Adaptive-Resolution Multi-Orientation Analysis of Complex Filamentous Network Images

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Abstract

Microscopy images of cytoskeletal, nucleoskeletal, and other filamentous structures contain complex junctions where multiple filaments with distinct orientations overlap, yet state-of-the-art software generally uses single orientation analysis to segment these structures. We describe an image analysis approach to simultaneously segment both filamentous structures and their intersections in microscopy images, based on analytically resolving coincident multiple orientations upon image filtering in a manner that balances orientation resolution and spatial localization.

Introduction

Our approach uses steerable ridge filters to enhance curvilinear structures in images^{1,2}. While most techniques using ridge filters identify the single best orientation from the filter response per point in space (e.g. pixel)²⁻⁶, our approach is based on the extraction of multiple orientations per point in space. This

allows the full segmentation of junctions without producing gaps, an inherent limitation of the commonly used single-orientation identification followed by non-maximum suppression (NMS)^{2,7} (Fig. 1A-F). A common approach to complete junctions in this case has been to close the gaps using inference methods^{3,8}, but these tend to involve heuristics. Additionally, unlike prior attempts to detect junctions explicitly, our approach does not need predefined angles² or symmetries⁹. Our approach applies generally to steerable ridge filters, including to a unifying parametric framework for steerable wavelets¹⁰.

Results and Discussion

The specific filter used in our work is based on that proposed by van Ginkel and van Vliet^{11,12}. This filter is well-suited for multiple orientation detection because its aspect ratio, and thus orientation resolution, can be controlled via an explicit parameter, K (Supplemental Notes 1-3; specifically Eqs 25-29). However, the original filter suffered from discontinuities for values of $K < 3$ (Supplemental Fig. S1). By redefining the angular part of the filter in terms of angular frequency space instead of direct

Figure 1: Multi-orientation detection and non-local maxima suppression (NLMS) segments simultaneously lines and junctions of arbitrary geometry

(A) Raw fluorescence intensity SIM image of Lamin A in a *Lmnb1*^{-/-} Mouse Embryonic Fibroblast. Magenta boxes, also shown in B-C, are magnified in D, E, and F. Scale bar is 380 nm, applicable to B and C as well. (B) Response to Canny-optimized steerable filter. (C) Non-maximum suppression of steerable filter response. (D-F) Zoom-in of binarized non-maximum suppression (using threshold = 0) for boxes in A. Magenta lines indicate the best orientation per pixel detected by the Canny-optimized steerable filter (response shown in B). Scale bar is 60 nm. (G) Kymograph of filter response curves across the parameter K, with each row false-colored between its minimum and maximum values (as shown in color legend to the left of the kymograph). 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. 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 (same as in R), the center pixel of which is being analyzed in the kymograph. The center pixel is at the intersection of the three colored dashed lines, indicating the detected orientations at K=8, using the same color-coding as in the kymograph. Inset scale bar is 200 nm. (H) Top: Orientations detected using a companion matrix root-solver approach, indicated by the blue, red, 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. 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 G. Bottom: Orientations detected in Regime 3 superimposed on the K = 3 filter response curve. (I) Orientation space construction 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. (J) Non-local maxima suppression (NLMS) applied to the orientation space in I. Magenta lines indicate non-suppressed pixels and their detected orientations. (K) Filter at K = 3 and K = 8. Scale bar is 200 nm. (L) Synthetic junction analyzed in I and J. Dashed cyan box indicates full extent of the representation in I and J. Solid cyan box indicates area depicted in M-Q. Scale bar is 200 nm. (M) Maximum response projection of orientation space in I with detected multiple orientations (magenta). Scale bar is 200 nm, applicable also to N-Q. (N) Maximum response projection of orientation space in J, in which the NLMS procedure has been applied. (O, P) Output of Step 1 (O) and Step 2 (P) of the minimal bridging segmentation algorithm, for junction in L. Bridges are shown as green pixels. (Q) Response-weighted segmentation of the y-junction (final output of the minimal bridging algorithm). (R) Raw fluorescence intensity SIM image of Lamin A immunofluorescence in a *Lmnb1*^{-/-} Mouse Embryonic Fibroblast (same as inset in G). Scale bar is 200 nm, applicable also to S-W. (S) Maximum response projection color-coded by orientation according to the color wheel in the lower left. Colors are blended proportional to the orientation response if multiple orientations are detected. (T-W) Same as N-Q but for the image in R.

