A principal skeleton algorithm for standardizing confocal images of fruit fly nervous systems

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ABSTRACT

Motivation: The fruit fly (Drosophila melanogaster) is a commonly used model organism in biology. We are currently building a 3D digital atlas of the fruit fly larval nervous system (LNS) based on a large collection of fly larva GAL4 lines, each of which targets a subset of neurons. To achieve such a goal, we need to automatically align a number of high-resolution confocal image stacks of these GAL4 lines. One commonly employed strategy in image pattern registration is to first globally align images using an affine transform, followed by local non-linear warping. Unfortunately, the spatially articulated and often twisted LNS makes it difficult to globally align the images directly using the affine method. In a parallel project to build a 3D digital map of the adult fly ventral nerve cord (VNC), we are confronted with a similar problem.

Results: We proposed to standardize a larval image by best aligning its principal skeleton (PS), and thus used this method as an alternative of the usually considered affine alignment. The PS of a shape was defined as a series of connected polylines that spans the entire shape as broadly as possible, but with the shortest overall length. We developed an automatic PS detection algorithm to robustly detect the PS from an image. Then for a pair of larval images, we designed an automatic image registration method to align their PSs and the entire images simultaneously. Our experimental results on both simulated images and real datasets showed that our method does not only produce satisfactory results for real confocal larval images, but also perform robustly and consistently when there is a lot of noise in the data. We also applied this method successfully to confocal images of some other patterns such as the adult fruit fly VNC and center brain, which have more complicated PS. This demonstrates the flexibility and extensibility of our method.

Availability: The supplementary movies, full size figures, test data, software, and tutorial on the software can be downloaded freely from our website http://penglab.janelia.org/proj/principal_skeleton

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Supplementary information: Supplementary data are available at Bioinformatics online.

Received on December 31, 2009; revised on February 9, 2010; accepted on February 17, 2010

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A related study was to straighten the strongly curved body of *et al.* 2004; Lam for data on the entire series of 1-pixel spacing cutting-planes orthogonal posterior axis of a worm, and then restacking the resampled image images (Peng *et al.* 1992; Malandain *et al.* 2008) by first detecting the curved anterior–posterior axis of a worm, and then restacking the resampled image data on the entire series of 1-pixel spacing cutting-planes orthogonal to such a detected ‘backbone’. This earlier method is very efficient for *C.elegans* confocal images and other similar cases where the backbone is a simple smooth curve, and the body of the pattern is not fat (wide) enough so that the slightly non-parallel cutting planes next to each other will not intersect within the pattern. Unfortunately, this method cannot be directly used in standardizing a fruit fly larval pattern because the articulated pattern cannot be described as a simple curve, and the fruit fly patterns are often fat/wide enough so that restacking the cutting planes may introduce artifacts especially in the articulated image regions. There are several other skeleton extraction methods (Brandt *et al.*, 1992; Chuang *et al.*, 2008; Lam *et al.*, 1992; Malandain *et al.*, 1998). However, they cannot easily produce skeletons of the same topological structure for different input images, and thus will not be very useful for the registration.

Therefore, in this work we proposed a new approach to standardize the confocal images of fruit fly LNS and adult VNC. We detected the principal skeleton (PS) of a shape using an automatic skeleton deformation algorithm (Section 2). Then we designed a smooth warping method to best align the PS of a pair of image patterns (Section 3). Our method introduces a minimum amount of degradation of the image quality during the standardization. It is also robust under a variety of conditions. In addition, our method is general. It is not limited to fly larval patterns; indeed it can be applied to any image patterns that have a reasonable PS1 (Section 4).

### 2 DETECTING PS

Intuitively, the PS of a shape can be understood as its ‘backbone’, which describes the basic structure as well as the major deformation of this shape. In this article, we define the PS as a set of connected polylines that spans the entire shape as broadly as possible and at the same time has the shortest overall length and sufficient smoothness. We call each polyline a segment. Each segment consists of multiple ordered control points.

Let us use the LNS (Fig. 1a) as an example. We view the nervous system as an articulated composition of three main parts: VNC, and left and right brain hemispheres. Despite the complexity in the articulation region, the deformation within each part can be approximated using a gentler curve. We define the PS of the LNS as a structure with six segments, including three branching segments, which represent the VNC, and left and right hemispheres, joined to another three segments of a triangle that models the ‘hole’ where the non-neuronal tissues (gut and heart) pass through the articulation area.

