**Extended Data for MosaicExplorerJ: Interactive Stitching of Terabyte-size Tiled Datasets from Lightsheet Microscopy**

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**S1 Datasets**

**All datasets can be downloaded from:** <https://bit.ly/37iocrP>.

**Emb3\_2x2\_Mosaic\_2CAMs\_12GB**

Mouse embryonic head, 2 columns x 2 rows mosaic, dual-sided camera, dividing nuclei in PH3-A488 fluorescence channel. Each 3D tile is stored as a series of 2D TIFF files. Cropped out volume from the original dataset. Should be opened with ***Dual camera*** mode in MosaicExplorerJ. Author: Maria Jose Barallobre.

**Emb4\_4x5Mosaic\_2CAMs\_595GB**

Mouse embryonic head, 4 columns x 5 rows mosaic, dual-sided camera, dividing nuclei in PH3-A488 fluorescence channel. Each 3D tile is stored as a series of 2D TIFF files. Cropped out volume from the original dataset (1.2 TB in size and two fluorescence channels). Should be opened with ***Dual camera*** mode in MosaicExplorerJ. Author: Maria Jose Barallobre.

**Brain2\_izq\_2x8Mosaic\_LeftSide\_300GB**

Mouse brain, left illumination side, 2 columns x 8 rows mosaic, blood vessels in Tomato-Lectin fluorescence channel. Each 3D tile is stored as a 3D TIFF image. The original dataset is 1.2 TB in size (dual-sided illumination and dual-sided camera detection). The right illumination side from the same camera view is provided as another dataset. Author: María Luisa Soto-Montenegro

**Brain2\_izq\_2x8Mosaic\_LeftSide\_BigStitcher\_300GB**

Same as **Brain2\_izq\_2x8Mosaic\_LeftSide\_300GB** but converted and aligned with BigStitcher.

**Brain2\_izq\_2x8Mosaic\_RightSide\_300GB**

Right illumination side from **Brain2\_izq\_2x8Mosaic\_LeftSide\_300GB**.

**Samples preparation**

The datasets used to test MosaicExplorerJ were acquired in the context of two currently unpublished scientific projects. Sample preparation and imaging conditions are provided here below.

Female C57Bl6/J mice were used in this experiment. Animals were maintained at a constant temperature (24 ± 0.5ºC) under a 12h-hour light/dark cycle, and permitted free access to commercial rodent laboratory chow and water. All experimental animal procedures were conducted in conformity with the European Communities Council Directive 2010/63/EU and approved by the Ethics Committee for Animal Experimentation.

**Mice embryonic heads with labeled dividing nuclei and Pax6 expression**

**(Emb3** & **Emb4)**

Mouse embryos were harvested from pregnant females at gestational stage 12.5. Heads were isolated, fixated in PFA 4% o/n and immuno-stained for PH3-A488 (anti-phospho-histone3 [pSer28] antibody, Sigma H9908, 1/500; mitosis marker) and Pax6 (anti-Pax6 antibody, BioLegend 901301, 1/100; radial glial cell marker) followed by optical clearing with the iDisco protocol (Renier 2014).

**Mice brains with labeled blood vessels**

**(Brain2\_izq)**

Tomato-Lectin (Lycopersicon Esculentum, Vector Laboratories, B-1175-1) was administered by tail vein injection (50ug lectin in 250ul volume, 0.2ug/ul concentration). After 20 minutes, animals were anesthetized with ketamine:xylazyne (10:1). Mice were first perfused with PBS solution for 3 minutes to clean the organs and later with 4% PFA for 10 minutes using a peristaltic pump (12-15 ml/min). After perfusion, the brains were removed and fixed in 4% PFA overnight. After 3 washes with PBS, brains were preserved in 70% EtOH. For clearing/mounting, the samples were first washed o/n in PBS and then washed again by 2x 5’ PBS washes and 1x 5’MilliQ water wash, then embedded in agarose blocks (0.8%) inside an Eppendorf (or 5 ml syringe). From now on the samples were covered with an aluminium foil. Dehydration was performed with MeOH:H20 (1h 30%, 1h 50%, 1h 70% then 1h 100%, o/n 100%, 6h 100% MeOH, o/n 100% MeOH) and the samples were finally transferred into BABB clearing solution (Benzyl Alcohol-Benzyl Benzoate 1:2) at RT until proper clearing (normally after 1 or 2 days).

