide and 1.5% potassium ferrocyanide in cacodylate buffer (100 mM, pH 7.2) for 1 h at RT. Wash with double distilled water (ddH$_2$O, or equivalent) for 5 x 3 min at RT.

Tannic acid staining ● TIMING 4 h 15 min

4 | Transfer the specimen to freshly made 1% tannic acid in 100 mM cacodylate buffer (pH 7.2) for 2 h at 4□C. Replace with fresh 1% tannic acid solution for another 2 h at 4□C. Wash the specimen in ddH$_2$O for 5 x 3 min at RT.

△ **CRITICAL STEP** Staining with tannic acid should be performed for at least 3 h because of slow penetration of most tissues.

? TROUBLESHOOTING

Second osmium staining ● TIMING 55 min

5 | Incubate with 2% osmium tetroxide in ddH$_2$O for 40 min, at RT. Wash specimen gently in ddH$_2$O for 3 x 5 min at RT.

△ **CRITICAL STEP** Before staining the sample with uranyl acetate (next step), the samples must be washed thoroughly because uranyl acetate reacts with the cacodylate buffer leading to a grainy contamination.

Uranyl acetate staining ● TIMING 16 h + 15 min

6 | Incubate with 1% uranyl acetate (aqueous) at 4°C for 16 h (overnight) then wash the specimen in ddH$_2$O for 3 x 5 min at RT.

△ **CRITICAL STEP** Uranyl acetate solution should be cleared before use by centrifugation 5000 x *g* for 5 min.

Dehydration, replacement and infiltration ● TIMING 1 h 30 min + 4 h

7 | Dehydrate the specimen in graded ethanol: 30%, 50%, 70%, 90% ethanol in ddH$_2$O for 10min each step. Final step is 100% ethanol; 4 x 10 min at RT.

8 | Transfer the specimen to propylene oxide for 10 min at RT.

9 | Infiltrate in graded series of Agar100Hard in propylene oxide at RT. First 4 h in 30% Agar100Hard, then overnight in 50% Agar100Hard. Then 1h in each of 70%, 90% and 100% Agar100Hard. Finally repeat 1h in 100% Agar100Hard twice more.

? TROUBLESHOOTING

Embedding ● TIMING 72 h

10 | Transfer samples to freshly made 100% Agar100 Hard in labeled molds. Allow to cure at 60°C for 72 h.

Electron microscopy

! CRITICAL Electron microscopy is a high-vacuum technology that requires specialist expertise and learning. The steps described below should be carried out under the guidance of experienced personnel.

11 | Researchers trained for TEM sectioning and imaging can use the same blocks for TEM and SBF-SEM. Choose Option (**A**) for TEM analysis of ultrathin sections. Choose Option (**B**) for 3View® SBF-SEM. Choose Option (**C**) to prepare samples for serial image reconstruction either after ssTEM or 3View®. Option C shows how to use IMOD to generate colored 3D reconstructions. The 3 main stages are: prepare a single file that contains the stacked 3View® images; generate a simple Step-Through movie that is playable by QuickTime; and generate a false-color 3D reconstruction. Although the protocol here uses IMOD it is possible to complete these steps with other image processing software. If the reader is familiar with a different image-processing package then option C can be used as an indication of processes that are be used to convert and filter the data and the order in which they should be run.

(A) Semi-thin and ultra-thin sectioning for TEM ● TIMING ~ 3 days for steps i-vi; 2h for steps vii-xvii

      i.   Remove blocks from molds.

      ii.   Using a diamond trimming knife cut semi-thin (2 µm thick) sections and place on a glass slide.

      iii.   Stain with 1% toluidine blue, rinse in distilled water, and dry the sections on a hot plate.

      iv. Examine the semi-thin sections using a light microscope to verify the specimen area, orientation and section quality (i.e. torn tissue or the presence of holes).

      v.   For ultra-thin sectioning, prepare 70-80 nm-thick (silver or gold interference colors on water) sections using an ultramicrotome.

      vi. Collect the sections on 200 mesh copper grids and examine, without additional staining, using a Tecnai 12 Biotwin electron microscope.

      vii. Automated measurement of fibril transverse sizes and shapes. Import images into ImageJ using File ☐ Open sequence.

viii. Set pixel size in nm using Analyze → Set Scale sequence. Set as global.

ix. Remove extreme intensity points using Image → Noise → Despeckle sequence.

x. Smooth noise but retain clear fibril edges using Process → Filter → Gaussian Smooth sequence.

xi. Set threshold level using interactive mode using Image → Threshold sequence. Adjust level to fill fibril with minimum holes areas but also minimize merging of fibrils.