Naturally, the PS can be viewed as a conceptual extension of the simple ‘backbone’ curve of a *C.elegans* body (Peng *et al.*, 2008), which is essentially the principal curve (Hastie, 1994) of a distribution of image pixels. However, the PS is more than a simple collection of multiple principal curves due to the connection of segments. In addition, instead of using a smooth curve to model each skeleton segment, without loss of generality, we model them by polylines. The control points in a polyline can be further used to fit a smooth cubic-spline curve (Bartels *et al.*, 1998), similar to the *C.elegans* case.

Similar to the case of LNS, the PS of a fruit fly adult VNC can be defined as an ¥-shape (Fig. 1c). The same algorithm is used to find the PSs for both cases. Throughout this article, we focus on LNS; experimental results on adult VNC and some other cases will be presented at the end of this article, and the corresponding movies can be found in the Supplementary Material.

#### 2.1 Shape prior of larval PS

The shape prior defines the initial PS including its topology and constraints of its segments and control points. It should be as simple as possible but complicated enough to capture the major topology of the shape. During the optimization, which is described below, the topology of a PS remains unchanged; however, the locations of all segment-control points will be iteratively updated.

The shape prior of LNS is defined in Figure 2. It contains totally 11 control points $c_1, \ldots, c_{11}$, which are arranged as 6 segments $B_1, \ldots, B_6$ in Equation (1). The control points $c_3$, $c_4$, and $c_7$ join

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1Some image patterns may not have a uniquely well-defined principal skeleton, such as a spherical cell that has the uniform intensity.
We minimize the length of each segment. Sometimes we may need to control points of skeleton. The V oronoi region to any other control points: $D_i$ this goal, in Equation (2) we define four optimization ‘domains’ would like to produce a very smooth skeleton for the junction area when their length is short enough. For example, for the LNS, we indicated by dotted eclipses, are used for the length constraint.

We minimize the length of each segment. Sometimes we may need to minimize the overall length of multiple segments, which indeed has a similar effect to maximizing the total smoothness of these segments when their length is short enough. For example, for the LNS, we would like to produce a very smooth skeleton for the junction area of the two brain hemispheres, which contains three segments $B_1$, $B_2$ and $B_3$. This is similar to forcing $B_1$, $B_2$ and $B_3$ to line up in an almost straight line; thus, their total length is as short as possible. To attain this goal, in Equation (2) we define four optimization ‘domains’ $D_1, D_2, D_3, D_4$, each of which is a polyline and may cover multiple segments (e.g. $D_1$).

\[
\begin{align*}
D_1 &= \{c_1, c_2, c_3\} \\
D_2 &= \{c_4, c_5, c_6\} \\
D_3 &= \{c_7, c_8, c_9, c_{10}, c_{11}\} \\
D_4 &= \{c_1, c_7\}
\end{align*}
\]

(1)

We define the internal term by aggregating all optimization domains defined in Equation (2). Since there are only a few control points in each domain (e.g. five in $D_2$ and six in $D_1$), minimizing their length has a comparable effect to smoothing. For simplicity, we only aggregate the length energy of each domain in the internal term. Let $U = \{D_1, D_2, D_3, D_4\}$ be the set of domain, we have:

\[
E_{\text{internal}} = \frac{1}{\sum_{D_i \in U} w(D_i)} \sum_{D_i \in U} w(D_i)\text{length}(D_i).
\]

(5)

where $w(D_i)$ is a coefficient that defines the contribution of domain $D_i$. For LNS, due to the vertical asymmetry of the shape prior, in order to avoid point $c_1$ and $c_4$ will be pulled downwards seriously by $D_1$ and $D_2$, we give $D_3$ more weight ($w(D_3) = 10$, all other weights equal 1). For a general case, the weights can be uniform. $E_{\text{length}}(D_i)$ in Equation (6) denotes the length of domain $D_i$:

\[
E_{\text{length}}(D) = \sum_{n=1}^{\text{length}(D)} |D[n] - D[n+1]|^2.
\]

(6)

where $D[n]$ is the $n$-th element (control point) in domain $D$.

The overall energy $E$ takes the form:

\[
E = aE_{\text{external}} + bE_{\text{internal}}.
\]

(7)

where $a$ and $b$ are two positive coefficients (both equal 0.5 in our experiments).

