**BigStitcher: Efficient reconstruction of high-resolution image datasets of cleared and expanded samples OR**

**BigStitcher: Efficient reconstruction of large multi-tile and multi-view image datasets**

**New methods for clearing and expansion of biological objects create large, transparent samples that can be rapidly imaged using lightsheet microscopy. Resulting image acquisitions are terabytes in size consisting of many large, unaligned image tiles that suffer from optical degradation. We developed the BigStitcher software that efficiently handles and reconstructs large multi-tile, multi-view acquisitions compensating all major optical effects, thereby making single-cell resolved whole-organ datasets amenable to biological studies.**

Sample clearing methods such as CLARITY or SCALE [chung, Hama] enable imaging of very large, fixed specimen without the need for physical sectioning (**Fig. 1a,b**). In combination with lightsheet microscopy (**Fig. 1c**) [Huisken, Keller, Truong] it is possible to scan entire volumes such as adult mouse brains at single-cell resolution within just a few hours [Tomer]. Since clearing protocols preserve endogenous fluorescent proteins and are compatible with most staining methods [chung, refs] these acquisitions are powerful tools for whole-organ and whole-organism studies (**Supplementary Fig. 1**).

However, to acquire an entire sample, many large, overlapping three-dimensional (3d) image tiles need to be collected that can amount to many terabytes in size (**Fig. 1d,e**) and require image alignment (**Fig. 1f-m**). Due to sample-induced scattering of the lightsheet in the direction of illumination [scat], 3d image tiles are typically acquired twice while alternating illumination from opposing directions to achieve full coverage (**Fig. 1d** and **Supplementary Fig. 2**). Similarly, emitted light is distorted by the sample, effectively limiting maximal imaging depth at which useful data can be collected (**Fig. 1n**). Additionally, sample-induced light refractions cause depth and wavelength dependent aberrations in the acquired images (**Fig. 1j,k**). We therefore developed the BigStitcher software that enables user-friendly import, interactive handling of multi-terabyte image data, fast and precise alignment, as well as deconvolution and real-time fusion of large, high-dimensional datasets. BigStitcher additionally supports the alignment of multi-tile acquisition taken from different physical orientations, so called multi-tile *views*, thus effectively doubling the size of cleared specimen that can be imaged (**Fig. 1n**).

Microscopy acquisitions are saved in a multitude of vendor-specific and custom formats that store the images along with important metadata. To address the challenge of generalized data import we developed the AutoLoader, an extendable, user-friendly interface based on BioFormats [bioFormats] and BigDataViewer (**Supplementary Note 1)** [bdv]. The AutoLoader automatically imports most formats that can be distributed over many files and extracts parameters relevant for reconstruction such as illumination directions, sample rotation, and approximate image positions whenever possible. Alternatively, image positions can be interactively defined using regular grids (**Supplementary Fig. 3**). BigStitcher accesses image data through memory-cached, virtual loading [imglib2] and offers virtual flatfield correction (**Supplementary Fig. 4** and **Supplementary Note 2**). BigStitcher works most efficiently when data is stored in a multiresolution, blocked, compressed format that enables interactive visualization, processing and interaction with terabyte-sized image datasets. The AutoLoader therefore supports resaving standard, single-block images into the BigDataViewer HDF5 format [bdv].

Although cleared samples are highly transparent (**Fig. 1b**), light scattering becomes an issue when imaging centimeters deep into fixed tissue (**Fig. 1c**). Therefore, dual-sided lightsheet illumination is able to significantly increase the sample size for which high resolution image data can be collected laterally (**Fig. 1d**). However, it usually requires to image each 3d image tile twice using left-sided and right-sided illumination. Since most tiles only hold useful information from either direction, the best illumination direction needs to be selected for each image tile. To address this problem, we estimate image sharpness at the lowest pre-computed resolution level to automatically suggest the best illumination direction (**Fig. 1d** and **Online Methods** and **Supplementary Fig. 2**).

To compute the location of each image tile we developed an optimized image stitching algorithm. It is tailored for very large datasets and can deal with acquisitions that contain empty tiles, are arranged in a non-regular grid (**Fig. 2a**), and that consist of multiple independent samples. The algorithm first computes all distances between pairs of overlapping image tiles, followed by outlier removal to identify wrong pairwise overlaps, and a globally optimal final determination of tile positions.

