**BigStitcher: Reconstructing high-resolution image datasets of cleared and expanded samples**

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**Abstract** (70 words)

**New methods for clearing and expansion of biological objects create large, transparent samples that can be rapidly imaged using light-sheet microscopy. Resulting terabyte-sized image acquisitions consist of many large, unaligned image tiles that suffer from optical aberrations. We developed the BigStitcher software that efficiently handles and reconstructs large multi-tile, multi-view acquisitions compensating all major geometric transformations introduced by optical effects, thereby making single-cell resolved whole-organ datasets amenable to biological studies.**

**Main Text** (1749 words)

Sample clearing1 and expansion microscopy (ExM)2 are powerful protocols that create large, transparent volumes of whole tissues and organisms (**Fig. 1a,b**). Using light-sheet microscopy (**Fig. 1c**) 3-5, these samples can be imaged with subcellular resolution in their entirety within a few hours6. These acquisitions have the potential to be powerful tools for whole-tissue and whole-organism studies since they preserve endogenous fluorescent proteins (**Supplementary Fig. 1**) and are compatible with most staining methods.

However, raw data acquired by the microscope is not directly suitable for visualization and analysis. Many large, overlapping three-dimensional (3d) image tiles are collected that amount to many terabytes in size and require image alignment (**Fig. 1d-n**). Due to sample-induced refraction and scattering of the light-sheet in the direction of illumination7, 3d image tiles are typically acquired twice with alternating illumination from opposing directions to achieve full coverage (**Fig. 1d, 2** 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, overlapping images suffer from spherical and chromatic aberrations (**Supplementary Fig. 3,4**). To reconstruct and make these complex datasets easily accessible to biologists and computer scientists, we developed the BigStitcher software. It enables interactive visualization using BigDataViewer8, fast and precise alignment, quality estimation, real-time fusion, deconvolution, as well as support for alignment of multi-tile acquisition taken from different physical orientations, so-called multi-tile *views*, thereby effectively doubling the size of specimens that can be imaged (**Fig. 1n**), while further orthogonal views can render the resolution isotropic.

BigStitcher features a new, user-friendly importer for a multitude of vendor-specific and custom formats and accesses image data through memory-cached, virtual loading10, optionally combined with virtual flatfield correction (**Supplementary Fig. 5,6** and **Supplementary Note 1,2**). Performance is optimal when images are initially converted to a multiresolution, blocked, compressed format such as HDF58 enabling interactive visualization, processing and interaction with terabyte-sized image datasets.

Accurate reconstruction of these large, complex datasets requires the compensation for different types of image and intensity transformations introduced by the acquisition process. We therefore developed an interactive, stepwise process that compensates for all relevant transformations while providing spatially localized feedback on the quality of the acquired image data (**Supplementary Fig. 7,8** and **Online Methods)**.

Firstly, overlapping 3d image tiles are acquired to cover the entire sample at each acquisition angle. Although approximate tile locations are typically known, translation stages usually show significant inaccuracies (**Supplementary Fig. 9**). To compute locations for every image tile, we developed an image stitching algorithm optimized for very large datasets that can deal with acquisitions arranged in non-regular grids (**Fig. 3a**) containing empty images and multiple independent samples (**Supplementary Fig. 10**). Since acquisitions often consist of hundreds of gigabyte-sized image tiles, each showing very different information content (**Fig. 3**), we initially compute each shift between all pairs of overlapping tiles (*links*) using the parameter-free Phase Correlation (PC) method11,13,14 on downsampled images. It computes all possible shifts between two images and intensity peaks in the resulting PC matrix correspond to shifts with high correlation that we localize with subpixel precision (**Supplementary Note 3** and **Supplementary Fig. 11**). Using simulations, we illustrate that our new pairwise stitching method achieves errors below 1 pixel while reducing computation times 100-fold (**Supplementary Fig. 12-15**). Since correlation-based approaches can fail for image pairs characterized by repetitive patterns, noise or low information content, computing final image tile locations requires global optimization, sometimes combined with manual curation (**Supplementary Fig. 16**). Our new global optimization method extends the concept of identifying tile positions by minimizing the distance between all links11,12, which, compared to computing a minimum spanning tree15, averages out normally distributed link errors (**Supplementary Fig. 15**) during optimization as tiles are typically linked to many neighbors (**Fig. 3a**). Incorrect links are filtered by quality and by iteratively removing the link disagreeing most with the global optimization result[stitching] using a new compound metric. In current implementations, unconnected tiles (e.g. empty images) and multiple independent objects in an acquisition are handled by ignoring them11,13, or assuming regular, 2d translational grids14. Here, we present a generic solution to this problem by introducing the concept of *strong* and *weak* links (**Supplementary Fig. 10**) that is independent of the original tile arrangement and not limited to translations. Strong links are equal to confirmed links, while weak links are derived from current transformations (e.g. approximately known tile positions). Optimizing both link types in an acquisition yields accurate registration results within strongly linked regions and as-good-as-possible alignments for weakly linked groups of tiles (**Fig. 3a** and **Supplementary Fig. 10** and **Supplementary Note 4**). However, correct tile placement (i.e. solving the classical stitching problem) represents the first step and is usually not sufficient to properly align such datasets (**Fig. 2**).

