

September 2, 2014

Dear PNAS Editor,

We are writing in response to the comments pertaining to MS 2014-10621. We thank the referees for their detailed and insightful comments and have revised our manuscript significantly to address many of the issues related to the general applicability of our method and its validation on model datasets.

The manuscript has been edited as follows:

1. We removed the first example (images taken during cellularization), as it could be ordered using PCA and did not illustrate the full advantages of our methods.

2. We removed the third example (images from different genotypes), choosing instead to focus on images collected during gastrulation where we observe significant morphological changes.

3. We added a data set from a live imaging study of gastrulation, which we use to validate our approach. This test was suggested by the referees.

4. We provide the results of a quantitative analysis of how our methods perform as a function of sample size and in data sets with interembryo variability.

5. We added a discussion of the requisite CPU time and the parameter tuning required for our methodology.

**In response to the editor comments:**

Our goal is to present an automated approach to the tasks of registration and temporal ordering of images. We acknowledge that these tasks could be done manually for the specific data sets presented. The main advantage of our proposed methodology is that it is fully automated and requires very little *a priori* knowledge of developmental dynamics, which is in contrast to any of the published works on image registration and ordering in context of embryogenesis. In contrast, manual and semi-automated analysis of images in developmental dynamics often relies on known image features that characterize the orientation and temporal order in a specific system.

For this approach to be applicable to general imaging data sets with unknown orientations and temporal order, it must first be validated on data where the true rotations and temporal order are known. We have provided evidence that our methods are robust, general, and computationally tractable so that they may readily be applied to larger, more complex imaging data sets in the future. We have removed the simple data sets that could be analyzed with standard PCA, and instead included a new data set collected from a live imaging study. In this live imaging data set, the true alignments and order are known, and we assess the accuracy of our methodology using this data. We also used this data set to perform statistical tests and to demonstrate the robustness of our proposed approach.

**In response to reviewer 1 comments:**

1. Capabilities and general usefulness of the proposed framework.

The reviewer was concerned about our methodology generalizing to other, more complicated data sets. We would like to comment that the data sets that we now analyze span gastrulation and show significant morphology changes. This is in contrast to typical analysis of cellularization data which exhibits no morphology changes, and no studies of developmental dynamics have been conducted spanning this stage of embryogenesis. The presented images cannot be analyzed using PCA and require more sophisticated, nonlinear approaches. Furthermore, the methodology requires very little *a priori* knowledge about the expected spatiotemporal dynamics. This is in contrast to other studies of developmental dynamics (such as Fowlkes *et al*, *Cell*, 2008) where researchers use knowledge of various expression patters to register and order images (e.g., aligning images using the peak of Dorsal or the Eve stripes, and timing images using membrane thickness). In addition to the cross-sectional images we have studied in this paper, we have also looked at lateral images of Eve stripe formation. In this case, diffusion maps could also successfully order these images and recover the correct dynamics for the emergence of the seven stripe pattern. Therefore, our algorithm is sufficiently general to apply to many types of images. We chose to focus only on the selected data sets in our manuscript to demonstrate the utility of our methods and we hope it is clear that the methods would generalize to other imaging data.

Our algorithms would directly generalize to 3D volume data (such as the data collected in Fowlkes *et al*, *Cell*, 2008), as computing pairwise alignments and distances between volumes is no different than computing pairwise alignments and distances for images. The computation of the three-dimensional pairwise alignments would be more computationally demanding, but 3D registration and temporal ordering of the entire data set would be straightforward. For data collected from arbitrary viewing directions of a three-dimensional object, we refer the reviewer to Singer and Wu*, Commun Pure Appl Math*, 2012, where they use the same methodology to analyze images of molecules taken from different viewing directions. In these examples, the embedding coordinates recovered by vector diffusion maps parameterize the viewing direction of the molecule, and the recovered rotations register images taken from the same viewing direction. Therefore, one can take a set of unregistered images which span multiple viewing directions, and register and organize the images according to viewing direction in order to reconstruct a three-dimensional view of the object.

1. Evaluation of performance and robustness of the proposed method

Using several data sets collected from live imaging studies, we quantitatively assessed the accuracy of our methods. We showed that the results are consistent across multiple data sets, require relatively few images to be accurate, and are robust to interembryo variability.

We also examined the required computational time for our methods. The live imaging data sets of 40 images required ~2 seconds to analyze on an Intel Core i7 2.93 GHz processor, and analysis of the 120 fixed images required ~26 seconds. In our manuscript, we now discuss the computational scaling, and also mention that the most computationally intensive portion of the computation is easily parallelizable for larger data sets.

We found that our results are robust to parameter selection. There are two parameters for our algorithms: the angular resolution used to compute the pairwise alignments, and the kernel scale in the diffusion maps calculation. For our fixed image example, we found that ε**2**=(median pairwise distance)2/3 - (median pairwise distance)2/26 all yielded rank correlations > 90%. We also found that angular resolution ≤ 36**°**yielded rank correlations > 90%. For our analysis, we used ε**2**=(median pairwise distance)2/10, and angular resolution 10**°**; these parameter values yielded accurate results for all data sets presented. In general, the parameter tuning required is minimal to obtain meaningful results for a general data set.

For the images presented, minimal preprocessing was required to obtain accurate results. We cropped the images (to remove size effects), normalized the nuclear signal intensity (as the absolute value of the nuclear signal is not meaningful), and blurred the nuclear signal (as the exact location of the nuclei is not important, but rather the overall shape that they delineate). These are relatively simple tasks that address specific features of the experimental system and imaging setup, and would most likely need to be performed for any image analysis algorithm.

**In response to reviewer 2 comments:**

We agree with the reviewer that the multiple uses of the term “cross-section” are confusing. As such, we have removed the use of “cross-sectional” to describe the data sampling scheme, to avoid confusion with referring to images taken along the dorsoventral cross-section of the *Drosophila* embryos.

Per the suggestion from the reviewer, we used live imaging studies to validate our proposed methodology. We validated that our algorithm recovers the correct orientation and temporal order for a live imaging data set that has been randomly shuffled and rotated. We also use several live imaging data sets to construct time courses with interembryo variability, and show that our methods can still recover the correct orientation and order with high fidelity.

We have made the code publically available at <http://genomics.princeton.edu/stas/publications.html>, under the link “Code for the `Temporal ordering and registration of images in studies of developmental dynamics’.” Included in this distribution are

* The five live imaging data sets presented in our manuscript.
* The data set of fixed images presented in our manuscript.
* Two example scripts, one for the analysis of the live imaging studies and one for the analysis of the fixed images, which demonstrate how to register and order a set of images using our methods.
* All functions needed for registration and ordering using vector diffusion maps. This includes
  + Code for computing the pairwise alignments and distances (rotations or translations and rotations) from a set of images.
  + Code for computing the optimal alignments and embedding coordinates from the pairwise alignments and distances using vector diffusion maps.
  + Code for registering all images using the optimal alignments computed from vector diffusion maps.
  + Code for computing an average developmental trajectory from a set of registered and ordered images.

Sincerely yours,



Stanislav Y. Shvartsman,

Professor, Princeton University

[stas@princeton.edu](mailto:stas@princeton.edu)