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Adaptive multiorientation resolution analysis of complex filamentous network images

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Abstract

Motivation: Microscopy images of cytoskeletal, nucleoskeletal and other structures contain complex junctions of overlapping filaments with arbitrary geometry. Yet, state-of-the-art algorithms generally perform single orientation analysis to segment these structures, resulting in gaps near junctions, or assume particular junction geometries to detect them.

Results: We developed a fully automated image analysis approach to address the challenge of determining the number of orientations and their values at each point in space to detect both lines and their junctions. Our approach does not assume any fixed number of orientations or any particular geometry in the case of multiple coincident orientations. It is based on analytically resolving coincident orientations revealed by steerable ridge filtering in an adaptive manner that balances orientation resolution and spatial localization. Combining this multiorientation resolution information with a generalization of the concept of non-maximum suppression allowed us to then identify the centers of lines and their junctions in an image. We validated our approach using a wide array of synthetic junctions and by comparison to manual segmentation. We also applied it to light microscopy images of cytoskeletal and nucleoskeletal networks.

Availability and implementation: <https://github.com/mkitti/AdaptiveResolutionOrientationSpace>.

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Supplementary information: [Supplementary information](#) is available at *Bioinformatics* online.

1 Introduction

Many biological structures, such as cytoskeletal and nucleoskeletal filamentous networks, produce light microscopy images that consist of curvilinear structures and junctions between them. Junctions could be due to true filament branching and intersections, or due to filaments crossing each other within the resolution limit of the microscope (which could be exacerbated by 2D imaging of what are in reality 3D structures).

Whichever is the case, while individual curvilinear structures can be described locally by a single orientation, junctions contain multiple orientations at the same location. As a result, automated analysis of filamentous network images must detect the local orientations of individual filaments, resolve multiple orientations at junctions and decide at every point in space whether there is one or multiple orientations to determine whether the point represents one curvilinear structure or a junction between multiple structures.

Segmentation approaches for these structures range from pixel-based digital morphological processing techniques such as skeletonization (Agamennoni *et al.*, 2010; Borgefors *et al.*, 2001; Chaudhuri *et al.*, 2012) to image filtering approaches in the image or frequency domains. While discrete morphological processing is efficient, it fails to take advantage of the continuous nature of images acquired via critically sampled high-resolution fluorescence microscopy (Unser *et al.*, 1993). To preserve the continuous nature of the data, we focus on convolutional filtering approaches in this work.

The prevalent approach in convolutional filtering for the detection of curvilinear structures in images is to perform single-orientation analysis based on ridge filtering, followed by non-maximum suppression (NMS) to identify line centers (Arganda-Carreras *et al.*, 2017; Canny, 1986; Gan *et al.*, 2016; Jacob and Unser, 2004; Peters *et al.*, 2018; Steger, 2000). This, however, inherently produces gaps at junctions (because they contain multiple orientations), which have been usually subsequently closed using

inference methods (Gan et al., 2016; Shimi et al., 2015). But these inference methods tend to involve heuristics. More recent convolutional filtering approaches detect junctions explicitly, but they require for this task predefined angles (Jacob and Unser, 2004) or symmetries (Puspoki et al., 2016), thus limiting their applicability.

Here, we propose an automated analysis approach that addresses the challenge of detecting single and multiple orientations, i.e. determining the number of orientations at each point in space automatically. It is based on analytically resolving coincident orientations revealed by steerable ridge filtering (Freeman and Adelson, 1991; Jacob and Unser, 2004) in a manner that balances orientation resolution and spatial localization. Our approach applies generally to steerable ridge filters, including to a unifying parametric framework for steerable wavelets (Unser and Chenouard, 2013). It is currently developed for analyzing 2D images, but can be generalized for 3D image analysis.

2 Methodology

2.1 Multiorientation analysis with adaptive orientation resolution/spatial localization

2.1.1 Filter definition

To identify local orientations and resolve spatially coincident multiple orientations, we modified the filter proposed by van Ginkel and van Vliet (Ginkel, 2002; Nieuwenhuizen et al., 2015). This filter is well-suited for multiple orientation detection because its aspect ratio, and thus orientation resolution, can be controlled in a straightforward manner via an explicit parameter K (Supplementary Notes S1–S3; specifically Equations 25–29). However, the original filter suffered from slope discontinuities at the periodic boundary for values of $K < 3$ (Supplementary Fig. S1). By redefining the angular part of the filter in terms of angular frequency space instead of direct angular space (Equation 3; Supplementary Note S2.3; specifically Equations 34–36), we were able to extend its applicability to the full range of K , namely $K > -0.5$ (note that K can be non-integer) (Supplementary Fig. S1). This redefinition allowed us to develop a line and junction analysis approach that integrates orientation information from multiple K values to balance orientation resolution and spatial localization (discussed in detail in Section 2.1.3; not to be confused with spatial resolution).