Acquisitions typically consist of tens to hundreds of image tiles, each of them many gigabytes in size and often showing very different information content (**Fig. 2**). We therefore compute the pairwise overlap using the parameter-free Fourier-based Phase Correlation Method [Kuglin]. It computes all possible shifts between two images at once and intensity peaks in the resulting Phase Correlation Matrix correspond to shifts between images with high correlation (**Supplementary Note 3** and **Supplementary Fig. 5**). To accommodate large image sizes, we support computing the Phase Correlation Matrix on precomputed, downsampled images while localizing high-correlation peaks with subpixel precision using a three-dimensional quadratic fit [sift]. Using simulations, we show that computing times can be reduced about 100-fold while still achieving pairwise alignment errors below 1 pixel (**Supplementary Fig. 6-9**). All computed shifts between pairs of images (*links*) can be filtered by minimum correlation and distance from metadata defined positions. Interactive display of remaining links allows fine-tuning of parameters and optionally manual removal of potentially wrong links (**Supplementary Fig. 10**).

To compute the final position of each image tile we developed a new optimization algorithm. It is based on identifying tile positions that globally minimize the distance between all links [bioinf, saalfeld]. Compared to computing a minimum spanning tree [terastitcher] this concept has the advantage that the small, normally distributed errors of the links (**Supplementary Fig. 9**) are averaged out during the optimization process since each image is typically linked to many neighbors (**Fig. 2a**). We detect potentially incorrect links that prevent a coherent placement of image tiles by iteratively removing the link that disagrees most with the result of the global optimization using a new compound metric (**Online Methods**). A common problem in image registration is the handling of images for whom no link could be computed (e.g. empty images), and with that the as-correct-as-possible placement of independent objects contained in an acquisition (**Supplementary Fig. 11**). Typically, not connected image tiles are simply dropped or ignored leading to incorrect results [bioinf]. Only for regular, two-dimensional, translation-only grids a solution has been proposed [mist]. Here, we present a generic solution to the problem by introducing the concept of *strong* and *weak* links that is independent of the tile arrangement and the transformation models used. Strong links are defined by computed and confirmed image overlaps, while weak links are derived from approximately known image positions (e.g. metadata). We first identify groups of images that are connected by strong links and compute their positions relative to each other for each group. Image transformations within these groups are then fixed and a final position of all tiles is computed by minimizing the distance between all weak links (**Fig. 2a** and **Online Methods** and **Supplementary Fig. 11** and **Supplementary Note 4**).

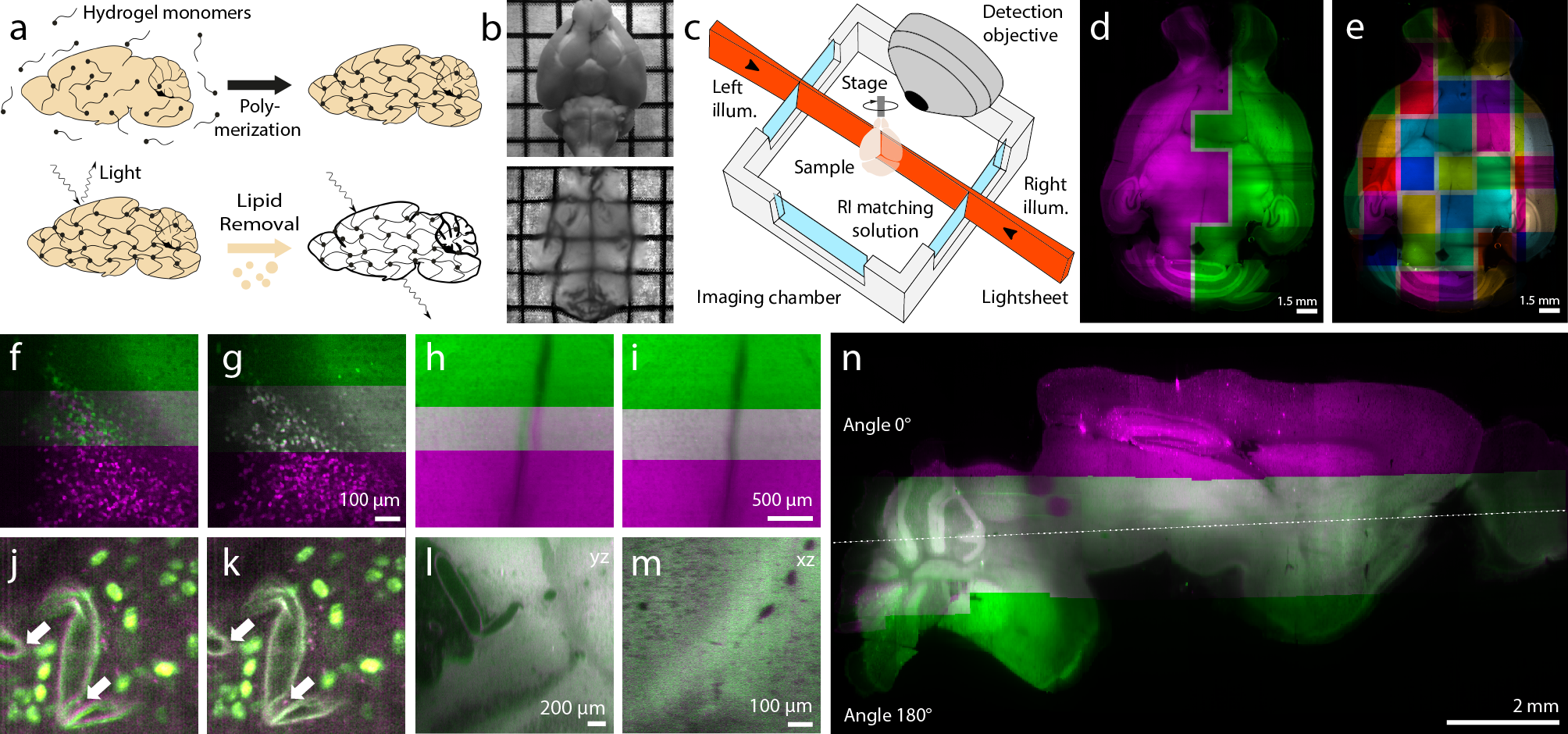
To estimate and correct for various forms of disturbances such as sample-induced light refraction, wavelength-dependent aberrations, as well as remaining small alignment we support an optional, easy-to-use interest point based alignment step supporting affine transformations. We automatically extract interest points and apply a variation of the iterative closest point algorithm [icp] combined with our new global optimization algorithm. This allows to correct for smaller rigid or affine distortions and even major effects of chromatic aberration if auto fluorescence levels are high enough (**Fig. 1i,j** and **Online Methods** and **Supplementary Fig. 12**).

