We are writing in response to the comments pertaining to “Temporal ordering and registration of images in studies of developmental dynamics.” We would like to thank the reviewers for their helpful and insightful comments. We have edited the manuscript significantly to address many of the issues brought to our attention. We hope that you will now find our submission acceptable for publication.

The manuscript has been edited as follows:  
1. We have 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 have 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 have added a data set coming from a live imaging study of gastrulation, which we use to validate our approach.

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

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

**In response to the editor comments:**

The goal of our paper 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 the developmental dynamics. 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 (see…..) which exhibits no morphology changes. 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 [[1]](#footnote-1) 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). Our algorithms would directly generalize to 3D volume data (such as the data collected in Fowlkes *et al*), 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[[2]](#footnote-2), where they use the same methodology to analyze images of molecules taken from different viewing directions.

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.

Reviewer 2:  
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.

To validate our approach, we used live imaging studies to show that out method can recover the correct orientation and temporal order of images.

We have made the code publically available at <http://genomics.princeton.edu/stas/publications.html>. This includes the data sets we have analyzed, two example scripts, and all the necessary functions to perform the analysis described in our manuscript.

1. Fowlkes, Charless C., *et al.* "A Quantitative Spatiotemporal Atlas of Gene Expression in the *Drosophila* Blastoderm." *Cell* 133.2 (2008): 364-374. [↑](#footnote-ref-1)
2. Singer, Amit, and H‐T. Wu. "Vector diffusion maps and the connection Laplacian." *Communications on pure and applied mathematics* 65.8 (2012): 1067-1144. [↑](#footnote-ref-2)