The final form of the filter used in our study is given in Equations 1–4 and Supplementary Note S2.4, Equations 43 and 44.

$$\hat{\Phi}(f, \theta; \phi, f_c, K) = \hat{\Phi}_{|f|}(f; f_c) \hat{\Phi}_{\hat{G}, \theta}(\theta - \phi; K) \quad (1)$$

$$\hat{\Phi}_{|f|}(f; f_c) = \sqrt{e} \left(\frac{f}{f_c} \right) \exp \left(-\frac{f^2}{2f_c^2} \right) \quad (2)$$

$$\hat{\Phi}_{\hat{G}, \theta}(\theta; K) = \sum_{k=-K_b}^{K_b} 2 \exp(-2\pi^2 k^2 s_a^2) \exp(2ik\theta) \quad (3)$$

$$s_a = \frac{\pi}{2K+1} \quad (4)$$

Table 1 lists the parameters used in the above equations.

2.1.2 Analytical identification of filter response maxima

To determine the single or multiple coincident orientations from the filter response, we treated the steerable filter response, which is a finite Fourier series, as a trigonometric polynomial (Freeman and Adelson, 1991). With this, the orientation(s) maximizing the filter response (i.e. filter response local maxima), along with all other extrema, were determined by solving a general eigenvalue problem using the polynomial companion matrix (Boyd, 2007; Marchant and Jackway, 2013) (Supplementary Notes S4 and S5). Unlike other eigensystem approaches, such as using a Hessian matrix (Frangi et al., 1998), our approach applies to any steerable filter response and is not constrained by the degree of the polynomial.

Table 1. Symbol definitions

Symbol	Description
f	Radial spatial frequency, $f \in (0, +\infty)$
θ	Polar angle in the Fourier domain, $\theta \in [-\pi, \pi)$
f_c	Central frequency of the spatial bandpass filter
ϕ	Orientation angle selected by the filter
K	Orientation resolution parameter
K_b	Highest orientation resolution parameter
s_a	Orientation resolution bandwidth
ρ	Orientation filter response
ϕ_m	Orientation local maximum

Note: See Supplementary Note S2 for details.

Additionally, the eigenvalues correspond directly with the orientations and only require trigonometric manipulation and scaling. This differs from approaches requiring eigensystem analysis of high-dimensional tensors of an order corresponding to the number of orientations (Muhlich and Aach, 2009). The analytical and global nature of our approach allowed us to identify orientation(s) much more precisely than would be achievable by sampling approaches, at a fraction of the computational cost (Supplementary Fig. S2).

2.1.3 Analytical mapping between filter responses at different orientation resolutions using the heat-diffusion equation

To detect multiple coincident orientations, the used filter must have sufficient orientation resolution. As mentioned above, the orientation resolution of our filter is controlled by the parameter K . Specifically, higher K produces longer filters that provide higher orientation resolution by integrating more signal along their length (Freeman and Adelson, 1991). However, by construction, this increased orientation resolution comes at the expense of spatial localization, because of the integration of signals over a longer spatial scale. Reduced spatial localization might lead to artifacts in the filter response, for example, in the cases of curved structures (i.e. not straight lines) or of nearby separate objects (which would get artificially linked to each other in the response).

It is worth pointing out here the interplay between orientation resolution, spatial localization and spatial resolution in the filter definition. The spatial resolution aspect of the filter (loosely its ‘width’) is controlled by the spatial frequency bandpass component (Equation 2). Thus, spatial resolution and orientation resolution are orthogonal properties of the filter. However, because the orientation resolution parameter K controls the filter’s aspect ratio, improved spatial resolution (i.e. smaller filter width) leads to shorter filter length for the same K . Thus, higher spatial resolution shifts the balance between orientation resolution and spatial localization, and allows higher orientation resolution for the same level of spatial localization. The interplay between spatial resolution and orientation resolution is discussed further in Supplementary Note S3.

The conflict between orientation resolution and spatial localization poses the question of what K to use for image filtering. To address this question in an objective manner and balance the conflicting requirements of orientation resolution and spatial localization, we did not use a single K for ridge filtering and subsequent analysis. Rather, we calculated the filter response, $\rho(\phi, K)$, as a function of orientation, ϕ , for a range of K , namely $-0.5 < K \leq K_b$ (K_b typically taken as 8, resulting in an orientation resolution = $\pi/8$; Supplementary Note S3).

For this calculation, we realized that steerable filter responses at different K values are related by the heat-diffusion partial differential equation with periodic boundary conditions:

$$\frac{\partial \rho}{\partial t} = D \frac{\partial^2 \rho}{\partial \phi^2}, \quad t = s_a^2 = \frac{\pi^2}{(2K+1)^2}, \quad D = \frac{1}{2} \quad (5)$$

$$\frac{\partial \rho}{\partial K} = \frac{\partial \rho}{\partial t} \frac{dt}{dK} \quad (6)$$

$$= \frac{-2\pi^2}{(2K+1)^3} \frac{\partial^2 \rho}{\partial \phi^2} \quad (7)$$

Therefore, here again, we used analytical methods to calculate the ridge filter response at any K from the response at the maximum value K_b (Supplementary Note S4). Here, t , which is time in physical diffusion, is equal to the square of the orientation resolution bandwidth (s_a^2), which is inversely related to the square of $2K+1$ (Supplementary Note S4). In essence, the filter responses at distinct K values are related by convolution/deconvolution with a known Gaussian kernel.