1 angular space (Supplemental Note 2.3;
2 specifically Eqs 34-36), we were able to
3 extend its applicability to the full range of K ,
4 namely $K > -0.5$ (Supplemental Fig. S1). This
5 redefinition allowed us to develop a line and
6 junction segmentation approach that
7 integrates orientation information from multiple
8 K values in order to balance orientation
9 resolution (Supplemental Note 3) and spatial
10 localization (discussed shortly). The final form
11 of the filter employed in our study is given in
12 Supplemental Note 2.4, Eqs 43 and 44.

13 To identify multiple coincident orientations
14 from the filter response, we treated the
15 steerable filter response, which is a finite
16 Fourier series, as a trigonometric polynomial¹.
17 Therefore, the orientation(s) maximizing the
18 filter response (i.e. filter response local
19 maxima), along with all other extrema, were
20 determined by solving a general eigenvalue
21 problem using the polynomial's companion
22 matrix^{13,14} (Supplemental Notes 4 and 5).
23 Unlike other eigensystem approaches, such
24 as using a Hessian matrix¹⁵, our approach
25 applies to any steerable filter response and is
26 not constrained by the degree of the
27 polynomial. The analytical and global nature
28 of our approach allowed us to identify
29 orientation(s) much more precisely than would
30 be achievable by sampling approaches, at a
31 fraction of the computational cost
32 (Supplemental Fig. S2).

33 As mentioned above, the orientation
34 resolution of our filter is controlled via the
35 parameter K . Specifically, higher K produces
36 higher aspect ratio filters that provide higher
37 orientation resolution by integrating more
38 signal along their length¹. However, by
39 construction, this increased orientation
40 resolution comes at the expense of spatial
41 localization. To balance the conflicting
42 requirements of orientation resolution and
43 spatial localization, we did not employ a single
44 K for ridge filtering and subsequent analysis.
45 Rather, we calculated the filter response for a
46 range of K values, namely $-0.5 < K \leq K_h$ (K_h
47 typically taken as 8, resulting in an orientation

48 resolution = $\pi/8$; Supplemental Note 3).
49 Noting that steerable filters at different K
50 values are related by the heat-diffusion partial
51 differentiation equation with periodic boundary
52 conditions, here again we used analytical
53 methods to calculate the ridge filter response
54 at any K from the response at the maximum
55 value K_h (Supplemental Note 4). In essence,
56 the filter responses at distinct K levels are
57 related by convolution/deconvolution with a
58 known Gaussian kernel.

59 Having the filter response as a function of K ,
60 we devised a procedure of adaptive-
61 orientation resolution ridge filtering (Fig. 1G,
62 H; Supplemental Fig. S3; Supplemental Note
63 4). First, we determined the regimes of K in
64 which different numbers of orientations are
65 identified, starting with the maximum number
66 in the highest regime (Regime H, which
67 includes K_h), and ending with asymptotically
68 diminishing orientation information in the
69 lowest regime (Regime 0, K in the open
70 interval -0.5 and 1). Second, within each
71 regime, we selected for each surviving
72 orientation its optimal K , defined as the K at
73 which the identified orientation changes the
74 least with respect to K . The orientation values
75 at their optimal K 's were then taken as the
76 identified orientations in each regime.
77 Altogether, adaptive resolution ridge filtering
78 provided us with multi-orientation information
79 at every pixel in the image in a manner that
80 balances orientation resolution and spatial
81 localization.