Convert threshold image to binary using Image → Binary → Make Binary sequence.

xii. Edit image to fill any holes by marking regions and filling using 'Ctrl+F'.

xiii. Separate any merged fibril regions using Process → Binary → Watershed sequence.

xiv. Set measurements using Analyze → Set Measurements: Area, Shape Descriptor, Perimeter, Fit ellipse.

xv. Run particle analysis using Analyze / Analyze Particles sequence. Set 'exclude objects over boundary' and 'display of masks'.

xvi. If the tendon sections are not transverse to the tendon long-axis, the cross-sectional shape of an otherwise cylindrical fibril will be an ellipse. In this case, measure the minor axis of the fitted ellipses.

xvii. For improved accuracy of perimeter measurements and associated shape measurements (e.g. circularity) run the 'ShapeFilter' Plugin after Analyze Particles. This can be run on the binary mask image generated by Analyze Particles using Plugins → Shape Filter. The order of fibrils measured is the same as for that for Analyze Particles.

? TROUBLESHOOTING

(B) Sample mounting and loading for 3View® ● TIMING 3 h for steps i-xi; 1.5h for steps xii-xvi

i. Attach the sample (~1-2 mm cube) to a cryo pin using cyanoacrylate glue and allow the glue to cure. Blocks can be pre-glued in batches to minimize waiting times.

△□**CRITICAL STEP** Users who are new to 3View® are advised to work alongside experienced personnel to ensure that the microscope performs at the manufacturer's specification. 3View® comes with a set of instructions for loading a sample and starting a run. The steps described here are a brief description of a procedure used in our laboratory and should be seen as complimentary to the standard instructions.

PAUSE POINT Samples can be stored for at least 2.5 years

ii. Once the glue has set, accurately and cleanly trim the block face with near vertical sides. Fragments of sections can affect image recording, therefore it is important to obtain edges that are clean. It is also important to trim the edges close to 90° vertical so that the block face does not become too large as the volume is cut away in the 3View®. A razor blade can be used, however, better results can be obtained by rotating a glass knife around the sample. If possible, trim into the sample to expose the stained tissue to expose a potential conduction path and prevent excess charging (for discussion see [10]).

iii. If the precise region of interest is not readily apparent or there is concern over stain penetration, it is advised to trim the trapezoid leaving a larger face than normal. The sample will still require trimming with a diamond knife to get a freshly cut face, but is possible to check stain gross morphology and stain quality in the scanning electron microscope. By using the back-scattered electron detector to examine the stain quality and the secondary electron image to locate the position in the block face it is possible to accurately trim the sample with the aid of the stereo-microscope. This technique is analogous to checking a semi-thin TEM section using a toluidine blue stain before trimming a smaller block face, but it allows examination of the final image quality.

iv. Use the Gatan conical shaped sample pin holder to trim a flat face using a diamond trimming-knife on an ultramicrotome set up to cut a face perpendicular to the mounting pin.

v. To minimize the time taken to approach the sample within the 3View® system, cut the face of the sample to match the face cut within the 3View®. A good starting place is to set all of the approach angles (sample and knife) to 0 degrees. With experience a more precise setting can be found to match a particular microtome with a particular 3View® system.

vi.   Coat with gold to give a conductive surface. In our hands the thickness of the gold layer does not appear to be critical. Any non-powdering conductive coating will be sufficient, as the face will be cut away before imaging. The remainder of the coating helps to dissipate electrical charges that can accumulate during imaging.

vii.   Using the main height adjusting screw move the sample close while watching the reflection in the stereomicroscope. If the block face is parallel with the knife cutting action it is possible to align to the second, paler, reflection (cutting face reflection), which means that the knife is approximately 10-15 µm away.

? TROUBLESHOOTING

∆ **CRITICAL STEP** It is important to move the 'stroke' up when bringing the sample towards the knife-edge. The 'stroke' is normally down in order to allow the knife to move over the sample without damaging it. When the sample is cut the 'stroke' moves up by more than 10 µm. If the adjustments are made with the stroke down it is possible to damage the knife by cutting a very thick first section.

viii.   Set the approach settings in Gatan DigitalMicrograph™ to: 100 nm, 100 cuts.

ix.   Start the approach and watch the knife cutting through the stereomicroscope in order to check that the knife is clearing the sample. It is possible to watch until the block face starts to cut. It might be necessary to start the approach for a second time if the sample has not started cutting within the 10 µm.

x.   Stop cutting when the block face is cut sufficiently and clear the knife out of the way.

xi.   Use a puff of dust remover to clear the block and knife-edge.