2.1.4 Balancing orientation resolution and spatial localization at each point in space

Having the filter response as a function of K allowed us to develop an analytical procedure to determine the optimal K at each point in space to balance orientation resolution and spatial localization (Fig. 1; Supplementary Fig. S3; Supplementary Note S4). Specifically, first, we determined the regimes of K in which different numbers of orientations are identified, starting with the maximum number in the highest regime (Regime H, which includes K_b), and ending with asymptotically diminishing orientation information in

the lowest regime (Regime 0, K in the open interval -0.5 to 1). Second, within each regime, we selected for each surviving orientation its optimal K , defined as the K at which the identified orientation changes the least with respect to K . In other words, for a local maximum in the filter response with respect to orientation, $\phi_m(K)$, we identified the K that minimized $|\frac{d\phi_m}{dK}|$. The expression for slope with respect to K is derived in Supplementary Note S4 and is stated below:

$$\frac{d\phi_m}{dK} = -D \frac{dt}{dK} \frac{\frac{\partial^3 \rho}{\partial \phi^3}}{\frac{\partial^2 \rho}{\partial \phi^2}} = \frac{2\pi^2}{(2K+1)^3} \frac{\frac{\partial^3 \rho}{\partial \phi^3}}{\frac{\partial^2 \rho}{\partial \phi^2}} \quad (8)$$

The orientation values, each at its optimal K , were then taken as the identified orientations in each regime. Altogether, adaptive orientation resolution ridge filtering provided us with multiorientation information at every pixel in the image in a manner that balances orientation resolution and spatial localization.

2.2 Adaptive orientation resolution non-local maxima suppression (AOR-NLMS)

In single orientation analysis, ridge filtering is often followed by NMS to identify the centers of the curvilinear structures in the image (Arganda-Carreras *et al.*, 2017; Canny, 1986; Gan *et al.*, 2016;

Fig. 1. Adaptive multiorientation resolution identification of filter response maxima. (A) Kymograph-like plot of filter response curves over a range of K values, which is related to diffusion 'time' as in Equation 5. Each row is false-colored between its minimum and maximum values (as shown in color legend above the plot). Filter response local maxima per K are depicted as blue, red, or yellow solid lines. Regimes of K containing distinct numbers of local maxima are demarked by horizontal dashed green lines. Filled-in circles indicate the selected filter local maxima according to the slope of the solid line, as derived from the heat-diffusion equation treatment of the filter responses at different K values. Empty squares indicate bifurcation points that occur at the intersection of the solid lines and the second partial derivative nullclines (green dot-dashed lines). Blue open diamond indicates location of asymptotic orientation maximum as K approaches -0.5 . Inset: Raw image, the center pixel of which is being analyzed in the plot. The center pixel is at the intersection of the three colored lines, indicating the detected orientations at $K=8$, using the same color-coding as in the main panel. Inset scale bar, 200 nm. (B) Top: Orientations detected using a companion matrix root-solver approach, indicated by the blue and yellow lines, compared to those detected by sampling with a fixed number of samples (indicated on the left axis) or with Halley's refinement method (see Supplementary Fig. S2 for more details). Middle: Filter response curves for Regimes 3, 2 and 1, numbered according to the number of detected orientations (i.e. filter response local maxima), which are shown with the same symbols used in A. Bottom: Orientations detected in Regime 3 superimposed on the $K=3$ filter response curve.

Jacob and Unser, 2004; Peters *et al.*, 2018; Steger, 2000). NMS however only considers the strongest detected orientation per point in space, i.e. it does not make full use of the multiorientation information revealed by the analysis described above. Therefore, we extended the traditional NMS concept to what we term Non-Local Maxima Suppression (NLMS; Fig. 2A–F; Supplementary Movies S1, S2). NLMS builds on the concept of orientation space (Chen *et al.*, 2000; Ginkel, 2002; vanGinkel *et al.*, 1997), and performs NMS separately in each plane where signal orientation (i.e. a filter response local maximum) has been identified. Performing a maximum response projection (MRP) along the orientation axis of all the NLMS outputs then produces a 2D image resembling NMS. However, unlike NMS, the projected NLMS retains multiple orientation information per pixel, and thus gaps do not appear at junctions (Fig. 2D–F). The concept of NLMS is independent of our specific filter, and is generally applicable to multiorientation information revealed by the response of any ridge filter.