Since emitted light is distorted by the sample, the maximum imaging depth is limited. To overcome this problem, we rotate samples and acquire them from opposing directions (**Fig. 1c**). We implemented a new multiview alignment algorithm that is able to efficiently register large multi-tile *views*, where each *view* consists of a large set of aligned image tiles from one physical orientation. We first segment interest points in virtually fused, downsampled images of each multi-tile view and identify corresponding interest points using an optimization of geometric hashing-based matching (**Online Methods**) [beads]. It improves matching speed drastically and robustly aligns these large volumes, effectively doubling the imaging depth of any sample (**Fig. 1c** and **Tab. 1** and **Online Methods**).

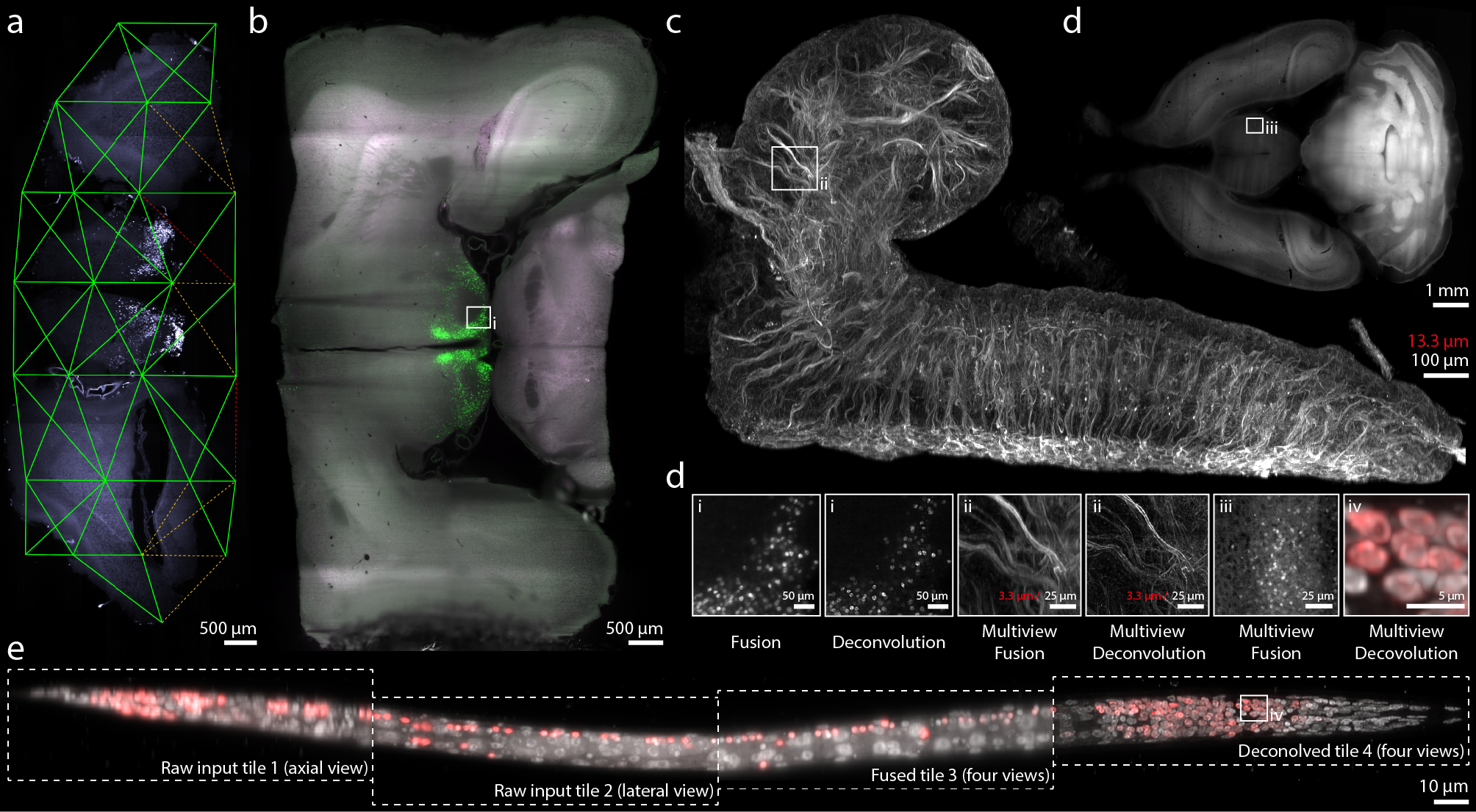
For downstream analysis of the data, BDV plugins can be used directly on the reconstructed dataset [mamut]. Fusion of the data into 3d images is enabled by automatically or manually defining bounding boxes on the dataset, which can comprise regions of interest or the entire dataset (**Supplementary Fig. 13**). We implemented an algorithm that allows fusion of bounding boxes at full resolution or downsampled in real-time by combining multithreaded fusion of the currently visible plane with blockwise multi-resolution loading (**Online Methods**). It enables fusion of terabyte-sized images even on machines with very little memory (**Supplementary Fig. 14**), while increased memory and compute power leads to very fast processing (**Tab. 1**).

