Quantification of fibre polymerization through Fourier space image analysis

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Quantification of changes in the total length of randomly oriented and possibly curved lines appearing in an image is a necessity in a wide variety of biological applications. Here, we present an automated approach based upon Fourier space analysis. Scaled, band-pass filtered power spectral densities of greyscale images are integrated to provide a quantitative measurement of the total length of lines of a particular range of thicknesses appearing in an image. A procedure is presented to correct for changes in image intensity. The method is most accurate for two-dimensional processes with fibres that do not occlude one another.

Keywords: fibre length measurement; cellular mechanics; automated image processing

1. Introduction

The total length of lines of a particular thickness appearing in an image is a metric which is important in a broad range of biological and mechanical applications including trabecular bone microfracture (Lee et al. 2000), microcracking in fibre-reinforced concrete (Nicolaides et al. 2006; Farhat et al. 2007; Stähli et al. 2008), crack growth associated with mechanical characterization of metals (Anstis et al. 1972; Saxena & Hudak 1978) and total internal reflection microscopy (Kim et al. 2007; Cooper & Sept 2008). Our interest lies in the remodelling of living cells, in which the total length of stress fibres within a cell can change in response to biochemical (Wakatsuki et al. 2000, Marquez et al. 2005a,b) and mechanical (Nekouzadeh et al. 2008) stimuli. Here, changes in the total length of stress fibres appearing in a cell are directly proportional to the mechanical force that the cell exerts on its environment (Nekouzadeh et al. 2008). This, combined with the orientation distribution of actin filaments is a primary determinant of the conformation and mechanical functioning of cells such as contractile fibroblasts (Kaunas et al. 2005; Deshpande et al. 2006, 2007; Nicolas et al. 2008; McGarry et al. 2009).

The typical problem involves identifying changes in the actin cytoskeletons of cells exposed to various conditions that promote remodelling of the actin cytoskeleton. Cytoskeletons can be viewed by fixing the cells with formalin, then staining the cytoskeleton-associated proteins using immunofluorescence

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Automated quantification of fibre length

2311
techniques (Cooper et al. 1988), or by inducing cells to express fluorescently labelled actin monomers (Colombelli et al. 2009). The measurement of fibre length is currently achieved through manual estimation (Kim et al. 2007). We developed a complete automated method of measuring changes in the total length of fibres within an image that relies on Fourier space analysis, abbreviated as Fourier-space automated band-pass length estimation (FABLE).

While Fourier space techniques are not yet well established for the measurement of fibre length, they have recently been developed for fibre orientation distributions (Marquez 2006; Sander & Barocas 2009). These latter efforts bin the amplitudes of the band-pass filtered power spectral density function of an image’s Fourier transform into radial segments that, when normalized, represent the orientation distribution of specific features in an image. These methods discard the amplitude information for points within a band-pass-filtered power spectral density function and estimate fibre orientations from the normalized angular variation of power. Here, we do the opposite: we estimate the quantity of fibre-like features in an image by discarding this angular variation and studying the total power within a band-pass-filtered power spectral density function.

This paper presents and assesses the accuracy of FABLE for estimating the change of length of fibres that appear in confocal microscopy images. We identified a filtering scheme that retains the information within a power spectral density corresponding to fibres of a certain width, and assessed the degree to which the filtered power spectral density scales with changes in the total length of fibres within an image. We further quantified accuracy through study of a series of images with defined fibre dimensions and orientation distributions. We concluded that FABLE can provide statistically meaningful information about fibre length changes when the fibres are long and slender and when the filter is designed adequately.

2. Background

The problem of quantifying information about a possibly three-dimensional structure from a two-dimensional image has a broad history in the field of quantitative stereology, which includes a spectrum of techniques that have been established in the form of American Society for Testing and Materials standards since the 1970s (e.g. Underwood 1972). The approaches most appropriate to estimating the total length of possibly branched curves in an image are closely related to those for edge detection, and the primary tool for these involves the Radon transform and its discrete implementations in the form of the Hough transform (Hart 2009). The Radon transform itself is suitable for detection of thin, straight lines and recent algorithms allow the detection and centring of thicker lines as well (Zhang & Couloigner 2007). In the Hough transform, the number of pixels that are located on any possible line in an image are counted and stored in an accumulator cell associated with that line. A high-concentration accumulator cell indicates many pixels along the associated line and therefore suggests a line along that direction in the image. The power of the method is that the concentration of an accumulator cell is proportional to the net length of the line (Akhtar & Atiquzzaman 1992). In the following, we will use the Hough transform as a basis for comparison of the method we propose.
3. Methods

(a) Synthetic images

A series of images was generated and analysed to determine the limitations of the approach outlined above. The 1024 × 1024 greyscale images consisted of a black background and edge-rounded rectangles representing fibres of prescribed characteristics (lengths, widths, intensities, positions, orientations and areal densities; e.g. insets, figure 2). To approximate physiological entities such as cells or stress fibres, edge rounding was implemented by reducing the fibre intensity from its peak value to zero (background brightness) over three pixels in the x and y (image) directions; fibre brightness was uniform elsewhere.