82 To then employ this information for image
83 segmentation, we extended the traditional
84 NMS concept in multiple ways, collectively
85 resulting in a novel algorithm that we term
86 Adaptive-Resolution Non-Local-Maxima
87 Suppression (AR-NLMS; Fig. 1I-N; Movies S1,
88 S2). NLMS is the core of this algorithm:
89 Building on the concept of orientation
90 space^{11,16,17}, the algorithm performs NMS
91 separately in each plane where signal
92 orientation (i.e. filter response local maximum)
93 has been identified (thus the term NLMS). The
94 adaptive-resolution feature of the algorithm

1 stems from (i) employing the adaptive-
2 resolution ridge filtering procedure described
3 above to identify signal orientations at their
4 optimal K values within a selected resolution
5 regime, and (ii) uncoupling the K used for
6 identifying signal orientations from the K used
7 to calculate the response to which NLMS is
8 applied. For example, Fig. 1I-K shows AR-
9 NLMS obtained by combining orientations
10 identified from high K ($K_h = 8$) with a medium
11 K response ($K_m = 3$), in order to balance the
12 conflicting needs of orientation resolution and
13 spatial localization. Performing a maximum
14 response projection (MRP) along the
15 orientation axis of all the AR-NLMS outputs
16 then produced a 2D image resembling NMS.
17 However, unlike NMS, the projected AR-
18 NLMS retained multiple orientation information
19 per pixel, and thus gaps did not appear at
20 junctions (Fig. 1L-N).

21 While the projected AR-NLMS already yielded
22 complete junctions without gaps, its output
23 was not guaranteed to be a one-pixel-wide
24 topological skeleton¹⁸ (Fig. 1N), as needed to
25 identify the centers of lines and their
26 intersections. Additionally, the AR-NLMS from
27 different orientation resolutions (and,
28 conversely, spatial localizations) may reveal
29 different information about the signal in the
30 image. Therefore, we devised a minimal
31 bridging algorithm to produce a parsimonious
32 segmentation of the centers of lines and
33 junctions by integrating the multi-scale
34 information available through AR-NLMS from
35 different orientation resolution combinations.
36 In a nutshell, starting with “simple” lines (i.e.
37 no junctions) as identified from lower
38 orientation resolution (but higher spatial
39 localization) filters, this algorithm determines
40 the shortest paths to bridge between these
41 lines by using multi-orientation information
42 revealed by higher orientation resolution filters
43 (Fig. 1O-W; Supplemental Note 6;
44 Supplemental Figs S4-S9). The output of the
45 minimal bridging algorithm is a 1-pixel wide
46 response-weighted segmentation (Fig. 1Q, W)
47 incorporating orientation information across
48 both low and high orientation resolution filters,

49 allowing the algorithm to balance the trade-off
50 between orientation resolution and spatial
51 localization. This output resembles the NMS
52 output of traditional single-orientation
53 analysis^{2,7}, and can be subsequently
54 thresholded to segment the lines and
55 junctions of interest (Supplemental Fig. S9).

56 To evaluate the performance of our AR-NLMS
57 analysis and minimal bridging algorithm, we
58 applied it to synthetic images of symmetric,
59 asymmetric, and curved junctions over the full
60 range of angles and at two distinct signal-to-
61 noise ratios (5 and 10) (Fig. 2A-I;
62 Supplemental Note 7; Supplemental Fig. S10;
63 Movies S3-S8). We compared the number of
64 identified orientations, their values, and the
65 junction location (if detected) to the ground
66 truth. We found that the algorithm performed
67 as expected from theoretical predictions
68 based on orientation and spatial resolution
69 (Supplemental Note 3). The detected junction
70 location deviated from the ground truth
71 location for small angles, but then was very
72 close to it when the intersecting orientations
73 were separated by about $\pi/8$ (22.5 degrees).
74 Performance for SNR 5 was slightly less
75 robust than for SNR 10, as reflected by
76 slightly larger performance metric standard
77 deviations (Fig. 2G, I; Supplemental Fig. S10).