Deconvolution is an established method to increase contrast and resolution in light microscopy acquisitions [rich & lucy]. Required point spread functions (PSF) are typically measured using fluorescent beads embedded with the sample [verveer], which is, however, challenging due to the complex clearing protocol. We therefore developed an embedding protocol for fluorescent beads in polymerization solution that enables measurement of realistic PSFs (materials). We extend existing deconvolution code to handle multi-tile acquisitions [mvdecon] enabling BigStitcher to perform deconvolution on selected bounding boxes and show that imaging quality can be significantly improved (**Fig. 2b-d**).

BigStitcher is a powerful software package that enables efficient and automatic processing of large cleared samples that can range up to many terabytes in size. It addresses major unsolved issues such as easy import, managing of very large images and datasets acquired in a non-regular grid (**Fig. 2a**), globally optimal alignment of sparse datasets, illumination selection, multiview alignment of multitile acquisitions, PSF extraction, and interactive fusion. The aligned dataset and all intermediate steps are interactively displayed. The user has the option to verify and interact with the alignment process at any time to confirm and potentially guide proper alignment of complicated datasets (**Supplementary Fig. 3,10,15,16**). Automatic reconstruction of even large datasets can be achieved within the matter of tens of minutes and BigStitcher clearly outperforms existing software in terms of performance, functionality, and user-interaction (**Tab. 1**) [tera, bioinf, xuv]. While BigStitcher was developed for cleared samples, it supports two-dimensional acquisitions, standard confocal and widefield image datasets, expansion microscopy samples (**Fig. 2c,d** and **Supplementary Fig. 17**) and any tiled, multiview lightsheet acquisition (**Fig. 2e**).It supports ImageJ Macro recording for most of its functionality and can thus easily be automated (**Online Methods**). BigStitcher is implemented in ImgLib2 [imglib2], open-source and provided as a Fiji [fiji] plugin with comprehensive documentation (<http://imagej.net/BigStitcher>). These properties make the BigStitcher a powerful and scalable tool for the reconstruction of tiled, high resolution image datasets acquired by new light microscopy technologies.



**Figure 1** | BigStitcher Principles. **(a)** schematic of the CLARITY sample clearing process. **(b)** adult mouse brain before (up) and after (down) clearing. **(c)** layout the type of lightsheet microscope used for acquisition. **(d)** single slice through an entire adult mouse brain acquired with dual-sided illumination. Pink (left illum.) and green (right illum.) image tiles highlight the illumination direction that was automatically selected for each tile. **(e)** overview of an entire section of an acquired adult mouse brain, different colors highlight individual image tiles (each 1920x1920x770 pixels). **(f-i)** illustration of the result of the image stitching from a BsxH2BeGFP brain using phase correlation before (f,h) and after stitching (g,i). **(j,k)** visualizes the effect of ICP refinement on two different channels with sufficient autofluorescence visible in both channels. Arrows highlight significant difference before (j) and after (k) refinement. **(l,m)** shows the quality of the multiview reconstruction for two overlapping multi-tile views at 0 (magenta) and 180 (green) degrees for an axial vs. axial (l) and lateral vs. axial (m) view. **(n)** Shows one slice through an entire adult mouse brain (2.24TB raw data), both views are shown in axial orientation looking along the rotation axis of the microscope. The dotted line illustrates the middle of the section.