Fibrosity was estimated from the discrete Fourier transform of each image. For instances in which some prescribed characteristics were selected randomly, the discrete Fourier transforms of the associated images varied stochastically. Monte Carlo simulations were performed to obtain a statistical characterization of how the predicted fibrosity depended upon the parameters used to generate the synthetic images. For each data point shown in each graph, 10 randomly generated images were studied.

(b) Image analysis

For each image considered, a two-dimensional Fourier transform \( F(u, v) \) was calculated and shifted so that the direct current (DC) peak was centred (e.g. Press et al. 2007):

\[
F(u_p, v_q) = \sum_{n=1}^{N} \sum_{m=1}^{M} f(m, n) \exp \left[ -2\pi i \left( \frac{(u_p - 1)(m - 1)}{M} + \frac{(v_q - 1)(n - 1)}{N} \right) \right], \tag{3.1}
\]

where the amplitude \( f(m, n) \) of the pixel at \((m, n)\) in an \(M \times N\) image is an integer from 0 to 255 for an 8-bit greyscale image, and here \(u_p\) and \(v_q\) denote discrete frequencies. The discrete form of the fibrosity is

\[
\Phi = \sum_{q=1}^{N} \sum_{p=1}^{M} (F(u_p, v_q))^2 B(u_p, v_q), \tag{3.2}
\]

where the discrete band-pass filter is \(B(u_p, v_q) = 1, \quad r_L \leq \sqrt{u_p^2 + v_q^2} \leq r_U, \) and \(B(u, v) = 0\) otherwise.

(c) Image processing

Two additional filtering steps were performed in cases in which variable intensity of fibres or background noise existed. To remove the background noise, a median filter was applied, in which the intensity of each pixel in an image was replaced by the median of its neighbours; pixel values at the edge of each image were repeated in the set of nearest neighbour values. To account for variable brightness in fibres, the Fourier transform of each image was normalized by the DC peak.

Automated quantification of fibre length

(d) Sample images

Tissue constructs containing chick embryo fibroblasts and collagen were synthesized using procedures described elsewhere (Wakatsuki et al. 2000). Briefly, fibroblasts isolated from 11-day-old chick embryos were incubated in Dulbecco’s modified Eagle’s medium (DMEM) at 37°C and 5 per cent CO2 for 7 days, split, then incubated in DMEM for another 7 days. Cultured cells (2.0 × 10^6 cells ml^{-1}) were mixed with DMEM and type I rat tail collagen (1 mg ml^{-1}), and the pH was brought to neutral using NaOH. One millilitre of cell suspension was poured into Teflon moulds. The final suspension was incubated at 37°C and 5 per cent CO2 for 3 days, with moulds topped with DMEM and 3 per cent fetal bovine serum (FBS) after the first hour of incubation. Ring-shaped samples were mounted on a force measurement apparatus (Pryse et al. 2003; Nekouzadeh et al. 2007) and kept in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-buffered DMEM (pH 7.4) containing 3 per cent FBS at 37°C.

Tissue constructs were stretched rapidly to depolymerize the actin cytoskeleton following protocols described elsewhere (Nekouzadeh et al. 2008). To study the actin cytoskeleton, samples were fixed at different phases using 1 per cent paraformaldehyde during relaxation tests and permeablized using 0.1 per cent Triton X-100. F-actin was stained with rhodamine phalloidin and 230 × 230 µm areas were imaged using confocal microscopy (1024 × 1024 pixels, 8-bit resolution). F-actin density and the prevalence of stress fibres were quantified for 10 randomly selected images at each of three time points in the relaxation response of the tissue construct using FABLE, and compared with estimates obtained by hand.