In addition to extending NMS to consider multiple local maxima that coincide in space, we also extended it to have adaptive orientation resolution. The adaptive orientation resolution stems from two features. First, using the adaptive orientation resolution analysis described above to identify signal orientations at their optimal K values within a selected orientation resolution regime. Second, uncoupling the K used for identifying signal orientations from the K used to calculate the response to which NLMS is applied. For example, Figure 2A–C shows adaptive orientation resolution-NLMS (AOR-NLMS) obtained by combining orientations identified from high K ($K_h = 8$) with a medium K response ($K_m = 3$), to balance the conflicting needs of orientation resolution and spatial localization.

2.3 Segmentation of curvilinear structures and junctions based on AOR-NLMS

While the projected AOR-NLMS yields complete junctions without gaps, its output is not guaranteed to be a one-pixel-wide topological skeleton (Lam *et al.*, 1992) (Fig. 2F), as needed to identify the centers of curvilinear structures and their junctions. Additionally, the AOR-NLMS from different orientation resolutions (and, conversely, spatial localizations) reveals different information about the signal in the image. Therefore, we devised an approach that combines the multiscale information available through AOR-NLMS at different orientation resolutions to produce a parsimonious segmentation of the centers of lines and junctions (Fig. 2G–O).

The main idea behind this approach (Supplementary Figs S4–S9; described in more detail in Supplementary Note S6) is to start with simple lines (i.e. no junctions), as identified from lower orientation resolution (but higher spatial localization) filters, and then to determine whether and where they should be connected to each other (i.e. whether there are junctions between them), using multiorientation information revealed by higher orientation resolution filters. For lines that do get connected to each other, the AOR-NLMS information at different orientation resolutions allows us to infer the shortest and most robust paths to connect them. This strategy reduces the occurrence of false-positive junctions, as would be the case if we relied entirely on high orientation resolution (but less spatially localized) information.

In specific terms, we integrate AOR-NLMS information from three different orientation resolution combinations that yield complementary information, as described below and in Supplementary Note S6:

Fig. 2. Non-local maxima suppression (NLMS) identifies simultaneously lines and junctions of arbitrary geometry. (A) Orientation space constructions where the x and y dimensions of a 2D image of a synthetic y -junction are augmented with an orientation dimension. Magenta lines indicate detected filter response local maxima at the indicated orientations. (B) Non-local maxima suppression (NLMS) applied to the orientation space in A. Magenta lines indicate non-suppressed pixels and their detected orientations. (C) Filter at $K = 3$ and $K = 8$. Scale bar, 10 pixels. (D) Synthetic junction analyzed in A–I. Dashed cyan box indicates full extent of the representation in A and B. Solid cyan box indicates area depicted in E–I. Scale bar, 10 pixels. (E) MRP of orientation space in A with detected multiple orientations (magenta: orientation with highest non-suppressed response, cyan: other orientations). Scale bar, 10 pixels, applicable also to F–I. (F) MRP of the NLMS orientation space in B. Magenta and cyan as in E. (G, H) The line and junction segmentation for junction in D after bridging the lines in the initial skeleton from Combination 1 with multiorientation information from Combination 2 (G) and after further bridging with the multiorientation information from Combination 3 (H). See text for details. Added bridges are shown as green pixels. (I) Response-weighted segmentation of the y -junction after the two bridging iterations. (J) Structured Illumination Microscopy (SIM) image of Lamin A immunofluorescence in a *Lmnb1*^{-/-} Mouse Embryonic Fibroblast (same as inset in Fig. 1A). Scale bar, 10 pixels = 316 nm, applicable also to K–O. (K) Filtered image MRP color-coded by the ratio of the filter response at the second highest maximum to the global maximum. Ratio of 0 (magenta) indicates one highly dominant orientation, while ratio of 1 (cyan) indicates at least two equally dominant orientations. (L–O) Same as F–I but for the image in J. Color-coding in L same as in K.