**Samples imaging**

All samples were imaged at ADMCF IRB Barcelona by the same custom light-sheet microscope (Kennel 2018) designed for whole mount organs. The microscope is equipped with an Orca Flash 4.0 V3 sCMOS camera (6.5 µm pixel size). The configurations used for the different samples are listed below:

**Emb3** & **Emb4**

5x objective + 5x zoom lens (15x overall zoom including tube lens), 1.720 Z slices (**Emb3**) and 1.840 Z slices (**Emb4**) at 5 µm step, 4x4 mosaic (**Emb3**) and 4x5 mosaic (**Emb4**).

**Brain2\_izq**

2x objective + 6x zoom lens (7.2x overall zoom including tube lens), 2.800 Z slices at 2.67 µm step, 8x2 mosaics for each illumination side (32 tiles in total).

**S2 - Comparison between MosaicExplorerJ with BigStitcher**

To compare the results and basic pipeline of operations of MosaicExplorerJ and BigStitcher, we performed the alignment of the dataset **Brain2\_izq\_2x8Mosaic\_LeftSide\_300GB** (**Section S1**) with both applications.

**Image exploration and alignment**

The TIFF tiles acquired by the microscope could be immediately explored with MosaicExplorerJ while it took about two hours to convert them to BigStitcher HDF5 format on a dedicated workstation (Intel Xeon E5-1650 3.5 GHz, 128 GB RAM, Disk I/O > 1.000 MB/s). While this conversion is not strictly necessary, BigStitcher 3D image viewer interactivity and automated alignment speed heavily rely on the multi resolution format, and are highly impaired when handling TIFF datasets in the hundreds of GBs to TB range. BigStitcher relies on the approximate manual placement of the tiles (with some automatic and interactive placement tools) prior to their automatic optimization. We laid the tiles at their approximate known positions (10% overlap) and the automatic alignment of the dataset took about 30 minutes with the settings we used. This operation was then performed manually in a comparable time (about 15 minutes) with MosaicExplorerJ. For this latter, the most time consuming step was to adjust the axial shifts of the tiles from first row and first column (eight tiles) with respect to the top left tile.

**Alignment accuracy**

To assess differences in the results, the X, Y, Z positions of the 3D tiles were compared after alignment by both tools. Concretely, we compared the distances from the top left tile to the three other tiles making up the corners of the mosaics (**Table S1**). The average of these three distances was computed as a simple measure of similarity between both mosaics and estimated to **1.82 pixels** (and **0.67 Z slice**).

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
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| **MosaicExplorerJ BigStitcher**   |  |  |  |  |  |  |  |  |  | | --- | --- | --- | --- | --- | --- | --- | --- | --- | | **Tile** | **X** | **Y** | **Z** |  | **Tile** | **X** | **Y** | **Z** | | X00-Y00 | 0 | 0 | 0 |  | X00-Y00 | 0 | 0 | 0 | | X00-Y07 | -7 | 12.628 | 146 |  | X00-Y07 | -8 | 12.626 | 147 | | X01-Y00 | 1.821 | 7 | 0 |  | X01-Y00 | 1.821 | 8 | 1 | | X01-Y07 | 1.814 | 12.635 | 146 |  | X01-Y07 | 1.812 | 12.634 | 146 | |

**Table S1.** Positions of the four corner 3D tiles of the mosaics after manual alignment with MosaicExplorerJ (left) and automatic alignment with BigStitcher (right)

Even though these figures and the visual inspection show that the results obtained by both tools are pretty similar for this dataset, it is to be noted that BigStitcher can achieve a higher accuracy for datasets suffering from sources of misalignments not taken into account by MosaicExplorerJ (e.g. optical artefacts produced by the sample, chromatic aberrations, non parallel lighsheet for dual-sided illumination, severely interleaved X,Y,Z motor axis wobbling).

**Operation**

MosaicExplorerJ relies on the visual inspection and the manual registration of matching features in the regions of overlap between the tiles (**S3-V1** & **S3-V2**). While this might look at first less attractive than automated algorithms, this is a fast process (the alignment is performed instantly after a limited number of tiles have been adjusted), and it is a simple way to ensure that a predictable correction model (camera tilt + lightsheet tilt + XY separable sample motor axial wobble) is sufficient to align a mosaic, which can help detecting acquisition flaws (e.g. sample deformation). Also, there is no risk that an automatic optimizer “hides” these artefacts or gets stuck in an incorrect configuration (potentially requiring to update the starting positions of the tiles before another round of optimization).