To minimize Equation (7), we solve the following equation for every $c_i$, $i = 1, \ldots, 11$:

\[
\frac{\partial E}{\partial c_i} = a\frac{\partial E_{\text{external}}}{\partial c_i} + b\frac{\partial E_{\text{internal}}}{\partial c_i} = 0.
\]

(8)

It is easy to derive an iterative optimization method from Equation (8) to estimate the new location of each non-tip control point, $c_i^{t+1}$, based on the Voronoi region’s center of mass of its current position $c_i^t$, and the positions of its connected neighbor control points:

\[
c_i^{t+1} = \frac{\sum_{c_j \in \text{left}(c_i^t)} w(D_j) c_j}{\sum_{c_j \in \text{left}(c_i^t)} w(D_j)} + \frac{\sum_{c_j \in \text{right}(c_i^t)} w(D_j) c_j}{\sum_{c_j \in \text{right}(c_i^t)} w(D_j)} + \frac{N(c_i^t)}{\sum_{c_j \in \text{left}(c_i^t) \cup \text{right}(c_i^t)} w(D_j)} (P(D(c_i^t)) - c_i^t),
\]

(9)

where $N(c_i) = \sum_{D_j \ni c_i \neq D_k} w(D_k)$ and $P(D(c_i))$ denotes the set of neighboring control points of $c_i$ in domain $D$ (in our case, since each domain is a polyline, this set includes both the left and right control points of $c_i$). Note that $P(D(c_i))$ is null if $c_i \notin D$.

For the end points of the PS, i.e. $c_1$, $c_4$ and $c_{11}$, we use an empirically more robust formula based on two respective neighboring control points. Let us use $c_1$ as an example, the simplified formula is:

\[
c_1^{t+1} = \frac{aO(\theta(c_1^t)) + b(2c_2^{t+1} - c_3^t)}{a + b}.
\]

(10)

Other end points can be updated similarly. Normally, the algorithm converges within 100 loops.

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**Principal skeleton algorithm**

**Fig. 2.** The shape prior of the fruit fly larva PS, where 11 control points $c_1, \ldots, c_11$ form six connected segments and four domains. The domains, indicated by dotted eclipses, are used for the length constraint.
TPS anchor points. Then, we use cubic spline to find a smooth curve. Thus, the DF should be smooth everywhere. (ii) There should be another, based on their PS information.

Peng et al. (2008) to standardize an LNS image. Instead, we generate a minimum amount of distortion to geometrically warp the PS, as well as the entire LNS. (iii) The algorithm should be extensible to other PSs; at the same time, when a PS consists of only one segment, the standardization should approximate the cutting-plane restacking method. We produce a DF using thin plate spline (TPS; Bookstein, 1989), which is defined as the least-bending smooth surface spanning a set of anchor points. Thus, the requirements (i) and (ii) are met naturally.

We have implemented the PS detection method as a plugin of the 3D software (http://penglab.janelia.org/proj/c3d; Peng et al., 2010). We provide a tutorial in the Supplementary Material on how to use the program.

3 ALIGNING PSs
Since the PS of a fly LNS has a more complicated topology than the simple curved ‘backbone’ of nematode C.elegans, we cannot simply reuse the cutting-plane restacking strategy in the earlier work (Peng et al., 2008) to standardize an LNS image. Instead, we generate a smooth displacement field (DF) to warp the fly larvae from one to another, based on their PS information.

There are three general requirements of a DF. (i) No singularity. Thus, the DF should be smooth everywhere. (ii) There should be a minimum amount of distortion to geometrically warp the PS, as well as the entire LNS. (iii) The algorithm should be extensible to other PSs; at the same time, when a PS consists of only one segment, the standardization should approximate the cutting-plane restacking method.

We produce a DF using thin plate spline (TPS; Bookstein, 1989), which is defined as the least-bending smooth surface spanning a set of anchor points. Thus, the requirements (i) and (ii) are met naturally. For requirement (iii), we first consider all control points of the PS as TPS anchor points. Then, we use cubic spline to find a smooth curve through each domain of the PS (as shown in Fig. 3a), and then for every consecutive pair of control points, we add the halfway point on the smooth curve as a TPS anchor point. We call the set of anchor points that consists of nicely spaced points on the PS as the PS-set. Next, we define additional anchor points based on this PS-set: for each PS-set anchor point, we compute the orthogonal cutting line that intersects at this anchor point location with the respective smooth curve in the PS, and then we define two anchor points on each of the left and right sides of the cutting line (spacing = 75 pixels for LNS). We call the set of anchor points that are not on the PS as the non-PS-set. If a non-PS-set anchor point falls into the intersection region of multiple cutting lines of different domains (as highlighted in Fig. 3a), we remove it from the set of the all anchor points, and thus avoid the non-smooth wrapping around of the DF.

While the choices of the number of anchor points of both PS-set and non-PS-set, as well as the spacing among anchoring points, are empirical, a general guideline is to make the anchoring points distribute evenly to cover the entire image pattern. The selected parameters are then used for all images.