4. Theory

We begin by proving that, for an idealized case, a radial band-pass filter of the Fourier transform of an image can be used to predict the total length of fibres in that image. Consider a continuous image f(x, y) of a single fibre centred at (0, 0), modelled as a rectangular white patch of dimensions a × b on a black background:

\[
 f(x, y) = \begin{cases} 
 1, & -\frac{a}{2} < x < \frac{a}{2} \text{ and } -\frac{b}{2} < y < \frac{b}{2} \\
 0, & \text{otherwise} 
\end{cases} \tag{4.1}
\]

The two-dimensional Fourier transform of f(x, y) is

\[
 F(u, v) = \int_{-\infty}^{\infty} \int_{-\infty}^{\infty} f(x, y) e^{-2\pi i (ux + vy)} \, dx \, dy = \frac{\sin(\pi u a)}{\pi u} \frac{\sin(\pi v b)}{\pi v}, \tag{4.2}
\]

where i = \sqrt{-1}. Translating the centre of the rectangle (x_0, y_0):

\[
 F^*(u, v) = e^{-2\pi i (u x_0 + v y_0)} \frac{\sin(\pi u a)}{\pi u} \frac{\sin(\pi v b)}{\pi v}. \tag{4.3}
\]

The amplitude of the Fourier transform remains unchanged when the fibre translates, but the phase does not.
(a) The Fourier transform amplitude scales with the number of identical fibres

If multiple rectangles are added at random, non-overlapping locations, then the Fourier transform of the whole image is the summation of those of each individual rectangle (Champeney 1987):

\[
F_{\text{tot}}(u, v) = \sum_{i=1}^{n} e^{-2\pi i (ux_i + vy_i)} \frac{\sin(\pi u)}{\pi u} \frac{\sin(\pi v b)}{\pi v} \sum_{i=1}^{n} \cos(2\pi ux_i) - i \sin(2\pi vy_i),
\]

(4.4)

where \(x_i\) and \(y_i\) are the centre of the \(i\)th rectangle added to the image and \(n\) is the total number of rectangles. The square of the amplitude of equation (4.4) is

\[
\|F_{\text{tot}}(u, v)\|^2 = \left( \frac{\sin(\pi u a)}{\pi u} \frac{\sin(\pi v b)}{\pi v} \right)^2 \left[ \left( \sum_{i=1}^{n} \cos(2\pi ux_i) \right)^2 + \left( \sum_{i=1}^{n} \sin(2\pi vy_i) \right)^2 \right].
\]

(4.5)

For rectangles distributed randomly, the expected value \(E\) is

\[
E[\|F_{\text{tot}}(u, v)\|^2] = \left( \frac{\sin(\pi u a)}{\pi u} \frac{\sin(\pi v b)}{\pi v} \right)^2 \left( E \left[ \left( \sum_{i=1}^{n} \cos(2\pi ux_i) \right)^2 \right] \right)
\]

\[
+ E \left[ \left( \sum_{i=1}^{n} \sin(2\pi vy_i) \right)^2 \right].
\]

(4.6)

This can be simplified using the following expansion:

\[
E \left[ \left( \sum_{i=1}^{n} \cos(2\pi ux_i) \right)^2 \right] = E \left[ \sum_{i=1}^{n} \cos^2(2\pi ux_i) \right]
\]

\[
+ E \left[ \sum_{i=1}^{n} \sum_{j=1}^{n} \cos(2\pi ux_i) \cos(2\pi ux_j) \right]
\]

\[
= \sum_{i=1}^{n} E[\cos^2(2\pi ux_i)] + \sum_{i=1}^{n} \sum_{j=1}^{n} E[\cos(2\pi ux_i) \cos(2\pi ux_j)]
\]

\[
\approx \frac{n}{2} + 0,
\]

(4.7)
Automated quantification of fibre length

Figure 1. (a) Two-dimensional spatial Fourier Transform of a rectangular fibre extended in the $x$ direction. The power density approaches zero much faster in the direction of increasing $x$ frequencies than in the direction of increasing $y$ frequencies. Therefore, most of the power is located between the parallel dashed lines. The mean square of the power located between the two circles is a quantitative measure of fibrosity. Note that the colorscale is logarithmic. (b) Sinc$^2$ function (solid black) and its average (solid grey). The integral of the sinc$^2$ curve varies in an oscillatory manner as the integral limits are scaled (dashed grey versus dashed black). The average variation of the integral (regardless of oscillations) may be approximated by integrating the grey curve. Black line, sinc$^2 (v)$; grey line, $1/(2\pi^2 v^2)$. (Online version in colour.)

where the last step is valid for $u$ sufficiently large that the random variable $2\pi ux_i$ is distributed almost uniformly over all angles. Noting that a similar relation can be derived for the sine series in equation (4.6), then substituting

$$E[\| F_{\text{tot}}(u, v) \|^2] = n \left( \frac{\sin(\pi ua)}{\pi u} \frac{\sin(\pi vb)}{\pi v} \right)^2,$$

indicating that if $u$ and $v$ are sufficiently large frequencies, the square of the amplitude of the Fourier transform of $f(x, y)$ is proportional to the number of rectangles within $f(x, y)$.