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**Figure 2** | Reconstructed samples. **(a)** One slice through an acquisition of an adult mouse BsxH2BGFP coronal slice encompassing the hypothalamus. Green lines indicate strong links between overlapping image tiles, dotted orange lines refer to links rejected because of low correlation, and red lines illustrate links that were determined to be inconcise. **(b)** One slice through an adult mouse brain expressing an H2B-GFP lineage tracing marker in BSX neurons, the box is highlighted in (d). **(c)** Maximum intensity projection of a central part of a 7.5-times expanded Drosophila 2nd instar larva stained with an Actin-dye, boxes are magnified in (d). **(d)** Zoom-ins to specific areas of (b,c) illustrating (sub-)cellular resolution and the advantage of (multiview) deconvolution over (multiview) fusion. **(e)** Fixed C. elegans dauer acquired in four tiles with four views each expressing mCherry in all neurons and stained with Hoechst. Boxes highlight the quality of axial and lateral raw input data, multiview fusion, and multiview deconvolution.

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| **Table 1 | Comparison of BigSticher features and performance against other available stitching programs.** | | | | | | |
| Data Size | 130 Mb / 63 Gb / 300 Gb | 130 Mb / 63 Gb / 300 Gb | 130 Mb / 63 Gb / 300 Gb | 130 Mb / 63 Gb / 300 Gb | 130 Mb / 63 Gb / 300 Gb | 1.67 Tb |
| Software | Illumination Selection | Stitching | ICP refine | Fusion | Virtual Fusion | MultiView Reconstruction |
| BigStitcher | <1 s / 5 s / 13 s | 1 s / 5 min / 32 min | 12 s / 2.5min / 3.15 min | <1 s / 1.17 min / 4 min | <1 s / <1 s / 2 s | 6 min |
| TeraStitcher | **X** | 7.6 s / 29 min / 2.15 h | **X** | 5s / 13 min / 2 h | **X** | **X** |
| ImageJ Stitcher | **X** | 7 s / 57 min / 6 h | **X** | 5s /1.6 h / 45.6 h | **X** | **X** |
| Xuv Tools | **X** | 2 s / n.a / n.a | **X** | 10 s / n.a / n.a | **X** | **X** |
| **Table 2 | BigStitcher benchmark for processing a sample terabyte-sized dataset** | | | | | | |
| Data Size | 1.67 Tb | | | | | |
| Software | Illumination Selection | Stitching | ICP refine | Fusion | Virtual Fusion | MultiView Reconstruction |
| BigStitcher | 96 s | 1h 15 min 22 s | 8 min 37 s | 1h 7 min 52 s | 40 s | 6 min |

**Table 1 & 2 |** Comparison of BigSticher features and performance against other available stitching programs. All benchmarks were performed on a HP Z840 workstation running Windows 10 Pro with two Intel Xenon E5-2667 CPUs (3.20 GHz) and 512 GB of DDR3 memory on-board. The latest stable version of each stitching program was used. BigStitcher dataset were stitched using a 4x4x1 downsampling, while fused images were generated 8x downsampled.

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**Methods**

*Animals*

This study used a previously generated BsxH2BeGFP mouse line [sakkou], where the exon 1 of the bsx gene is replaced starting at the ATG with the coding sequence for histone2B eGFP. Brains from 10-week old female BSXH2BeGFP/+ mice were used for tissue clearing and imaging. Experiments were conducted according to institutional guidelines of the Max Delbrueck Center for Molecular Medicine in the Helmholtz Association after approval from the Berlin State Office for Health and Social Affairs (LAGeSo, Landesamt für Gesundheit und Soziales, Berlin, Germany).

*Clearing*

Tissue clearing was performed using the CLARITY protocol [chung]. Mice were deeply anesthetized by intraperitoneal injection of 100 mg/kg Ketamine and 15 mg/kg Xylazine. Mice were exsanguinated by transcardial perfusion with 25 ml cold PBS followed by whole body perfusion with 25 ml cold monomer solution (4 % v/v acrylamide, 4 % w/v Paraformaldehyde (PFA), 0.25 % w/v VA-044 in PBS). The brains were collected and fixed in monomer solution for further 2 days. Next, the whole brains were placed in fresh monomer solution and oxygen was removed from the tubes by vacuum and flushing the tube with nitrogen gas for 15 minutes. The samples were then polymerized by placing the tubes in a 37 °C water bath under gentle shaking for 2 hours. Polymerized brains were placed in clearing solution (4% SDS in 200 mM Boric acid). Brains were incubated in clearing solution for 1 week at 37 °C with daily solution change. Then, the brains were actively cleared for 24 hours using the X-Clarity setup from Logos Bioscience for 24 hours with a current of 1 A at 37 °C. Cleared brains were washed twice overnight with 0.1 % v/v Triton X-100 in PBS and once with PBS. Before imaging, brains were placed overnight in FocusClear for refractive index matching.