1. **Combination 1: Regime 0 orientations combined with response at medium orientation resolution (K_m , usually taken as 3; red branch in [Supplementary Fig. S4](#)).** Regime 0 orientations come from the asymptotic regime, the limit as $K \rightarrow -0.5$. They are the most spatially localized, and there is typically only a single orientation (i.e. response maximum) at each point in space. Combining Regime 0 orientations with the medium orientation resolution response allows for determination of a single local orientation at each pixel in the image. In a sense, this orientation is a weighted average of all the orientations that may exist around a pixel. The output of this combination represents the most localized and conservative estimation of local orientations, and is thus a good starting point for integrating higher orientation resolution information, as described next.
2. **Combination 2: Regime H orientations combined with response at medium orientation resolution (green branch in [Supplementary Fig. S4](#)).** Regime H orientations are from the highest regime, where the number of maxima matches the number of maxima initially detected at the highest orientation resolution used (K_h , typically taken as 8), but with potentially increased spatial localization because of K adaptation within the regime. Combining Regime H orientations with the medium orientation resolution response yields multiorientation information (in a manner balanced with spatial localization) that complements the conservative orientation information of Combination 1. This information is thus used to connect the lines obtained from Combination 1 above (in Bridging Step 1; [Supplementary Figs S4 and S7](#)). The output of this bridging is then the starting point for the second and final bridging steps, which integrates even higher orientation resolution information.
3. **Combination 3: Regime 0 and Regime H orientations combined with response at the highest used orientation resolution (blue branch in [Supplementary Fig. S4](#)).** The last combination uses the previously identified orientations in Regime H and Regime 0, but now evaluates these orientations with the highest resolution filter response at K_h . The strength of this combination stems from the longer aspect ratio of the filter at K_h , which makes its response at junctions more robust to noise and helps with overcoming spatial resolution issues in the direction perpendicular to the investigated orientation because of integrating information over longer spatial scales parallel to the investigated orientation. The additional multiorientation information provided by this combination is used to further connect the lines resulting from Bridging Step 1 above (in Bridging Step 2; [Supplementary Figs S4 and S7](#)). It enables the resolution of very small angles, potentially even below the orientation resolution limit, as shown e.g. in [Supplementary Figure S9](#), where the junction (with an angle of 6°) gets fully connected but only after Bridging Step 2. Note that away from junctions, the above attributes of this response and filter combination become a liability because the response values have poor spatial localization. However, because this combination is used solely for bridging between lines at junctions, the effect of lack of spatial localization is kept to a minimum.

The AOR-NLMS integration approach described above yields a 1-pixel wide, response-weighted segmentation of the image ([Fig. 21, O](#)), incorporating orientation information across both low and high orientation resolution filters, allowing us another layer of balancing the trade-off between orientation resolution and spatial localization. This output resembles the NMS output of traditional single-orientation analysis ([Canny, 1986; Jacob and Unser, 2004](#)) (but

with intact junctions), and can be subsequently thresholded to segment the lines and junctions of interest ([Supplementary Fig. S9](#)).

3 Results

3.1 Evaluation on synthetic images

To evaluate the performance of our multiorientation analysis and segmentation scheme, we analyzed synthetic images containing a wide variety of junctions. Specifically, we investigated the performance as a function of junction geometry and complexity, the length of the lines coming together at a junction and the distance between nearby but not intersecting objects (to address the question of false positives).

3.1.1 Performance as a function of geometry and complexity

To investigate the performance as a function of junction geometry, we used three types of junctions: two intersecting lines, three lines coming together at a junction and two intersecting arcs, where each configuration went through its full range of possible angles. These examples allowed us to test performance as a function of the angles between lines, and in the presence of curved structures. We evaluated the performance at five signal-to-noise ratios (SNRs), ranging from high (SNR = 10) to low (SNR = 1) ([Fig. 3; Supplementary Note S7; Supplementary Movies S3–S11](#)). We defined signal-to-noise ratio as the mean intensity over background versus the standard deviation under a Poisson noise model ([Supplementary Note S7](#)). We compared the number of identified orientations, their values and the junction location (if detected) to the ground truth.

We found that the scheme performed as expected from theoretical predictions based on orientation and spatial resolution ([Supplementary Note S3](#)). In the case of straight lines, the errors generally became negligible ([Fig. 3A–R](#)) when the inscribed angles between the lines exceeded the orientation resolution of the filters used ($\pi/8$ or 22.5° ; [Fig. 3](#), dashed lines). In the case of two intersecting lines ([Fig. 3A–I](#)), the errors were overall symmetric as the inscribed angle approached 0 or 180° . In the case of three lines meeting at the junction ([Fig. 3J–R](#)), the errors increased near 90° as well because two of the three lines had very similar orientations.

The intersecting arcs example ([Fig. 3S–AA](#)) provided a challenge because it introduced curvature, whereas the filters used only extend along a straight line. In this case, as in the straight line-based examples, the errors decreased when the inscribed angle exceeded the orientation resolution of the filter, but the decrease was more gradual. Curiously, the junction location error seemed to decrease with decreasing SNR when the inscribed angle between the arcs was below the orientation resolution limit of the used filter (region to the left of the dashed line in [Fig. 3AA](#)). Investigating the source of this counterintuitive result revealed that it was due to increased false line (and thus junction) detection with decreasing SNR for that range of inscribed angles. We reasoned that this is because the filter response in the case of arcs is lower than that for straight lines because of their curvature, making it more challenging to distinguish between true and false lines. These issues can be overcome by selective thresholding, or, if needed, explicit curvature or scale detection. Nevertheless, when the inscribed angles were above the orientation resolution limit, the junction location error was very similar for all SNRs and it decreased, as expected, as the inscribed angle increased.

In all of these tests, performance became less robust as the SNR decreased, but remained reliable down to an SNR of 2. Performance also decreased slightly with decreasing spatial resolution ([Supplementary Fig. S10](#) versus [Fig. 3 F–I; Supplementary Movie S12](#)), as expected, because of the interplay between spatial resolution and orientation resolution ([Supplementary Note S3](#)).