(b) Relative numbers of fibres in images can be estimated using a radial band-pass filter

In the Fourier transform of an image containing fibres (e.g. power spectral density in figure 1), information about fibre length is contained far from the intensity peak at the origin. This intensity peak is dominated by information about lower frequency phenomena; for instance, in an image of actomyosin stress fibres clustered in living cells, the information in the lower (central) frequencies will be dominated by the cells rather than the stress fibres. Farther from the centre of the image, information about the length of stress fibres persists, while the information about the cells dies off. The strategy is thus to filter out the lower frequencies.
A radial band-pass filter was used. Rotation of a fibre alters the amplitude of the Fourier transform at a particular point \((u, v)\) in the frequency domain, but does not alter the integral of amplitudes along any circular path centred at the origin of the frequency domain: rotating an image simply rotates the Fourier transform (Champeney 1987). To quantify the lengths of fibres independent of their angular distribution, we considered the summation of the squares of Fourier transform amplitudes in a radial band-pass filter located at high frequencies. The highest frequencies must also be filtered: these appear in the ‘corners’ of the discrete fast Fourier transform (FFT) of an image, and must be filtered out through a radial band-pass filter to avoid providing an undesired bias to fibres oriented at 45° to the image axes.

The scaling in equation (4.8) holds for arbitrary locations and orientations of fibres within an image, provided that location and orientation are independent variables, and that at least one of the frequencies is sufficiently large that at least one of the random variables, \(2\pi u x_i\) and \(2\pi v y_i\), maps uniformly over the domain of angles \((0 - 2\pi)\) for a continuous range of frequencies, independent of the exact distribution of \(x_i\) and \(y_i\). This can be achieved if \(|u x_i| \gg 1\) or \(|v y_i| \gg 1\); note that frequencies and displacements can be negative. In this work, we found \(|u x| > 50\) or \(|v y| > 50\) to be sufficient.

For pixelated images, the number of pixels is the unit of length and pixels\(^{-1}\) the unit of frequency. The FFT of a \(1024 \times 1024\) image is itself a \(1024 \times 1024\) image, with \(u\) and \(v\) ranging from \(-511/1024\) pixels\(^{-1}\) to \(+512/1024\) pixels\(^{-1}\). For a \(1024 \times 1024\) image, a band-pass filter with a lower cut-off frequency (radius) greater than \(u_c \equiv 50/1024\) pixels\(^{-1}\) generated a measure proportional to the number of fibres in an image. We term this measure the ‘fibrosity,’ \(\Phi\), of the image:

\[
\Phi = \int_{\text{Ring Region}} \|F_{\text{tot}}(u, v)\|^2 \, dv \, du. \tag{4.9}
\]

\((c)\) Fibrosity is proportional to fibre length, but relatively insensitive to fibre width

The fibrosity measure in equation (4.9) is also appropriate for fibres whose dimensions change with respect to time. Fibrosity varies in proportion to fibre length, but is relatively insensitive to changes in fibre width. For a rectangular fibre, fibrosity \(\Phi_f\) is

\[
\Phi_f = \int_{\text{Ring Region}} \left( \frac{\sin(\pi u a)}{\pi u} \frac{\sin(\pi v b)}{\pi v} \right)^2 \, dv \, du. \tag{4.10}
\]

For \(a \gg b\), the power spectrum is highly extended towards \(v\) frequencies. For example, in the Fourier transform of a rectangle with \(a = 100\) and \(b = 5\), power is concentrated within the range \(-u_c < u < u_c\) (parallel dashed lines, figure 1), where \(u_c = 50/1024\) pixels\(^{-1}\) is the cut-off frequency below which the amplitude of the Fourier transform is very small along the horizontal axis (for the case shown, approx. 95% of the power lies within \(\pm u_c\)). A band-pass filter \((B(u, v) = 1, \ r_L \leq \sqrt{u^2 + v^2} \leq r_U; \ B(u, v) = 0, \ \text{otherwise})\) can be chosen such that power within
the oscillatory nature of the sinc² function in term changes the integral in equation (4.13) in an oscillatory manner owing to

where

an acceptable approximation for both

changes, but increases in direct proportional to fibre length. As described below,