? TROUBLESHOOTING

(B) ● TIMING 1.5h

xii.   **3View® set up and image acquisition.** For studies of collagen fibrils *in situ* use the settings: accelerating voltage = 3.8 kV; spot size = 3.5; aperture = 30 µm.

xiii.   Within Gatan DigitalMicrograph™ use search parameters (512 x 512, 1 µs) at a low magnification to select a region to check the focus.

? TROUBLESHOOTING

∆ **CRITICAL STEP** The stigmator setting is one of the most critical settings for consistent imaging. It is worth spending some time here at high magnification and adjust the settings using a live fast Fourier transform (FFT: chosen from the **Process** menu in Gatan DigitalMicrograph™) to check for a clear circle. Adjust the focus and check for a circularly symmetric power spectrum.

xiv.   Set the acquisition parameter cutting-step to 100 nm and set imaging parameters.

xv.   It is useful to make a note of the pixel size and cut thickness to aid future data manipulation away from the acquisition software. The pixel size can be found in **ImageDisplay>Calibrations** within Gatan DigitalMicrograph™.

xvi.   Start the acquisition. Watch the first few images to check that all is well. If the sample does not cut for a few frames it is worth leaving it for up to 10 cuts (1µm) to check if problems with accumulation of imaging dose during the uncut period are removed and the resin starts to cut without any further problems.

(C) Image processing for ssTEM and 3View® TIMING 10 min for step i; 30 min for steps ii-xiii; 10 min for steps xiv-xxiv; timing for step xxv-xxvi is open ended.

i.   Download and install the recent beta version of IMOD. The latest version at the time of publication was IMOD 4.6.15, which is able to read the **.dm4** files produced by 3View®. Up-to-date information about installing the software on a variety of operating systems is available on the IMOD website (http://bio3d.colorado.edu/imod/).

ii.   **Preparing the image stack.** From command line (e.g. 'Terminal' in Mac OS X/Linux or 'Cygwin' on a Windows PC) change directory to the Desktop (e.g. in Mac OS X key $ cd ~/Desktop)

iii.   Make a new directory called 'My3ViewProject' (e.g. $ mkdir My3ViewProject)

iv.   Change directory to My3ViewProject e.g. cd ~/Desktop/My3ViewProject

v.   Copy the 3View .dm4 files to My3ViewProject.

vi.   Stack the .dm4 files into a single .mrc file by running the following Shell Script.

```
#!/bin/bash
#
#Shell Script to stack .dm4 files
#
mkdir temporary
cd temporary
dm2mrc ../My3ViewProject/*_*.dm4 initial.mrc
newstack -float 2 initial.mrc floated.mrc
clip smooth -2d floated.mrc smoothed.mrc
```

∆ **CRITICAL STEP** The Shell Script floats the images to the same mean and standard deviation to compensate for changes in image intensity caused by changes in the room temperature affecting detector sensitivity during long runs. The output file is 'floated.mrc'. The file 'initial.mrc' is the unprocessed image stack. *newstack* is used here to float the images to a common range or mean of density however it is a powerful stack editor within IMOD capable of binning images, applying specified rotation or expansion, and pulling out a range of images from a previously-formed stack. To learn more about the capabilities and options within *newstack* type 'man *newstack*' from a terminal. *Clip* is used here to smooth images but has additional functions including image averaging, contrast adjustment and false coloring. To learn more about the capabilities and options within *clip* type 'man clip' from a terminal. If desired, to remove some of the noise in the images, smooth the files in 2D to produce 'smoothed.mrc'. This processing is run on each 2D image separately because each voxel in the image is incomplete as the depth of imaging does not match the depth of cutting with our current imaging protocol.