**Dual-sided camera datasets**

MosaicExplorerJ is designed to stitch the images coming from two opposing cameras (**S3-V4**) by 2D registering them in a user defined Z slice (union slice) and by deterministically keeping the slices coming from the closest camera (Figure 1). Handling dual-sided cameras in BigStitcher is a bit more tricky since the images need to be flipped manually and 3D registered from automatically detected interest points. Also, BigStitcher relies on image dependent quality metric to merge the best images from two overlapping views. While this strategy is more versatile (it is for instance also used to merge illumination sides or multi-angle views), it is also more computationally intensive and MosaicExplorerJ strategy guarantees that residual misalignments do not propagate beyond the union slice. This can be advantageous when no image quality improvement is expected from blending the images from two cameras.

**Dual-sided illumination datasets**

The accurate stitching of the mosaics from two illumination sides can only be achieved accurately with MosaicExplorerJ when the lightsheets from both illumination sides are approximately parallel (**S3-V3**). If this condition is not met, the mosaics must be 3D registered (typically from interest points) and 3D rotated, two operations that are currently only supported by BigStitcher.

**Intensity correction**

Both tools can perform flat field intensity correction. A dark and a bright image has to be provided for BigStitcher while MosaicExplorerJ directly relies on an externally computed intensity correction image (multiplicative weights). When no reference images are available, MosaicExplorerJ additionally enables to interactively adjust XY separable linear shading correction masks to correct for non homogeneous illumination (**S3-V5**).

**Nonrigid registration and chromatic aberrations correction**

MosaicExplorerJ applies the same transformations to all channels while BigStitcher supports channel dependent transformations (for the correction of chromatic aberrations) and nonrigid transformations to account for residual mismatches (e.g. by subdividing the image tiles in virtual blocks).

**Summary**

We summarize the main features of MosaicExplorerJ and BigStitcher (stitching only) in Table **S2**.

|  |  |  |
| --- | --- | --- |
| **Features** | **MosaicExplorerJ** | **BigStitcher** |
| Process 3D TIFF and 2D TIFF series interactively | **YES** | **Limited[[1]](#footnote-0)** |
| Mosaic geometry | **Regular XY grid** | **Arbitrary** |
| Tiles grid position from file names | **XY numeric fields** | **Configurable** |
| Tiles dimensions | **Same XYZ size** | **Arbitrary** |
| Maximum XY size of reconstructed mosaic (pix) | **47.000 x 47.000** | **None** |
| Maximum number of channels | **8** | **None** |
| Maximum number of Z slices | **None** | **None** |
| Manually align mosaic from physical correction model | **YES** | **NO** |
| Automatically align mosaics from tile correlations | **NO** | **YES** |
| 2D Register opposing cameras at union Z slice | **YES** | **NO** |
| Register dual-sided illumination | **Limited[[2]](#footnote-1)** | **YES[[3]](#footnote-2)** |
| Alternate tile color coding | **YES** | **YES[[4]](#footnote-3)** |
| Flat field intensity correction | **YES** | **YES** |
| Adjustable linear intensity shading | **YES** | **NO** |
| Signal quality dependent image fusion | **Limited[[5]](#footnote-4)** | **YES** |
| Refinement / chromatic aberration correction | **NO** | **YES** |
| Easily customizable features / user interface | **YES** | **NO** |

**Table S2.** Main features of MosaicExplorerJ and BigStitcher (stitching only)

**S3 Software Documentation**

A complete documentation of MosaicExplorerJ can be found from the GitHub repository: [**https://github.com/SebastienTs/MosaicExplorerJ**](https://github.com/SebastienTs/MosaicExplorerJ) and five video tutorials illustrate the main operations described in the documentation (referenced in the corresponding sections):

**S3-V1** Aligning tiles from a mosaic and exporting the images

**S3-V2** Correcting for the axial shift between the tiles

**S3-V3** Registering dual-sided camera detection datasets

**S3-V4** Registering dual-sided illumination datasets and balancing intensity between both sides

**S3-V5** Intensity correction

The videos can all be streamed from: **bit.ly/2KY0BDN**

**References**

Renier N *et al* (2014). iDISCO: A Simple, Rapid Method to Immunolabel Large Tissue Samples for Volume Imaging, Cell 159:896–910.

Kennel P *et al* (2018). Toward quantitative three-dimensional microvascular networks segmentation with multiview light-sheet fluorescence microscopy.  J Biomed. Optics 23(8): 1-14.

1. Processing and 3D interactions speed heavily impaired [↑](#footnote-ref-0)
2. Lightsheets from both sides must be parallel for optimal stitching [↑](#footnote-ref-1)
3. Robustness is sample dependent since relying on the detection of interest points [↑](#footnote-ref-2)
4. Tile Colors are assigned randomly (even though deterministically) [↑](#footnote-ref-3)
5. Only maximum intensity blending supported [↑](#footnote-ref-4)