Therefore, for long, slender fibres, a thickened or narrowed fibre with dimensions

a thickened or narrowed fibre with dimensions

oscillations are smaller. As an estimate of the effect of width changes on

the limits of integration towards the right in figure 1

larger fibre widths and for higher filter cut-off frequencies, because both move

integrate over an averaged function of sinc²,

oscillations are smaller. As an estimate of the effect of width changes on

where

an elongated or shortened fibre with dimensions

an elongated or shortened fibre with dimensions

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Changing fibre width from

to

f is then approximately:

where

Unlike the case of the

term changes the integral in equation (4.13) in an oscillatory manner owing to

the oscillatory nature of the sinc² function in

(figure 1b): scaling the limits

can increase or decrease

relative to

The oscillations are smaller for

larger fibre widths and for higher filter cut-off frequencies, because both move

the limits of integration towards the right in figure 1b, where the amplitude of

oscillations are smaller. As an estimate of the effect of width changes on

we integrate over an averaged function of sinc²,

solid grey curve, figure 1b).

where

φ₁ is then approximately:

and

φ₂ is approximately:

Therefore, for long, slender fibres, φ does not change on average with fibre width

changes, but increases in direct proportional to fibre length. As described below,

computer simulations showed this to be valid for

What inner filter radius

is appropriate? The following simple computation suggests that equation (4.11) is a reasonable approximation provided that the

inner arc of the band-pass filter, the arc spanning

, covers less than

radians, or

Since about 95 per cent of the power of the

sinc² function lies within its first three domes, a safe choice of $u_c$ is $u_c a_{\min} > 3$, where $a_{\min}$ is a lower limit for the lengths of the fibres of interest. Combining these yields the lower limit $r_L > 1024(3/(a_{\min} \sin(\pi/8)))$.

5. Simulations

(a) Fibrosity correlates with the total length of fibres in an image

Ten images of simulated fibres were generated under each of a series of prescribed conditions, and statistical analyses were performed to assess the accuracy of FABLE in predicting differences in fibre length among images.

For a filter $B$ with $r_U = 1$ pixel⁻¹ and $r_L = 0.5$ pixel⁻¹, the integrated power within a band correlated linearly with the total length of fibres within an image (figure 2). Here, 0 to 110 aligned, non-overlapping fibres of dimensions 5 × 200 pixels were placed on a 1024 × 1024 pixel black background, and relative fibrosity $\Phi^*$ was defined as the ratio of the fibrosity computed for a specific image to that computed for the image with 110 fibres. When compared with the correct total length of fibres in the images (figure 2, solid line), the relative fibrosity was found to underpredict the total length of fibres very slightly, but the correlation in this case was excellent, with $R^2 = 0.998$. Note that the normalization $\Phi_0$ of $\Phi^*$ is arbitrary: the linear trend would be recovered by calibrating $\Phi_0$ to the average value of $\Phi$ from sets of images with any specific total fibre length. The only caveat is that a sufficient number of images should be sampled to ensure that the standard error in $\Phi$ is low.

(b) Weak dependence on lengths of individual fibres, fibre thickness, and fibre curvature and fibre orientation

For long, slender fibres (aspect ratio greater than 5), the relative fibrosity is insensitive to the way that the total length of fibres is distributed. The fibrosity of 10 images with 20 white, aligned, randomly positioned, non-overlapping fibres of dimensions 5 × 300 pixels was the same as that for images with 30 5 × 200 fibres ($p = 0.19$) and 60 5 × 100 fibres ($p = 0.69$) (figure 3a). Increasing fibre thickness yielded an approximately constant fibrosity for fibres with aspect ratio up to 0.2 (figure 3b, normalized standard deviation less than 5%); thereafter, for reasons described in the discussion, the error increased linearly in a predictable way (figure 3c).