∆ **CRITICAL STEP** A beta version of a more complete Shell Script that includes all of the steps vi-xiii can be downloaded as Supplementary Data 4 3view-dm4-script.sh. The script floats the images, converts to 8-bit, and corrects the header information and removes all of the intermediate files. The beta script also flips the images about the X-axis to compensate for the way that the MRC file orders the images. The script can be edited using a text editor such as *vim*. To run the script put the dm4 files into folders with a suitable name with no spaces. Place the script in the same top folder and make sure it is executable using the command:

```
$ chmod +x 3view-dm4-script.sh
```

Then run as the data folders and run the script with the command:

```
$ ./3view-dm4-script.sh foldername1 foldername2
```

This should generate a separate folder containing MRC files named after the data folders (in this case foldername1.rec foldername2.rec)

vii.   If desired, half the file size by converting the files from 16 bit to 8 bit. 8 bit (0-255) is suitable for most analyses. To convert the file: open the smoothed data set in IMOD cached. Select cache 1 image in the option window, or type:

```
$ 3dmod –C 1 smoothed.mrc
```

viii. Check the contrast and brightness through the data set and make a note of your favored black and white values (see the bars in the 3dmod window). Input the values into the following command:

```
$ newstack –c [black],[white] –mode 0 smoothed.mrc my3view.rec
```

ix.   Check that the voxel size is correct in order that subsequent models give realistic results. To check the header information type

```
$ alterheader smoothed.mrc
```

x.   Now check the 'Pixel spacing (Ångstroms)' . If the information has not been successfully transferred from the **.dm4** file the correct values can be added by typing 'del', then typing X Y Z in Ångstrom. As an Ångstrom is 1/10 of a nanometer, it is necessary to multiply all of the nanometer values by 10. Thus for our typical data sets we would type

```
Enter option: del

Alter delta - changes cell sizes to achieve desired pixel
spacing
```

```
            Current delta x, y, z:  1.000       1.000        1.000

            New delta x, y, z: 100 100 1000
```

xi.   To finish editing the header, type 'done'. This example will set the voxel size to 10 nm x 10 nm x 100 nm.

xii. If you have not converted the stack to 8bit (step viii) then change the name of the image stack to something meaningful, e.g.

```
$ mv smoothed.mrc my3view.rec
```

xiii. Delete the other intermediate files (initial.rec and floated.rec).

xiv. **Making a step-through movie.** Make the movie by writing .jpg files from `my3view.rec` and reading the files into ImageJ:

From a command line type:

```
$ 3dmod -C 1 my3view.rec
```

∆ **CRITICAL STEP** If a model has been generated using the MRC file it is possible to open the model file within IMOD in order to overlay the model points and contours on the slice images. It is worth spending some time to familiarize yourself with the model overlay options within the Objects menu (found from within the model view). We would typically adjust the color of the objects and the 2D line thickness when overlaying a model on a slice view.

xv.   Set a folder in which to save the individual movie frames: From the File menu, select 'Set Snap Dir...'

xvi. Create a folder called 'movie'.

xvii. Choose your region of interest by selecting the image (Zap) window and adjusting the level of magnification.

xviii. When the region of interest is selected press 'Shift-T' to remove the small cross-hair (pressing T on its own will toggle the model points on and off if this is needed).

xix. From the 3dmod menu, select 'File>Movie/Montage...'

A control panel appears. Select Start/End, Increment, Round Trip or One Way, JPEG, Start/end. Do not click 'Done'.

xx.   Middle, or Right click on the image to start scrolling through the slices.

The frames for the movie will be saved to the selected folder as Zap_xxx.jpg.

xxi. When the frames have been created, launch Fiji.

xxii. Import 'Image Sequence'.

xxiii. Test movie speed (frames per second) Image>Stacks>Tools>'Animation Options…'

xxiv. Save as .AVI (or other suitable format).

∆ **CRITICAL STEP** There are several variations to the above listed procedure. For example, it is possible to change the magnification during the image output phase by pressing + or – on the keyboard. Explore the options in IMOD to produce the desired image sequence. In ImageJ it is possible to select alternative numbered images and to alter the frame rate. These choices influence the file size, which might be important when importing the movie into a presentation package or when sending the file electronically to another computer.

xxv. **Segmentation.** Data segment as desired. There are many packages that offer tools for 3D data segmentation. Free packages that can be used include TrackEM2, which is included as part of the Fiji (Fiji is ImageJ: http://fiji.sc/TrakEM2). Currently we are using IMOD as it includes a host of utilities that allow you to generate models. There is a comprehensive guide to using IMOD (man pages from command line and on the IMOD website (http://bio3d.colorado.edu/imod/doc/3dmodguide.html). For modeling cells and organelles it is possible to trace the features using a closed contour. If the cell cross-sectional shape remains much the same through each section, it is possible to generate a relatively quick model of a cell by tracing around the plasma membrane every 10 sections (every 1 µm). This crude model can be meshed by selecting the SKIP option in the meshing menu. The benefit of this system is that if a higher resolution image is required at a particular region it is possible to fill in the missing section contours and re-mesh the volume. When we track individual collagen fibrils we use open contours and put a

single point on each section. These contours can be meshed as tubes in order to be seen in the 3-dimensional model.