*Imaging*

Cleared brains were acquired using the Zeiss lightsheet Z1 microscope. The samples were fixed using a cyanoacrylate-based glue on the sample adapter. Mounted samples were placed a FocusClear pre-filled imaging chamber. Images were acquired using the EC Plan-NEOFLUAR 5×/NA 0.16 objective together with the LSFM 5x/NA 0.1 illumination objectives. The data was acquired using dual side illumination and from different angles. Images were collected with two 1920 X 1920 pixels sCMOS cameras and stored in the Zeiss CZI file format.

*Bead Embedding and PSF Extraction*

In lightsheet microscopy measured PSFs often differ significantly from measured ones due to variable precision of lightsheet alignment in every experiment. Therefore, lightsheet deconvolution relies on the extraction of PSF’s from the actual experiment [Verveer, mvdecon] and we developed a protocol for bead-based PSF estimation in cleared samples. Estapor Fluorescent Microspheres (F-XC 030) were diluted 1:20000 in monomer solution containing bis-acrylamide (0,05 % v/v bis-acrylamide, 4 % v/v acrylamide, 4 % w/v Paraformaldehyde (PFA), 0.25 % w/v VA-044 in PBS). The monomer solution was polymerised under constant vacuum and shaking at 37 °C for 2 hours.  The formed hydrogel was incubated in FocusClear overnight and imaged using the Zeiss lightsheet Z1 microscope with the same experimental settings used to acquire previous samples. The PSF was extracted by detecting interest points in the acquired bead images. Potential bead aggregates were excluded by manual removal on the maximum intensity projection using the BigStitcher module “Manage Interest Points > Remove Interactively”.

*Data processing pipeline*

All data shown in this paper was processed using the BigStitcher Fiji plugin. Zeiss CZI files and TIFF files exported by custom microscopes were imported using the AutoLoader and converted it to the HDF5 format. For Zeiss CZI file approximate tile positions and rotation angles were imported automatically, for other files is was specified by hand using BigStitcher tools (**Supplementary Fig. 3, 16**). For each tile the best illumination was selected. Tiles were aligned using the phase correlation method together with two-round global optimization, followed by ICP refinement. Interest point detection for each multi-tile angle was performed. The fast descriptor-based rotation invariant algorithm was used to register the interest points of each angle, followed by another round of ICP refinement of all image tiles of the acquisition. Fused and deconvolved images were exported as TIFF files.

*Illumination selection*

When imaging large samples with multiple illumination directions, a lot of unnecessary images are acquired since typically, only illumination from one direction provides optimal images. We therefore implemented a simple *illumination selection* functionality in BigStitcher. It starts by *combining* all (selected) images by their Illumination attribute, i.e. it groups images that share all other attributes besides Illumination. In each of the resulting groups we select a best image. We do this by loading the pixel data for all images in the group at the lowest resolution level (in the case of non-multiresolution images, this corresponds to the original image) and calculating a *quality metric*. We currently offer mean intensity and mean gradient magnitude as quality metrics. The image with the highest score is kept, while all other images are marked as *missing* in the SpimData, which will lead to them being ignored in subsequent processing steps. Prior to applying automatic illumination estimation, the user has the option to verify and potentially change the result.

*Downsampling and Simulations*

To assess the effect of downsampling on the pairwise stitching we use simulations of spheroid-like objects at different signal-to-noise ratios (SNRs) as ground truth. We create realistic images by mimicking image creation in lightsheet microscopy including optical sectioning, 3-fold anisotropy, light attenuation, convolution, and pixel intensity generation using Poisson processes [mvdecon]. Importantly, pairs of overlapping images that we use for benchmarking the subpixel phase correlation method are created using different Poisson processes and are additionally rendered with half a pixel offset of the full resolution images to avoid nearly identical overlaps at high SNRs due to the simulation process (**Supplementary Fig. 6**). We simulate 500 pairwise overlaps each at SNRs ranging from 1 to 32, and lateral downsamplings ranging from 1x and 8x where axial downsampling is matched as good as possible to achieve near-isotropic resolution as in the actual software. We illustrate that across SNRs downsampled images yield a constant registration quality, which even exceed that of registration at full resolution for low SNRs. This is achieved through a combination of the smoothing effect during downsampling (**Supplementary Fig. 6**) and precise subpixel-localization (**Supplementary Fig. 7-9**). Constant registration quality with an average error of below one pixel can be computed at a fraction of the computing time of full resolution, typically 32 - 256 times faster. Existing outliers are filtered during the global optimization and overall registration quality can further be improved during the ICP refinement step.