$\Phi$ was relatively insensitive to moderate fibre curvature for sinusoidally bent fibres with a centreline defined as $y_i = C(\lambda, a) \sin(\pi x_i/\lambda)$, where $x_i$ and $y_i$ are the Cartesian coordinates of pixel $i$, $\lambda$ is a constant, and $C(\lambda, a)$ is the maximum deflection from the straight line (figure 4); fibre thickness was truncated in the vertical direction as illustrated in the inset to figure 4a. $\lambda$ varies between 0 and fibre length, $a$; $C(\lambda, a)$ varies between $a/2$ (infinite curvature, $\lambda = 0$) and 0 (no curvature, $\lambda = a$). For very small $\lambda$ fibres fold at their midpoints, with the resulting overlap of the fibre’s two halves halving the fibrosity. This effect is small for moderate levels of curvature ($\lambda/a = 0.5$). The orientation distribution had little effect on the relative fibrosity (figure 4b, identical but potentially overlapping white fibres on a black background, $p < 0.27$); effects owing to fibre overlap, detailed in the next section were within sampling error for this image.
(c) Overlapping fibres introduce artefact in a predictable way

Overlapping fibres could not be distinguished from connected fibres. When extrapolating the fibrosity estimated from 10 images of 10 identical, randomly oriented, overlapping white fibres (aspect ratio = 20) to images of higher numbers of fibres placed in the same region, the result was an over-prediction of fibrosity (figure 4c). The fibrosities measured from these images with more dense overlapping networks were well-predicted by the following model that accounted for area that appeared to be removed by an overlap.

Overlapping fibres appear in a two-dimensional image as a meshwork, and areas of overlap are not double-counted by FABLE. This leads to errors of the order of 10 per cent for images with moderate density, but errors must be compensated for in images with significant overlap (figure 4c). The fibre length correction in this case can be estimated from estimates of the number of fibre crossings, \( n_c \), and the average area \( \bar{A}_c \) occluded by each crossing:

\[
L_{\text{actual}} = L_{\text{measured}} + \frac{\bar{A}_c}{b} n_c, \tag{5.1}
\]

where \( L_{\text{actual}} \) is the fibre length assuming that all perceived fibre intersections are in fact fibres overlapping fibres in parallel planes, \( L_{\text{measured}} \) is the total fibre length estimated using FABLE, and \( b \) the fibre width. For a uniform orientation distribution of rectangular fibres, the number of fibre crossings expected on each fibre, \( n_c \), is

\[
n_c = \frac{\bar{c}^2}{2\pi} \left( \frac{A_n}{b^2} \right) (1 + \exp(-\bar{c})), \tag{5.2}
\]

where the dimensionless fibre concentration is \( \bar{c} = n_f L_f b / A_n \), where \( L_f \) is the fibre length, and \( A_n \) the area containing \( n_f \) fibres (Kallmes & Corte 1960). The average area occluded by a crossing for such a fibre distribution is \( \bar{A}_c = \pi b^2 / 2 \) (Perkins 1980). Expressions are available for non-uniform planar distributions of
Figure 3. Fibrosity estimates (a) the total length of fibres in the image regardless of length or (b) width of individual fibres up to (c) the aspect ratio of 0.2. Grey dashed line, model.
Automated quantification of fibre length

Figure 4. Effect of fibre curvature, fibre orientation and fibre overlap on the fibrosity. (a) Moderate fibre curvature and (b) random fibre orientation have little effect on fibrosity. When there is a significant fibre overlap the fibrosity under-predicts the total length of fibres, as the regions of overlap cannot be distinguished from interlocking fibres. (c) Solid line, FABLE result; dashed line, adjusted for overlap using equation (5.3). (Online version in colour.)

Figure 5. Comparison of line length estimation via FABLE to length estimation using the Hough transform. (a) The Hough transform of a single fibre has a characteristic shape, but this is masked or adjusted by the presence of additional fibres. (b) This leads to inaccuracy in length estimation for images containing multiple fibres.

fibres (Bronkhorst 2003); note that the occluded area per crossing increases with increasing alignment of fibres. Combining

\[ L_{\text{actual}} = L_{\text{measured}} + \frac{c^2}{4} \left( \frac{A_n}{b} \right) (1 + \exp(-c)). \]  \hspace{1cm} (5.3)

To fit this to the numerical data, the model was normalized to the mean level of fibrosity associated with images containing 10 fibres in a defined fraction of the image (insets, figure 4c). The fibrosity was increasingly underpredicted by FABLE for denser populations of overlapping fibres. However, applying the model, allows a nearly perfect \( R^2 > 0.99 \) recovery of the linear trend (dashed line, figure 4c). As it is impossible to determine from a two-dimensional image whether the fibres that comprise the image are continuous or overlapping, equation (5.3) serves as a bound on error rather than as a corrective formula.
Automated quantification of fibre length

Figure 6. The background noise is a significant source of error if not corrected. However, median filtering can remove the background noise without distorting the fibre images in a way that restores the correct value of fibrosity.