Δ **CRITICAL STEP** Currently IMOD defaults to treating a volume as though image 1 is the bottom image and you are viewing the stack from within the volume. To examine 3View® data where image 1 is the top image and you are viewing from above the stack you need to select the option to invert Z (With the Model View window selected, choose **View>'Invert Z'**). Failure to do this will invert the handedness of features that rotate as they pass through the volume, but this can be corrected by flipping any images generated (NB: the attached beta script compensates for this by flipping the images about the x-axis, so inverting Z is not required if this script is used).

xxvi. After features have been modeled, get information on the volume, lengths and separation distances using an **open contour** to track between two points of interest. Alternatively, obtain information on cell volumes, or fibril lengths are found using the program **imodinfo**

(D) Estimating the length of a collagen fibril TIMING 30 min

i. Open an image stack in IMOD (If the data set is too large it may be necessary to cache the data, e.g.

```
$ 3dmod –C 1 myimagestack.mrc mymodel.mod
```

ii. Click 'model' in the mode panel of the IMOD window.

iii. Select a fibril to model. Change object type to **open** (or start a new open object) Edit>Object>type

iv. Select to display a circle at each point and set a suitable diameter (this can be re-adjusted later). Track the fibril by clicking the middle mouse button once on each slice. It is possible to start tracking in the middle of the fibrils and work to one end, and then go back to the other end. If this is done the model will be incorrect as it will have long connections from the first end found back to the middle. The points can be sorted into the correct order using **Edit>Point>'Sort by Z'**.

v. If desired change the color in the model window by selecting model view (**Shift-V** to open (or **Image>'Model View'**)), then the object window (while model window is in front Shift O, or Edit Objects). The model can also be meshed as a tube at this point, but this will not affect any calculations based on the contours.

vi. Find a single fibril length by selecting the contour corresponding to the fibril of interest and choosing **Edit>Contour>info** The output appears in the main information window. Generally it is more efficient to model multiple fibrils using separate contours for each fibril and separate objects to group fibrils by type (such as totally internal fibrils, or fibripositor fibrils). The lengths of these fibrils can be found by interrogating the model file using **imodinfo**. A typical command would be:

```
$ imodinfo –l –f output_data.txt –input INPUTMODEL
```

This command will save a text file containing information about the model, which includes the lengths of the open contour objects calculated based on the voxel information within the MRC file header.

vii. If desired, use the '-l' option to print lengths of contours. Alternatively other options are available including 'Print ratio of length to area for closed contours' (option –r). For a complete list of options type:

```
$ man imodinfo
```

TABLE 1 | Troubleshooting table.

Step	Problem	Possible reason	Solution
2	Some tissues might shrink when excised. This is particularly noticeable in embryonic tendon. It might be desirable to keep the tissue at its physiological length during the fixation process.	Cell-mediated contraction of the extracellular matrix.	For tendon, immerse the entire limb or tail in glutaraldehyde-cacodylate fixative, remove the skin, and leave the tendon attached to bone and muscle. In older tissue, cut down the middle of the tendon to expose the tissue to fixative.
4	Low contrast of cellular structures.	Insufficient stain.	Tannic acid is a mordant and can improve the staining.

9	Selecting an appropriate resin. We have evaluated different resins for studies of collagen-rich tissues by TEM and 3View®, including CY212, TAAB LV and Agar100Hard.	Our experience is that CY212 is sticky and difficult to handle, it is difficult to change to 100% resin and infiltration is poor. TAAB LV-embedded specimens are too soft for serial sectioning by 3View®.	Agar100Hard is conveniently premixed and easy to handle. Infiltration and embedding is good throughout. It is ideal for routine sectioning and for processing large numbers of samples. Blocks can be used both for TEM without additional staining and 3View®.
11 (A)	Individual collagen fibrils are not accurately detected.	The automated steps are most effective on images with high contrast in which the edges of the fibrils are clearly discernable.	Manually segment.
11 (Bvii)	Difficulty adjusting the rotation of the sample.	The knife located over the sample.	If you cannot fit your fingers between the knife and microtome in order to nudge the grub screw, it may be easier to adjust the rotation with the knife in the 'clear' position.
11 (Bxi)	Contamination of the knife.	This might be because the propellant contains oils.	Use only a brief puff of dust remover to clean the cutting area. If possible, use dust-negative propellant.
11 Bxiii)	We have found that some samples shrink slightly when first exposed to the microscope vacuum.	Unknown for certain, however, confirm that the sample face is at the cutting position before focusing.	Take a few test cuts e.g. 10 x 100 nm sections before completing the focusing.