*Iterative Closest Point Refinement*

Although the Phase Correlation based image stitching is quite precise, small errors can remain due to its inaccuracy (**Supplementary Fig. 7-9**) or because of non-translational effects such as chromatic aberration or sample-induced light refraction. These effects can be better approximated using affine transformations. We therefore automatically detect interest points and run an Iterative Closest Point algorithm [icp] for each overlapping pair of images, where the assignment of correspondences is limited by a distance threshold. We use the identified corresponding points of all pairwise links and compute a globally optimal affine transformation for each tile using our new global optimization algorithm. To avoid scaling of datasets, we regularize the affine transformation using a rigid transformation [saalfeld]. The resulting alignment usually improves the alignment quality and can be applied to multichannel alignment if sufficient autofluorescent signal is available (**Supplementary Fig. 12**).

*Geometric Local Descriptor Matching*

To identify corresponding interest points in between two point clouds, geometric local descriptor matching has been proven to be a powerful technique [beads, mfm]. The basic idea to express each interest point as a geometric constellation using its *n* nearest neighboring (typically three) interest points. The minimal vector difference between two descriptors then describes how similar the local area of two points is. A geometric local descriptor (GLD) is assumed to be a correspondence candidate it is at least *m* times (typically one to ten) more similar than second most similar GLD [sift]. True corresponding interest points between two point clouds are finally identified using the random sample consensus algorithm [ransac] on an affine transformation model. However, fast GLD matching using the rotation-invariant technique based on geometric hashing [beads] requires relatively randomly oriented points to robustly identify correspondences, while the non-accelerated, redundant, translation-invariant counterpart [mfm] identifies correspondences reliably in non-rotated point clouds of up to 1000 points in reasonable time. Here, we extended both techniques to better suit the requirements when attempting to identify corresponding interest point in between clouds derived from imaged structures that are potentially rotated relative to each other.

Redundancy is a powerful mechanism for GLD matching. It uses additional nearest neighbors but excludes some of them sequentially during matching making it more robust to potentially misdetected interest points [mfm]. We therefore extend the fast rotation-invariant technique based on geometric hashing [beads] with the capability for redundancy. This significantly increases the chance of being able to align randomly oriented point clouds very fast, albeit at low inlier ratios (ratio of true correspondences to total number of correspondence candidates).

Rotation invariance is not desired if both point clouds are known to be approximately in same orientation, for example if the rotation of the sample performed by the microscope was known and has been applied to the dataset. Checking for potential rotations simply increases the chance for wrong correspondence candidates. We therefore implemented a fast translation-invariant GLD based on geometric hashing that supports redundancy. All four versions of GLD is available in BigStitcher to enable robust multiview alignment.

*Virtual Image Fusion*

A set of overlapping, transformed image tiles is fused into one output image using a per-pixel weighted average that minimizes boundary artefacts and can increase contrast by incorporating entropy estimation (**Supplementary Note 6**) [beads]. The memory requirements for the fusion of large volumes can easily exceed the available RAM on a machine due to the size of the output and the combined size of the input images. We therefore developed a framework based on ImgLib2 *RandomAcessibleIntervals* [imglib2], intensity transformations and coordinate transformations that virtually fuses all pixels of a defined bounding box using all input images and their associated weights. Since the input images are provided through virtual image loading, the size of a virtually fused image is close to zero, irrespective of the size of input and output images. Ideally, input images are available in blocks so that affine transformations that slice input images in arbitrary orientations do not require to load the entire image [bdv]. The output image can now be rendered on a pixel-by-pixel basis with minimal memory requirements. Additional caching of the input image and the output images allows an efficient multithreaded fusion for as-fast-as-possible processing given the available memory. Therefore, more RAM will effectively speed up the fusion process (**Tab. 1**), but even machines with very low RAM will be able to fuse even very terabyte-sized volumes. Images can be saved by choosing cached or virtual fusion and subsequently saving the ImageJ virtual stack using “Save as image sequence…”.

Downsampling of the output can easily be incorporated by scaling the bounding box and pre-concatenation of the downsampling transformation with each image transformation. If the input files are multi-resolution, we automatically compute the optimal resolution level at which the input needs to be loaded. To optionally further reduce the image size of the fused image, the GUI offers to conserve the original anisotropy between lateral and axial of the acquired sample, which is a sensible choice if the dataset contains a single or opposing (e.g. 0 and 180 degrees) multi-tile views.