(d) The Hough transform is difficult to use on images containing multiple fibres

The Hough transform estimate for line length is accurate if the lines are thin and few. As detailed in §7, several major obstacles make application of the Hough transform to the images of interest difficult. As an example, consider images of isolated, aligned fibres (figure 5). The Hough transform of a single fibre involves a bright patch at $\theta = \pi/2$, with power concentrated in isosceles triangular regions emanating in the directions of both greater and smaller angles (figure 5a). The Hough transform of 20 such fibres is clearly not a superposition of the individual contributions (figure 5a). Increasing numbers of fibres increases the number of high-concentration regions; in such overlap regions the contributions of individual fibres can be masked or amplified, leading to inaccuracy in line length estimation. For example, an image of 20 randomly located fibres yielded an overestimation in total length of approximately 75 per cent (figure 5b).

(e) Image noise and irregular brightness introduce artefacts that can be removed

Background noise superimposed on an image can lead to over-prediction of fibrosity. For the images of 10 randomly distributed fibres considered in figure 6, superimposing a pattern of salt and pepper noise tripled the fibrosity (figure 6); here, noise was added by adding randomly distributed bright pixels to the image, truncating the intensity at the maximum of 255. Reducing this noise by applying a median filter (see §3) eliminated artefact owing to this white noise, but reduced the fibrosity slightly compared with the control images ($p = 0.12$).

Identical images of moderately aligned, identical fibres with randomly selected brightnesses following different intensity distributions yielded significantly different estimates of image fibrosity (dashed lines, figure 7, in which fibres in 10 identical images were assigned brightnesses from the three different distributions
Normalizing fibre brightness by the average brightness of fibres within an image compensates for this effect. Variations in the mean value of the brightness distribution, as occurs in photobleaching, affect fibrosity significantly, but this can be virtually eliminated. Variations in the standard deviation of distribution will affect the fibrosity only slightly, but are difficult to remove. Open bars, before filtering; grey bars, after filtering. Normalizing the FFT by the average fibre brightness reduced the standard deviation of the measured fibrosity among the images corresponding to the same intensity distribution (solid lines compared with dashed lines, figure 7). Additionally, doing so resulted in compensating measurements of fibrosity among all three intensity distributions (grey bars, figure 7). After correction, shifts in the brightness distribution (e.g. as in photobleaching) yielded statistically insignificant effects ($p = 0.27$), while scaling of the brightness distribution affected $\Phi$ in a small but statistically significant way ($p = 0.03$).

6. Application to sample data

When applied to images of the actin cytoskeletons of fibroblast cells repolymerizing following stretch-induced degradation, FABLE was able to identify a nearly linear increase in polymerization over time (figure 8). Estimates by hand taken by four individuals blinded to the temporal image order show significant scatter; however, the linear trend is consistent with these data, and consistent with some regimes of actin dynamics that might be expected following mechanical activation (Carlsson 2010).
7. Discussion

FABLE is effective for estimating the total length in images of long, non-overlapping slender fibres (figure 2), independent of individual fibre length (figure 3a), orientation distributions (figure 4b) or curvature (figure 4a). We discuss here cases in which higher error is expected: images with thick fibres, images in which fibre overlap is unknown, images containing noise and images in which the fibres have differing brightnesses. Each of these error sources can be bounded or eliminated.

(a) Comparison with existing techniques

The Hough transform has some limitations analogous to those of FABLE: it cannot determine whether a line is continuous or has multiple segments, and it cannot identify the end points of a line or its segments. As shown above, this obstructs the ability of the Hough transform to estimate total line length, while FABLE is unaffected.

The method is further limited because, by definition, it is intended for black and white images only. Greyscale images must be thresholded into black and white images, and this discretization limits the accuracy of length and orientation estimates: discretization leads to a spreading of ‘votes’ in accumulator cells over a few adjacent cells (Akhtar & Atiquzzaman 1992). Hough transforms can provide an estimate of the line length if spreading votes and relation between number of pixels and line length at different angles are considered. The limitation concerning overlapping lines when using the Hough transform is the opposite of that encountered when using FABLE: with the Hough transform, fibres are all assumed to overlap rather than intersect, because pixels located at the intersection of two lines are counted in both lines.