ANTICIPATED RESULTS

3View® protocol development for collagen applications – Figure 4 shows a 3D reconstruction of embryonic tendon with cells and bundles of collagen fibrils displayed in different colors. The sample was fixed, *en bloc* stained (osmium tetroxide and uranyl acetate) and embedded for conventional TEM as previously described[8]. 3View® was used to cut 795 x 100 nm-thick sections. The Gatan DigitalMicrograph™ images were stacked using IMOD and segmentation done manually to track individual cells and bundles of collagen fibrils. The results showed that IMOD was highly effective for analysis of the image stack. We found that using a Wacom® Cintiq tablet to trace the outlines of objects greatly simplified the segmentation tasks. Regarding the specimen preparation, we found that the contrast obtained from our microscope was sufficient to identify the outlines of individual cells and fibril bundles but too low to provide sufficient confidence to track single collagen fibrils. It should be noted that good contrast from conventional staining and embedding protocols has been obtained using serial section scanning electron microscopy combined with focused ion beam milling[41], although milling thin layers (~15 nm, thereby increasing the number of images collected) might be necessary to see the tapered tips of collagen fibrils that are ~30 nm at their thickest point. The curvilinear organization of the cells and ECM of the tendon as shown by 3View® agrees with the curvilinear crimp structure of tendon as seen by plane polarized light microscopy [42,43], and would be lost when serially aligning to the previous section as in ssTEM.

Studying collagen fibril assembly *in vivo* using 3View® – Detailed studies of collagen fibril assembly *in vivo* require the ends of fibrils to be located with high precision because the ends contain sites of molecular accretion during fibril elongation[44]. The extreme length of the fibrils *in vivo* makes identifying fibril ends by conventional serial section EM extremely challenging. In this regard, the speed of 3View® permits relatively large z-depths of tissue (~100 μm per 2 working days) to be imaged in a reasonable time frame and the resolution is sufficient to identify fibril tips. During new fibril formation, such in embryonic development[7] or wound healing[45], the ends of fibrils often occur in plasma membrane recesses. To illustrate this point, Fig. 5 shows the course of a fibril from within a plasma membrane recess to within a bundle of collagen fibrils in the ECM where the other end of the same fibril is located. See also Supplementary Data 1 ('complete-fibril.mod', which is an IMOD model file) and Supplementary Video 2 ('complete-fibril.mov'). To view Supplementary Data 1, install IMOD and use the following command:

```
$ 3dmodv complete-fibril.mod
```

Within IMOD it is possible to zoom in and out of the model and rotate the volume. The length of the short fibril is 5.1 µm, which is in good agreement with the length range of short fibrils isolated from embryonic tendon by mild homogenization[21]. Figure 6 shows screen shots of Supplementary Data 1.

Estimating the mean length of collagen fibrils by 3View® – As shown in the above section, it is possible to trace the entire path of relatively short fibrils by 3View®. However, especially in aged tissues, the fibrils are too long to be enclosed within a 100 µm-z-depth volume of a typical 3View® run. Therefore, it is only possible to *estimate* a value for mean fibril length (L_m). One approach for estimating mean fibril length is to generate an image stack from a 3View® run on 100 x 100 nm-thick sections (z-axis, parallel to the fibrils, of 10 µm) and to count the number of fibril tips and the total length of the fibril shafts in a bundle of fibrils. The mean fibril length (L_m) is estimated according to Equation 4 (corresponding to that used for the mean length of isolated fibril length fragments). Applying this approach to E15.5 mouse embryonic tendon produced a value for the mean fibril length of 587 ± 86 µm. From the same dataset we showed that the average cell length was 53.1 ± 8 µm, which is ~ 1/10th the average fibril length.