The entire algorithm for LNS standardization is as follows:

(1) Define/initialize the shape prior of PS.
(2) Find the Voronoi region of each control point in an input image (called ‘subject’ image below for simplicity).
(3) Update the positions of control points using Equations (9) and (10).
(4) Check whether or not the positions of control points have converged (i.e. the maximal distance between the new and old positions of control points is <0.01). If yes, go to Step 5. Otherwise go back to Step 2.
(5) Use cubic spline to interpolate PS and produce a smooth skeleton according to the defined domains.
(6) Define both the PS-set and non-PS-set of TPS anchor points, remove some non-PS-set points if they fall into the cutting line overlapping/intersection region.
(7) Compute the TPS DF of this image using the corresponding anchor points between this image and a predefined target image.
(8) Warp the subject image to a ‘standard’ shape using the DF.
(9) Set the warped (standardized) subject image as the input image and repeat Steps 1–8 until the PS of the image does not vary significantly (defined as the average displace of control points between two consequent iteration <3 pixels).

Our standardization method is general; it can be applied to both 2D and 3D image patterns. For fruit fly LNS, although our image stacks are 3D, the major variation of the shape is in the 2D plane of two brain hemispheres and the VNC. Therefore, we simplify the processing using 2D maximum intensity projection, and detect the PS in 2D. Accordingly, the TPS DF is produced in 2D; all z-sections of a fly LNS image stack will share the same DF.

Of note, in our algorithm it is necessary to consider Step 9, i.e. the iterative alignment of a PS. Because TPS warping is non-linear and will cause some regions expand or shrink smoothly, the redetected PS of a warped image is slightly different from that of target image. Several iterations of the optimization will produce nicely aligned image patterns whose PSs match well. Normally, it takes about three loops to generate satisfactorily aligned PSs (Section 4.4).

4 EXPERIMENTS AND DISCUSSION
We first evaluated the robustness and consistency of the PS detection algorithm, and then compared our method with the commonly used morphological thinning and affine-transform-based alignment method. In the end, we show some PS detection results of other cases.

For the fruit fly larva data, N-cadherin-labeled LNS was imaged in 3D using a confocal laser scanning microscope (Zeiss LSM 510) in the laboratory of J. Truman. The voxel resolution is 0.46 × 0.46 × 2.0 µm. For the adult fly VNC data, NC-labeled VNC was imaged using a confocal microscope (Zeiss LSM Pascal 5) in J. Simpson’s laboratory. The voxel resolution is 0.58 × 0.58 × 0.8 µm in 3D.
4.1 Robustness

We tested the robustness of detecting the PS when the LNS patterns in images have different orientations, scales, contrasts, articulated and twisted parts, as well as other noises.

Figure 4a shows robustness test results for the twisted LNS. We simulated the deformation by rotating the right brain hemisphere from $-45^\circ$ to $45^\circ$, with a $15^\circ$ interval, to produce seven rows from top to bottom. For every row, we rotated the VNC from $-45^\circ$ to $45^\circ$ similarly to produce seven columns. The detected PSs (red) from the same initialization (green) are correct, indicating that our method is robust for twisted patterns. We further added noise to the test images and changed the scale of the initialization. The results (Supplementary Material) were also robust.

Figure 4b shows a perturbation test based on real LNS images. We randomly selected four LNS images that have differently twisted nervous systems. Similarly to Figure 4a, we rotated them with different angles ($-45^\circ$, $-30^\circ$, $-15^\circ$, $0^\circ$, $15^\circ$, $30^\circ$ and $45^\circ$). From exactly the same initialization (green), our algorithm successfully detected the PSs (red), except two errors in the left-bottom corner and in the last column of the third row, which correspond to $-45^\circ$ and $+45^\circ$ rotations, respectively. The errors occurred when the angle between the longest axis (along the brain-VNC direction) of an LNS and the initialization of the PS is bigger than $45^\circ$, the VNC part of the larva would more likely be interpreted as $B_1$ or $B_2$ rather than $B_3$ according to the shape prior we assumed in Section 2. In other words, the PS was initialized too poorly. However, practically speaking, this situation is rare in the real data. We can also avoid it completely by preprocessing an image so that its longest axis is roughly aligned with the $B_3$ segment. We also changed the contrast of the test images and found that our method robustly produced meaningful results (Supplementary Material).

4.2 Consistency

We quantified the consistency of PS detection given different initializations. For four LNS images, we rotated the respective initial shape prior of the PS in the range of $\pm 30^\circ$ (interval = $15^\circ$). For any pair of the five PSs detected for each image, we computed the mean square error (MSE) of the corresponding control points of this pair of skeletons. Table 1 shows the maximal and average MSE scores for all possible pairs. The MSE scores are much < 1 pixel, indicating that PSs were detected very consistently from different initializations.