Since the Hough transform is intended to identify individual lines or a particular curve in an image, it has difficulty identifying and thus measuring the total length in images with multiple fibre shapes (Hart 2009); FABLE does
A. Nekouzadeh and G. M. Genin

not have this limitation (figure 3). The Hough transform is known to be sensitive to the background noise, as noise is interpreted as numerous lines in different locations and different angles (Zhang & Couloigner 2007); non-fibros elements should be removed from the image prior to processing. This is desirable in FABLE as well, but less critical, as stains on an image cause error proportional to half of their perimeter. Finally, estimates of fibre length using the Hough transform are sensitive to fibre thickness (Zhang & Couloigner 2007), which leads to difficulty in images containing multiple fibre thicknesses. Increasing the thickness of fibres causes votes to spread over a larger region of the transformed domain, and simultaneously increasing numbers of fibres increases the number of high-concentration regions; both factors act in concert to increase the probability of overlap between regions and in turn lead to inaccuracy in line length estimation via the Hough transform.

(b) Thick fibres

For thick fibres (aspect ratio greater than 0.2), equation (4.15) no longer holds, and \( \Phi \) varies with fibre width. If fibre thickness varies little, these effects can be minimal; however, when a filter is chosen for fibres of aspect ratio 7.5:100 and applied to fibres of increasing aspect ratios up to 50:100, the average error increases nearly linearly (figure 3c). The reason is evident from the images inset into figure 3c, obtained by performing an inverse Fourier transform after bandpass filtering the Fourier transform of an image containing fibres of prescribed dimensions. For sufficiently thick fibres, the thickness of the fibres becomes a significant contributor to the fibre ‘outline’ that survives the band-pass filter. Note that a consequence of this is that two fibres must be separated by at least a single pixel to contribute to the fibrosity measure.

Error was estimated by modelling fibres as rectangular fibres, and noting that filtered fibres appear as boxes whose height introduces error into \( \Phi \) (figure 3c):

\[
\text{Relative fibrosity} = \begin{cases} 
1, & \frac{b}{a_0} \leq \frac{b_0}{a_0} \\
1 + \frac{b - b_0}{a_0}, & \frac{b}{a_0} \geq \frac{b_0}{a_0}
\end{cases}, \quad (7.1)
\]

where \( b \) is the fibre width, and \( a_0 \) and \( b_0 \) are the dimensions of the rectangular fibre to which the filter was optimized (\( a_0/b_0 = 0.075 \) in figure 3c). As the model fails to account for corner effects, which cause some of the fibrosity associated with the thickness to be masked by that of the length, the model overpredicts error. Note that the artefact caused by single spurious pixels (aspect ratio = 1) differs from equation (7.1) because such artefacts have no outline; this error is addressed in §8.

(c) Image noise and fibres with irregular brightnesses

The final two sources of error in FABLE can be reduced through filtering. Background noise contributes to \( \Phi \); the frequency data associated with a single spurious white pixel on a black background produce noise throughout
Automated quantification of fibre length

Fourier space, and noise associated with random clusters of such pixels can appear as short fibres. Median filtering eliminated this source of error without the sacrifice of image clarity that occurs using low-pass filters.

Variation in brightness between fibres increases the mean and standard deviation of error. The amplitude of the Fourier transform increases with increasing fibre brightness, and without normalization, FABLE is not capable of distinguishing between a power spectral density map that arises from a few bright fibres and one that arises from many dim fibres. Normalization of the FFT by the average fibre brightness nearly eliminated this problem.

\[(d)\text{ Out-of-plane fibre orientations}\]

Three-dimensional effects lead to errors because of both out-of-plane fibre orientations and overlapping of fibres. Out-of-plane fibre orientations lead to an underestimate of the overall fibre length measured in an image. Measurement errors associated with changes in the length of individual fibres that are straight and not directly normal to the image plane will scale with measurement errors associated with the initial lengths of such fibres (cf. Underwood 1972). In such cases, measurements of relative changes of the fibre length are insensitive to fibre orientation. However, for fibres that are curved fibres out of plane this will not be the case. Further, for fibre distributions that are non-uniform in the out-of-plane direction, changes to the relative fibrosity measures will be skewed towards in-plane fibres because of this effect.

8. Conclusion

FABLE is well-suited to the automated estimation of changes of total fibre length in images that contain non-overlapping, slender fibres. Pre-processing in the form of median filtering must be done to eliminate the background noise, and normalization of the Fourier transform is required to minimize artefact from variations in fibre brightness. Care must be taken when analysing images containing non-slender fibres, including large globular artefacts and fibres that are sufficiently dense to appear as a single fibre. While overlapping fibres cannot be distinguished from interlocking fibres, the error associated with this ambiguity can be estimated.

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A. Nekouzadeh and G. M. Genin