Example 3View® datasets – Two example datasets are available for download. These are:

> Supplemental Data 2 (example_bin4.zip);

> Supplemental Data 3 (example_cropped.zip);

The datasets are folders of 100 images in tiff format. To convert the images back into an MRC stack by using the commands:

```
$ tif2mrc example_bin4/*.tif example_bin4.mrc
```

```
$ tif2mrc example_cropped/*.tif example_cropped.mrc
```

The image stacks can be viewed in IMOD using the following commands:

```
$ 3dmod -C 1 example_bin4.mrc
```

```
$ 3dmod -C 1 example_cropped.mrc
```

example_bin4 – To highlight some of the artifacts that can arise, we generated the images using an old 3View® knife that introduced marks on the block face. The images show some clear knife marks running vertically within the images. The knife marks are more noticeable in regions surrounding the sample where the resin is softer because of the lack of stain. Stepping through the volume it is possible to see horizontal lines, especially in the empty resin. These lines are caused by knife chatter. The stained region shows reduced chatter because of the stain density. Another artifact is a fleck of gold near the top of the 22nd image. The gold fleck probably originated from the outer coating and has fallen on the block face. The 56th image in the dataset appears to have a ghost section. This has arisen because a section has landed on the block face near the end of the acquisition phase. Presumably it was sitting off to one side and was drawn in by electrical charging during the imaging phase.

The protocol typically produces datasets with good alignment, as shown by the example datasets. Alignment problems can arise if the electron dose is increased. We suspect this is because variable charging induces shifts and pin-cushion affects in the beam that change from slice to slice.

To illustrate the quality of the images that can be obtained, yet keeping the file size low to facilitate download, we cropped the original dataset without binning. The images and assembled mrc file can be downloaded from Supplemental Data 4 and 5, respectively.

Alternative views of the image stack – IMOD can provide alternative views of the image stack. Figure 7 shows an example in which the y-plane and z-plane of an image stack are superimposed.

Smoothing in IMOD – IMOD can remove some of the noise in the images using the `clip` program. Figure 8 shows a comparison of an unsmoothed and a smoothed image using the default smoothing kernel.

Figure legends

Figure 1

Embryonic chick metatarsal tendon prepared using the reduced osmium protocol and imaged using 3View® and TEM. (a) a typical 3View® image of the block face. The inserts show the boxed area in serial sections. Collagen fibrils (red arrows) and fibrillin microfibril bundles (black arrows). (b), a TEM image of a 60 nm-thick section from the same block and imaged with an FEI BioTwin TEM. Insert, boxed region shown at a higher optical magnification to show collagen fibrils (red arrows) and fibrillin microfibrils (black arrows).

Figure 2

TEM of postnatal tendon. (a) A tendon fibroblast flanked by *D*-periodic collagen fibrils. Scale bar, 500 nm. (b) Enlarged view of (a) showing fibril *D*-periods. Scale bar, 100 nm. (c) Tendon sectioned transversely to the long axis of the tissue. Scale bar, 1 μm. (d) Enlarged view of (c) showing near-circularity of fibril profiles. Scale bar, 200 nm.

Figure 3

Semi-automated measurements of fibril diameter from transverse sections. (a), A typical TEM image of the transverse section of adult rat-tail tendon. (b) The binary image was derived from the image in (a) by thresholding to identify the dark fibril regions followed by application of the Watershed algorithm to separate close fibril boundaries. (c) The outline of each object fully contained within the image area in (b) is fitted to an ellipse. The long axis of the ellipses in (c) are aligned, which is consistent with a slight departure of the fibril images from precise transverse section. (d, e, f), Measurements from the binary image using the Analyze Particles routine of ImageJ. (d) Fibril diameters based on the fitted ellipse minor axes. The distribution has been fitted to two Gaussian distributions (solid line). (e) Area distribution computed from binary image (b) and corrected for fibril tilt indicates the relative area contribution of fibrils of different sizes. (f) The area-weighted version of the histogram shown in (e). Scale bars = 200 nm.

Figure 4

3View® analysis of a conventionally *en bloc* stained resin-embedded sample of a newborn mouse tendon. The z-depth of the reconstruction is 795 x 100 nm-thick 3View® sections. 3D reconstruction and segmentation were completed using IMOD. The 3D reconstruction is viewed 'side on' to the long axis of the tendon. Colors represent different cells and bundles of collagen fibrils. The fibril bundles are continuous throughout the 3D reconstruction.