*Deconvolution*

In addition to image fusion, we offer deconvolution of the fused volume using a MultiView formulation of the iterative Richardson-Lucy deconvolution algorithm [rich & lucy] with Tikhonov regularization and various optimizations [mvdecon]. The PSFs required for deconvolution can be extracted from interest points detected in the images (e.g. when subdiffraction fluorescent beads were incorporated with the sample) or supplied as TIFF-stacks by the user. We offer GPU acceleration of the deconvolution on CUDA-capable Nvidia GPUs.

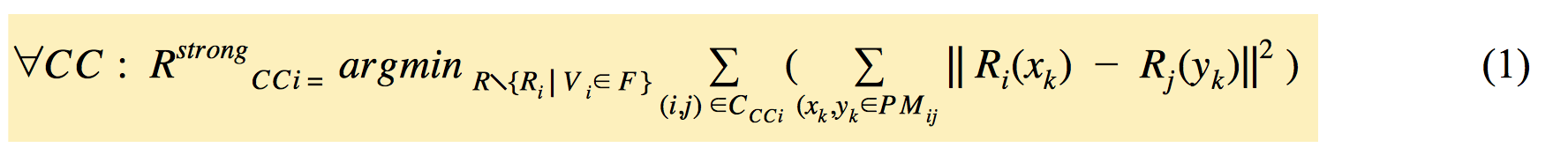
To allow deconvolution of multi-tile views, we extended the original deconvolution [mvdecon] to be based on the *virtual fusion*. Thereby, any number of input image tiles are virtually fused and serve as one of input views for the multiview deconvolution. Proper multiview deconvolution of partly overlapping samples requires sophisticated weight normalization in between views [mvdecon], which we implemented to be computed virtually. Since also the input views are virtually loaded, the memory requirement of the deconvolution solely depends on the output image size and thus shows a significantly increased memory-efficiency. All virtual inputs and weights are additionally cached, ensuring highest-possible processing performance for systems with large amounts of RAM.

*Macro automation and headless operation*

In addition to the graphical user interface (GUI), we offer ImageJ Plugins for most of the individual steps, such as data import, illumination selection, pairwise shift calculation, link filtering, global optimization and image fusion/deconvolution. The results will not be displayed interactively but saved to the XML project file or output files immediately. The individual steps can be recorded as ImageJ *macros* and easily combined into a script for headless batch processing [schmied].

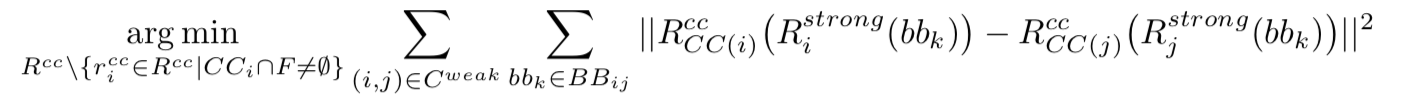
*Global optimization*

We calculate the final image registrations using an algorithm for globally optimal registration by iterative minimization of square displacement of point correspondences (**Supplementary Note 4**) [bioinf, saalfeld]. We express pairwise shifts as point correspondences between the bounding box vertices of the overlap region of the images and the same points transformed by the inverse pairwise shift. We globally optimize the registrations R of all connected components CC of the link graph of images V and (strong) links C, given point correspondences PM and fixed images F by minimizing:



In some cases, erroneous pairwise links might not have been filtered out, e.g. due to low cross-correlation, repetitive patterns, or a low number of correspondences in the ICP refinement. This leads to persistently high registration errors after global optimization, which manifests in a large distance error, i.e. the difference between the individually computed distance between of images (link) and the actual distance between them after global optimization. Iterative removal of the link with the highest highest distance error from the link graph and repeating the global optimization leads to convergence to user-defined thresholds [bioinf]. We extend this concept to affine transformations, introduce a new heuristic that additionally incorporates link quality and implement it in an extendable framework required for the two-round global optimization (**Supplementary Note 4**).

If the dataset contains multiple disconnected objects with image tiles that do not have links between them (e.g. empty images), the final transformations will not be propagated between them (sup-fig-globalopt b). We therefore developed a two-round global optimization that is capable of aligning independent connected components of the link graph using weak links defined by pre-existing transformations (e.g. approximate locations from metadata or manual alignments). We therefore use the corners of the bounding box of their overlap region, transformed using the results Rstrong of the first round (eq. 1), as point correspondences. The between-component transformations can then be determined by minimizing:

(2)

The final transformations are given by concatenating the in-component and between-component registrations. Using the two-round strategy, registrations are propagated between connected components and distances between neighboring objects are preserved as-well-as-possible (**Supplementary Note 6** and **Supplementary Fig. 11b**).

Our global optimization is agnostic to the nature of the point correspondences and transformation model, which allows us to use the same algorithm for translation-based alignment of for example tiled datasets using phase correlation, as well as affine registrations of multi-tile multi-view datasets based on ICP refinement or geometric descriptor matching.

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