To investigate further the effect of junction complexity on performance, we considered two aspects of complexity: the number of lines meeting at the junction, and the symmetry (or lack thereof) at the junction ([Supplementary Movie S13](#)). We found that the performance depended on the extent of symmetry at junctions, with little dependence on the number of incoming lines, as long as the average angle between the lines was above the orientation resolution limit of

Fig. 3. Performance evaluation of orientation analysis and junction detection for various configurations. (A–E) Examples of synthetic junctions composed of two intersecting lines with indicated inscribed angles and SNRs. Scale bars (throughout figure), 20 pixels. (F–I) Performance analysis, as captured by comparing the detected orientations with the ground truth orientations in F [total Root Mean Square Error (RMSE) over all inscribed angles] and G (RMSE per inscribed angle), the number of orientations at the detected junction in H, and the distance between the detected junction and the ground truth junction in I. In F, the bar graph shows the RMSE mean and standard deviation over all inscribed angles in 10 trials [standard deviation is calculated using Equation 14 in (Nicholls, 2014)]. In G–I, solid lines indicate the mean and shaded areas indicate the standard deviation per inscribed angle and SNR over 10 trials. Vertical dashed lines delimit the range of frames where the angles between the lines forming the junction are greater than the orientation resolution of $K=8$ filter ($\pi/8 = 22.5^\circ$). Because junction detection is based on morphological criteria (branchpoints), it is possible to have a detected junction with only 1 orientation in H. (J–R) Examples of synthetic junctions composed of three lines coming together at the junction with indicated angles and SNRs. (O–R) Performance evaluation for three-line junctions. Description as in F–I. (S–W) Examples of synthetic junctions composed of intersecting arcs at indicated angles and SNRs. (X–AA) Performance evaluation for intersecting arcs junctions. Description as in F–I. Further examples and performance analyses of these junction configurations are shown in Supplementary Movies S3–S11.

Fig. 4. Comparison of multiple and single orientation analysis. (A–D) SIM image of Lamin A immunofluorescence in a *Lmnb1*^{-/-} MEF. Magenta squares in A indicate areas of magnification shown in B–D. Scale bar in A (applicable to all images in left-most column), 500 nm = 16 pixels. Scale bars in B–D (applicable to all but the left-most column), 60 nm = 2 pixels. (E–H) MRP at $K = 3$ for images in A–D, respectively. In F–H, multiple detected orientations are shown, where magenta lines indicate the orientation with the largest non-suppressed response, and cyan lines indicate other orientations. (I–L) AOR-NLMS-based segmentation. The multiple detected orientations are overlaid in J–L as in F–H. (M–P) Single orientation detection and Non-Maximum Suppression (NMS). In N–P, magenta lines indicate the single orientation detected at Regime 0.

the filter (Supplementary Fig. S11). Specifically, the performance was best when junctions were symmetric, and deteriorated as they deviated from symmetry, especially if the angle between any of the incoming lines was below the orientation resolution limit (as expected from the performance analysis above). The deterioration was faster when junctions had an odd symmetry (e.g. 5-fold or 7-fold), while the performance was more robust around junctions with even symmetry (e.g. 6-fold or 8-fold). This trend held for all SNRs, with lower SNRs generally performing worse, especially at junctions with odd symmetry. Nevertheless, for junctions of arbitrary geometry, our multiorientation detection strategy was overall more accurate than a junction detection strategy based on assuming m -fold junction symmetries (Puspoki *et al.*, 2016) (Supplementary Fig. S12). The advantage of our approach increased as the number of lines meeting at the junction decreased, presumably because the room for deviation from symmetry is greater when the number of lines is smaller.

3.1.2 Performance as a function of line length

Next, we investigated the performance as a function of the length of the intersecting lines, to determine at what length the analysis is able

to resolve multiple coincident orientations. First, we investigated the case of 6 lines coming together at a junction, with angle = 30° between neighboring lines and with line length varying between 0 and 64 pixels (Supplementary Movie S14). The angle between neighboring lines was just above the orientation resolution limit of our filter at $K_b = 8$, and was well below the orientation resolution limit of the filter at $K_m = 3$. We found that our analysis scheme could resolve the six coincident orientations starting with a line length of 18.5 pixels at the highest SNR of 10, but that the minimum length to resolve the six orientations increased as SNR decreased (Supplementary Fig. S13). At SNR 1, the six orientations were rarely resolved, and only for the longest lines. Given the effect of spatial resolution on the balance between orientation resolution and spatial localization, we expect the minimum length to decrease with increasing spatial resolution (and vice versa). We also expect the performance to improve with increased angle between the coincident orientations.