78 To demonstrate the broad applicability of our
79 approach, we applied it to complex networks
80 formed by nuclear lamins (Fig. 2J-Q), the actin
81 cytoskeleton (Fig. 2R-Y), and vimentin
82 filaments (Fig. 2Z-AG) in samples of fixed
83 mouse embryonic fibroblasts acquired through
84 structured illumination microscopy (Fig. 2J-Q)
85 or confocal microscopy (Fig. 2R-AG). We
86 found that our approach was able to segment
87 successfully both the lines and junctions in
88 these complex networks, without producing
89 gaps at junctions (e.g., compare Fig. 2K-M vs.
90 Fig. 1D-F).

91 Overall, our work demonstrates that
92 orientation response curves produced by
93 ridge-like steerable filters contain sufficient
94 information to completely segment complex
95 curvilinear structures including junctions of

SNR = 5

SNR = 10

— Orientations / Detected / Ground Truth Jxn

Lamin A

Intensity Segmentation

Response at K = 3

Actin

Intensity Segmentation

Response at K = 3

Vimentin

Intensity Segmentation

Response at K = 3

Figure 2: AR-NLMS and minimal bridging based segmentation of synthetic images and light microscopy images of biological filaments

(A-D) Examples of asymmetric, three radial line synthetic junctions with the indicated intervening angles. Scale bar in A is 600 nm, applicable to Panels B-D as well. **(E)** Ridge filters at $K = 8$ (top) and $K = 3$ (bottom). Scale bar is 600 nm. **(F-I)** Algorithm performance analysis for the three radial line junction case, as captured by comparing the detected orientations with the ground truth orientations (**F** for SNR 5 and **H** for SNR 10), the number of orientations at the detected junction in (**G**; SNR 5 in pink, SNR 10 in green), and the distance between the detected junction and the ground truth junction in (**I**; same color coding as in G). The results are the mean (dark lines) $\pm$ standard deviation (shaded area around dark lines) over 10 tests per angle and SNR. See Fig. S10 for further discussion of performance analysis results. **(J-Q)** Lamin A analyzed in a *Lmnb1*^{-/-} MEF, corresponding to images in Fig. 1A-F. In **J-M**, the segmentation (magenta) is overlaid on the raw images. In **N-Q**, the filter response (brightness) and detected orientations (colored vectors) are shown, with colors corresponding to color wheel in N. Scale bars: 380 nm in J and N; 50 nm in K-M and O-Q. **(R-Y)** Orientation analysis and segmentation of phalloidin-stained actin in a MEF imaged via confocal microscopy. Panel descriptions as in J-Q. Scale bars: 11.4 μm in R and V; 1.4 μm in S, T, W and X; 0.72 μm in U and Y. **(Z-AG)** Orientation analysis and segmentation of vimentin immunofluorescence imaging via confocal microscopy. Cell same as in R. Panel descriptions as in J-Q. Scale bars: 11.4 μm in Z and AD; 1.5 μm in AA and AE; 2.2 μm in AB and AF; 2.7 μm in AC and AG.

1 arbitrary geometry without the need for
2 inference and heuristics. Our method for
3 extracting multi-orientation information with
4 adaptive orientation resolution could thus be
5 useful for deep learning approaches for image
6 segmentation¹⁹. Of note, because the
7 orientation resolution of our filter is determined
8 by its aspect ratio, improved spatial resolution
9 (e.g. in SIM images vs. conventional light
10 microscopy images) allows the use of
11 narrower filters and thus improved orientation
12 resolution. Furthermore, this multi-orientation
13 information could be used to localize lines and
14 junctions with sub-pixel precision⁴. Our
15 approach is generic, and can be applied to
16 segment network-like biological structures
17 other than cytoskeletal and nucleoskeletal
18 filaments, such as stained membranes in cell
19 sheets or network-like organelles such as the
20 endoplasmic reticulum.