Figure 5

Complete collagen fibril at the plasma membrane of a fibroblast in embryonic mouse tendon. (a) Images produced using the 'Snap Tiff As …' command in IMOD selected from 'complete-fibril.mov'. A short fibril at the plasma membrane is highlighted with a red circle. The proximal tip is within a plasma membrane recess. The distal tip is within a bundle of fibrils. The length of the fibril is 5.1 μm. (b) Schematic showing the relative position of the fibril (red) with the plasma membrane of the cell (grey) and extracellular collagen fibrils (yellow). Scale bars, 500 nm.

Figure 6

Screen shots of an IMOD model window from 'complete-fibril.mod'. Blue, nucleus. Green, plasma membrane. Gray, the plasma membrane recess. Red, the extracellular aspect of the short fibril. Yellow, collagen fibrils. Arrow, fibril ends. Scale bar, 1 μm.

Figure 7

Alternative views of an image stack produced in IMOD. The image is based on the original data set used to prepare Supplemental Data 2. To generate the image the entire volume was opened in IMOD. The image was generated by examining the volume in the model view (Press V, or select Image> Model View). The Z and Y planes were opened by selecting the Image option within the model view (Shift I, or Edit > Image). The background color was changed to white (Shift B, or Edit > Background) and a 2 x 2 montage was saved (File > Movie/Montage).

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Figure 8

Example output images from *dm2mrc* and *clip* showing the effects of smoothing on image appearance. The dataset was the original dataset used to prepare Supplemental Data 2 prior to binning. (a) a non-floated and non-smoothed image produced by *dm2mrc*. Insert shows enlarged area containing two fibripositors surrounded by collagen fibrils. (b) the same image stack was floated (produced by *newstack*) and smoothed (produced by *clip*). The image shows the same two fibripositors and surrounding fibrils. Scale bar, 1 μm. Inserts, 5x magnified views to make voxels visible.

SUPPLEMENTARY INFORMATION

Supplementary Data 1: complete-fibril.mod

IMOD-generated model file of a cell (green, day 13 embryonic chick metatarsal tendon fibroblast), three extracellular matrix collagen fibrils (yellow) and a short entire fibril (red) within a recessed fibripositor (gray). Nucleus, blue. To view this file, install IMOD and use the following command:

$ 3dmodv complete-fibril.mod

Supplemental Data 2: example_bin4.zip

An example dataset of 3View® images. The dataset shows 100 images from 400 x 100 nm slices through embryonic mouse tail tendon. The images have been binned x4 to reduce the file size from 4096 x 4096 to 1024 x 1024. The voxel dimensions are 45 x 45 x 100 nm. The DigitalMicrograph™ images were converted to TIF format using `mrc2tif`. N.B. the images in this data set are flipped about the x-axis. The flipping means that the scanning line order is inverted, i.e. the image is generated from the bottom of the screen. The reversed image line order affects the appearance of some of the section fragments that fall onto the surface during image acquisition.

Supplementary Data 3: example_cropped.zip

A cropped volume (at original resolution) of the same image stack that was used to generate 'example_bin4'. The dataset shows 100 images from 400 x 100 nm slices through embryonic mouse tail tendon. The images have been cropped at full resolution. The voxel dimensions are 11 x 11 x 100 nm. The DigitalMicrograph™ images were converted to TIF format using `mrc2tif`.

Supplementary Data 4: 3view-dm4-script.sh

A Shell Script for producing an image stack from 3View® images. Requires pre-installation of IMOD. The Shell Script produces the image stack, floats and smooths the images in the stack, and flips the images about the x-axis.

Supplementary Video 1: 3DMOD-movie.mov

Step-through movie of 250 images produced by 3View® showing the range of size of elements that can be segmented. The sample was embryonic mouse-tail tendon cut at 100 nm intervals. IMOD was used to generate the 3D reconstruction of a cell (green) and individual collagen fibrils in the extracellular matrix (blue), a fibril within a fibripositor (yellow), and a fibril within a fibricarrier (purple). Other elements in the image stack that have not been segmented are nuclei, endoplasmic reticulum, mitochondria, and the Golgi apparatus.

Supplementary Video 2: complete-fibril.mov

Step-through movie of 117 images produced from 'complete-fibril.mod'. The movie shows a short complete fibril (circled in red) in a plasma membrane recess (recessed fibripositor). The movie demonstrates the high quality imaging that is possible with reduced osmium staining and 3View®. Scale bar, 500 nm.

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