Next, we investigated the case of unequal line lengths. Specifically, we considered the case of two intersecting lines, in both a ‘plus sign’ configuration and a ‘T’ configuration (Supplementary Movies S15 and S16). For both configurations, the vertical line had

Fig. 5. Multiple orientation analysis and segmentation of actin and vimentin filamentous networks. (A–P) Analysis and segmentation of phalloidin-stained actin (A–H) and immunostained vimentin (I–P) in a MEF imaged via confocal microscopy. In A–D and I–L, the segmentation (magenta) is overlaid on the raw images. In E–H and M–P, the filtered image MRP is color-coded by the ratio of the filter response at the second highest maximum to the global maximum. Ratio of 0 (magenta) indicates one highly dominant orientation, while the ratio of 1 (cyan) indicates at least two equally dominant orientations. Scale bars, 10 μm in the left-most column and 1 μm elsewhere.

a fixed length (64 pixels on each side of the junction), while the horizontal line varied from 0 to 64 pixels (per side in the case of the ‘plus sign’ configuration). For SNR 10, the coincident orientations were resolved when the horizontal line was 3.5 pixels on each side in the ‘plus sign’ configuration, and when the horizontal line was 4.5 pixels in the ‘T’ configuration (Supplementary Fig. S14). As observed above, the minimum horizontal line length needed to resolve the coincident orientations increased with decreasing SNR. Once detected, the estimated horizontal line length was within 1–2 pixels of the ground truth length for $\text{SNR} \geq 2$.

3.1.3 Performance at gaps between neighboring objects

The above performance tests were focused on our scheme’s ability to detect true junctions. However, for a full understanding of its performance, it is important to also investigate whether it creates false junctions when objects are close but do not intersect. For this, we investigated the case of one vertical line and one horizontal line with a variable distance between them (Supplementary Movie S17), and the case of one vertical line and a point object with a variable distance between them (Supplementary Movie S18). We found that indeed the scheme can create false junctions when objects are very close to each other, with an inverse dependence on SNR (Supplementary Fig. S15). The minimum separation at which the objects were never observed to get falsely connected was 18.5 pixels for the line case and 13.9 pixels for the point object case at SNR 10, and it increased slightly as SNR went down to 1. These separations are smaller than the length of the used $K=8$ filter (see Fig. 2C;

Supplementary Fig. S16), highlighting the advantage of our approach, which combines low orientation resolution and high orientation resolution in an adaptive manner to balance orientation resolution and spatial localization, thus minimizing false junction detection.

3.2 Application to real micrographs

To demonstrate the broad applicability of our approach, we applied it to complex networks formed by nuclear lamin A (Fig. 4), the actin cytoskeleton (Fig. 5A–H), or vimentin filaments (Fig. 5I–P), in samples of fixed mouse embryonic fibroblasts (MEFs) acquired through structured illumination microscopy (SIM) (Fig. 4) or confocal microscopy (Fig. 5). It is important to keep in mind here that these are in reality 3D samples. Therefore, what might appear as junctions could be filaments in different z-planes that do not in reality intersect. This issue, while biologically important, is beyond the scope of this work, and in fact applies to any 2D imaging and image analysis technique. Keeping this caveat in mind, we found that our approach was able to analyze and segment both the lines and junctions in these complex networks (as they appear in 2D), without producing gaps at junctions, in contrast to single-orientation analysis (Fig. 4I–L versus Fig. 4M–N; Fig. 5).

3.2.1 Comparison to manual segmentation

To evaluate the segmentation resulting from our automated analysis of these real images, we compared it to manual segmentation of the

Fig. 6. Analysis of junction characteristics in Lamin A, actin and vimentin networks. (A) Frequency of junctions with indicated number of angles for each of the three networks. (B–D) Histogram of angle values for junctions with three angles (B), four angles (C) and five angles (D). Dotted vertical lines indicate angle expected from 3-fold symmetry (B), 4-fold symmetry (C) and 5-fold symmetry (D). Dashed lines indicate orientation resolution limit of used filter. $N = 928$ junctions from 2 cells for Lamin A, 879 junctions from 3 cells for actin and 637 junctions from 3 cells for vimentin.

three networks (Supplementary Fig. S17; Supplementary Note S8). For both the overall segmentation and the identification of junctions, we found that the difference between the automated solutions and the manual solutions was contained within, and was sometimes even smaller than, the variation among manual solutions (Supplementary Fig. S17P, Q). There were, however, differences in performance between the different networks, for both automated and manual solutions. Specifically, for lamin A and vimentin, 95% of the automatically identified pixels and junctions were within a distance of 2–4 pixels from the manually identified pixels and junctions, with inter-manual variability being very similar. For actin, on the other hand, the 95% threshold was only reached at 6–7 pixels, for both automated and manual solutions. The greater variation in actin segmentation and junction identification compared to lamin A and vimentin is most likely due to the presence of faint lines in the actin images that were segmented in an inconsistent manner (and often ignored) manually (arrows in Supplementary Fig. S17H, J). In Supplementary Note S8, we elaborate upon these findings and suggest parameter changes for the automated analysis to more closely resemble the manual segmentation if desired, such as lowering K_b .