4.3 Comparison with morphology thinning

Morphology thinning (Lam et al., 1992) is a commonly used algorithm to extract the ‘backbone’ of an image object that has closed contour. In order to use thinning, one needs to first segment the object from the image background. Unfortunately, the thinning algorithm is very sensitive to the segmentation. As shown in Figure 5a, direct thinning on a binary image generated too many artificial branches due to spurs in the contour of the object and unstained areas (holes). Here, we simply took the foreground as the set of the pixels whose intensity was above the mean value of all pixels in the image. After we carefully smoothed the contour and filled the holes using additional morphological opening and closing operations (with morphological element set to be ‘disk’ shape, with 10 and 20 pixels in diameter, respectively), we achieved better thinning results in Figure 5b and c, which however were still not as good as the detected PS in Figure 5d.

4.4 Standardization of real LNS images

As a real application, we used the PS method to standardize a randomly selected set of 237 3D confocal images of the third instar
Fig. 5. Skeletons detected using image thinning (a–c) under different conditions and using the PS method (d).

Fig. 6. Comparison of LNS standardization using the PS method and the affine-based alignment. Overlaid patterns (a–c), as well as the respective PSs (d–f): before standardization (a and d), after a global alignment using affine transformation (b and e), and after our PS-based warping method (c and f). The skeletons are periodically colored with eight different colors, and the images are periodically colored with three colors (red, green and blue) (g–i). The density map of overlaid PSs after 1, 2 and 3 iterations of optimization. The radius of skeleton indicates the standard deviation of PSs of images after 1, 2 and 3 iterations of standardization.

Fig. 7. Comparison of the image content before (a and b) and after (c and d) standardization. (b and d) are the zoom-in view of the red boxes in (a and c). Red image channel: GFP-tagged GAL4 patterns. Green: neurotactin and blue channel: N-cadherin.

larval stage LNS. The comparison results of overlaid patterns are shown in Figure 6a-c, as well as the respective PSs are shown in Figure 6d-f, before and after standardization. Obviously, the affine transform-based standardization (Fig. 6b and e) did not align LNS patterns well, especially the big deviation in the VNC of an LNS. This was due to that the articulated LNS could not be well approximated by an affine transformation. On the contrary, the PS-based standardization scheme (Fig. 6c and f) successfully registered all images, with the PSs almost exactly overlapping on top of each other.

We also quantified the spatial variations of the aligned PSs. As explained at the end of Section 3, the iterative optimization of the PS standardization, i.e. Step 9, is important. Figure 6g–i show that with 1, 2 and 3 iterations, the standard deviation of the entire set of PSs became smaller and smaller. Indeed, the average ‘pair-wise distance’ of the entire population of PSs dropped from 50.225 pixels before standardization to 5.661, 3.220 and 2.323 pixels in three iterations. For a better visualization, see the Supplementary Material for large frame-size movies of this experiment.

4.5 Quality of standardized images

We also visually inspected the image quality of an LNS pattern before and after our standardization process. Figure 7 shows that all the local features of an image before standardization (Fig. 7a and b) were well preserved in the post-standardization image (Fig. 7c and d). No visible artifact was seen. The processed image was a little bit smoother, due to the interpolation process during the TPS warping. The overall loss of information was negligible.
VNC is to detect the PS robustly. Figure 8b shows the results of a C.elegans PS. (upper row) and thinning (lower row) on five adult VNC images (background optimization domains (Fig. 8a), than an LNS. more complicated shape prior, which has nine segments and seven standardize an adult fruit fly VNC. Indeed the adult VNC has a

in Figure 1c and d, we show that the PS method can be used to easily adapted to other shape analysis applications. For example, by modifying the shape prior of the PS, our algorithm can be more robust by first optimizing the skeleton of the main trunk (i.e. the horizontal domain in Fig. 8a) so that we can get a good estimation of the scale, position and the orientation of the VNC, and then optimizing the remaining domains/segments to complete the entire skeleton.

ACKNOWLEDGEMENTS

We thank James Truman for providing the larva image dataset and comments on the manuscript. We thank Julie Simpson for the adult VNC test data. We also thank Zongcai Ruan, Wayne Perenau, Fuhui Long, Gene Myers and other people in Gene Myers lab and Hanxuan Peng lab for discussion. We thank Margaret Jefferies for help of proofreading of the manuscript.

Funding: Howard Hughes Medical Institute.
Conflict of Interest: none declared.

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