21 Online methods

22 Please see Section entitled "Online methods"
23 at the end of the main text file.

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33 Author contributions

34 M.K. and K.J. conceived of the project and
35 wrote the manuscript. M.K. developed the
36 software, tested it and applied it to
37 experimental data, and prepared the figures.
38 A.V., T.S. and R.D.G. provided the microscopy
39 images.

40 Competing interests

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48 Additional information

49 This manuscript is accompanied by a
50 Supplemental Information PDF file consisting
51 of 8 notes and 10 figures, and by 8 movies
52 (movie legends are Note 8 in Supplemental
53 Information PDF).

54 Custom software written in MATLAB to
55 implement the algorithms described in this
56 work is available online on Github at the
57 following URL:
58 [https://github.com/mkitti/AdaptiveResolutionOr](https://github.com/mkitti/AdaptiveResolutionOrientationSpace)
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Online methods

Cell culture. The cell samples for actin and vimentin staining were Mouse embryonic fibroblasts (MEFs) provided by John Eriksson (Abo Akademi University, Turku, Finland). For lamin staining studies, the MEFs were provided by Yixian Zheng (Carnegie Institution for Science, Baltimore, MD, USA). Cells were maintained in DMEM with 25 mM HEPES and sodium pyruvate (Life Technologies; Grand Island, NY) supplemented by 10% fetal bovine serum, 1% penicillin streptomycin, and nonessential amino acids. All cell cultures were maintained at 37°C and 5% CO₂.

Immunofluorescence and Antibodies. Cold methanol fixation was used for 10 min at -20°C to fix the MEFs. Following fixation, initial blocking was done with 1% bovine serum albumin (BSA) in phosphate buffered saline (PBS; ThermoFisher 70011044) for 30 min at RT. Primary antibody incubation was done either at room temperature for 1 hr or overnight at 4°C for single or double label immunofluorescence. Antibody dilutions: chicken anti-vimentin (Biolegend 919101) at 1:200; rabbit anti-Lamin A (#323¹) at 1:500. Cells were washed in PBS at RT and then incubated with secondary antibodies: goat anti-chicken Alexa Fluor 488 (ThermoFisher A-11039) at 1:1000 dilution; goat anti-rabbit Alexa Fluor 488 (ThermoFisher A-11008) at 1:1000 dilution. Cells were washed and stained with Hoechst 33342 (ThermoFisher H-1399) to help locate cells, and in some cases Alexa Fluor 647 phalloidin (ThermoFisher 8940S) for 1 hr according to manufacturer's instructions.

Confocal Microscopy. Images of MEFs stained for vimentin and actin were imaged using a scanning confocal Zeiss LSM 510 META microscope (Carl Zeiss, Jena, Germany) using an oil immersion objective lens (PlanApoChromat, 63x, 1.40 numerical aperture).

Structured Illumination Microscopy. Images of nuclear lamin A were acquired using a Nikon Structured Illumination Microscope (N-SIM; Nikon, Tokyo, Japan) using an oil immersion objective lens CFI SR (Apochromat TIRF 100x, 1.49 NA, Nikon). N-SIM images were reconstructed using Nikon Elements with the following reconstruction parameters: Illumination Contrast Modulation 1.00, High Frequency Noise Suppression 0.75, and Out-of-Focus Blur Suppression 0.15.

Image Analysis and Computing Environment. Images acquired by microscopy were loaded into MATLAB 2017a (Natick, MA) using Bioformats². All acquired images are shown with linear contrast between the maximum and minimum value unless otherwise noted. The steerable filter code based on Jacobs and Unser was implemented by Francois Aguet, and was previously distributed³. Image analysis code was executed on the UT Southwestern Biological High Performance Computing (BioHPC) cluster. Custom software written in MATLAB to implement the algorithms described in this work is available online on Github at the following URL: <https://github.com/mkitti/AdaptiveResolutionOrientationSpace>.

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