3.2.2 Analysis of junction properties

Once such filamentous networks are analyzed, and both lines and junctions are identified, many analyses can follow the automated segmentation, such as those presented in the studies by Shimi *et al.* (2015), Gan *et al.* (2016), Kittisopikul *et al.* (2020) and Zhang *et al.* (2017). Here, to illustrate the rich information provided by our

analysis, we calculated two properties of the identified junctions. First, the number of lines coming together at each junction (Fig. 6A), allowing us to determine what fraction of junctions contained 3, 4, etc. coincident orientations (termed 3-way, 4-way etc. junctions). Second, the distribution of intervening angles between the lines (Fig. 6B–D), allowing us to characterize the geometry of the junctions. We then compared these properties between the different filamentous networks.

This analysis revealed that the junction properties are different between the three filamentous networks, both in terms of number of lines coming together at the junctions and the angles between lines at the junctions. All networks exhibited mostly 3-way junctions, although actin and vimentin networks had a slightly higher fraction of 4- and 5-way junctions than lamin A networks (Fig. 6A). The fraction of junctions with >5 lines coming together was negligible, which is a biologically meaningful result, as our analysis can handle up to at least 8-way junctions (Supplementary Fig. S11). In terms of intervening angles at the junctions (Fig. 6B–D), all filamentous networks had broad distributions, indicating that these junction geometries deviate highly from m -fold symmetry, highlighting the need for (and advantage of) our analysis, which does not make such assumptions. Interestingly, the intervening angle distributions for 3-way junctions differed between the different networks (Fig. 6B): the distribution was unimodal, with a peak at 120° , for lamin A, but bimodal for actin and vimentin. In the case of actin, the two peaks were at 90° and 170° (suggesting many ‘T’-like configurations), while the vimentin junctions peaked at 110° and 160° . The

intervening angle distributions were similar between the different networks for 4- and 5-way junctions (the histograms were also noisier for 5-way junctions because 5-way junctions were infrequent). These analyses and comparisons highlight the new insight that multiorientation analysis and segmentation of curvilinear structures and junctions of arbitrary geometry can bring.

The intervening angle distributions also allowed us to assess the reliability of the detected orientations, as reflected by what fraction of intervening angles were close to or lower than the orientation resolution limit. For all networks, only 3% of intervening angles was close to or below the orientation resolution limit. This suggests that most lines at the junctions are separated by angles well within our analysis' orientation resolution limit, increasing our confidence in the identified orientations and junctions. This performance metric can be used in future applications of our analysis and segmentation scheme to other filamentous networks to assess, from the data, the reliability of the orientation and junction identification.

4 Discussion

Our work demonstrates that combining orientation responses produced by ridge-like steerable filters in an adaptive manner that balances orientation resolution and spatial localization allows advanced analysis and extraction of the multiorientation information contained at junctions of arbitrary geometry, without the need for inference, heuristics, symmetries or templates. Beyond the AOR-NLMS segmentation presented here, the multiorientation information extracted by our analysis could be used to localize lines and junctions with sub-pixel precision, by reconstructing the continuous image through interpolation (Kittisopikul et al., 2020; Steger, 2000). This multiorientation information could be also useful for deep learning approaches for image segmentation (Depeursinge et al., 2017). Our approach is generic, and can be applied to analyze network-like biological structures other than cytoskeletal and nucleoskeletal filaments, e.g. stained membranes in cell sheets or network-like organelles such as the endoplasmic reticulum.

Our approach builds upon the decades-long success of steerable filters and wavelets by devising a method to continuously vary the orientation resolution parameter, K . With this, rather than selecting a single orientation resolution parameter for the entire image, we adapt K to the complexity of the neighborhood using the heat equation. Importantly, K is orthogonal to the spatial scale parameter, σ , which determines the filter width and is related to spatial resolution (Simoncelli and Freeman, 1995; Unser et al., 2011). However, because the orientation resolution of our filter is determined by its aspect ratio (controlled by K), improved spatial resolution allows the use of narrower filters and thus improved orientation resolution for a filter of the same length (Jacob and Unser, 2004). Additionally, it may be possible to adapt K and σ simultaneously for the neighborhood around each pixel within an orientation-scale space to determine multiorientation and multiscale features. Because the adaptive process itself is general to all steerable filters, such adaptive strategies can be applied to the entire parametric framework of steerable filters and wavelets described in Unser and Chenouard (2013) to provide rich features such as multiple orientations without the need for prior assumptions.

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Author contributions

M.K. and K.J. conceived of the project and wrote the manuscript, with input from A.V., T.S. and R.D.G. M.K. developed the software, tested it and applied it to experimental data, and prepared the figures. A.V., T.S. and R.D.G. provided the microscopy images. A.V. and M.K. also assisted with manually segmenting the images.

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