Secondly, microscopy images suffer from spherical and chromatic aberrations, which can be approximated reasonably well by affine transformations if distortions are in the range of a few pixels (**Supplementary Fig. 3,4**). For these cases, we implemented an easy-to-use interest-point-based alignment step that automatically extracts interest points and applies a variation of the iterative closest point algorithm (ICP)16 on affine transformations combined with our new global optimization to compensate small affine distortions that arise from spherical, and also chromatic aberrations if the same autofluorescent structures are visible across channels (**Fig. 1i,j** and **Supplementary Fig. 3,4,9** and **Online Methods/Limitations**).

Thirdly, although samples are highly transparent (**Fig. 1b**) light scattering is an issue when imaging centimeters deep into fixed tissue. Although improved designs were recently proposed17, dual-sided light-sheet illumination (**Fig. 1c,d**) remains the most prominent method to double the sample size for which high resolution image data can be collected laterally. Prior to alignment, we automatically suggest the best illumination direction for each tile by estimating image sharpness (**Fig. 1d**) using newly development methods (**Supplementary Fig. 2 and Online Methods**). Unexpectedly, we observed non-rigid image deformations in-between image tiles of different illumination directions (**Fig. 1d,2a**). To understand how these transformations are created, we performed simulations of light propagation in tissue using raytracing (**Fig 2b-e** and **Online Methods**). They show that refractions within the illumination light path can lead to different parts of the sample being illuminated, which can both lie in focus of the same detection objective due to typical depths-of-field in the range of several tens of microns (**Supplementary Table 1**). To compensate for these transformations we implemented a virtual, non-rigid alignment method based on identified corresponding interest points as well as piece-wise, ICP-based affine alignment through virtual splitting of image tiles into smaller blocks (**Online Methods & Fig. 2f-m**). Depending on the magnitude of refraction, affine, split-affine or non-rigid is the right choice for precise alignment (**Fig. 2g,h,m**). While the lightsheet remains within focus, images can be aligned. However, once the lightsheet is out-of-focus, blurred image data is acquired that cannot be reconstructed using BigStitcher. Such first-order defocusing can, however, be minimized by employing autofocusing during the acquisition process18.

Finally, since emitted light is distorted by the sample, maximum imaging depth is limited. To overcome this problem, we acquire samples from opposing directions by rotation (**Fig. 1c**) or by simultaneous acquisition with two objectives4. We therefore developed an optimized method for registration of large multi-tile *views*, where each *view* consists of a set of aligned image tiles from one physical orientation (**Online Methods**). It robustly aligns large volumes using affine transformations, effectively doubling the imaging depth of any sample (**Fig. 1n** and **Supplementary** **Table 2**). Subsequently applying ICP-based non-rigid, split affine, or affine registration allows precise *multiview* alignment that accounts for additional light refraction in the excitation light path. Using example data, we quantify theoretically possible and practically achievable registration performance (**Fig. 2ghm**, **Supplementary Fig. 3,4,17** and **Online Methods**), which illustrate that translations alone are not sufficient to achieve high-quality image reconstructions.

As image quality is not constant across the sample it needs to be quantified to ensure that every part of the reconstructed dataset was acquired with high-quality. However, manual inspection at the highest resolution for the entire sample is impossible due to its size. We therefore developed the relative Fourier Ring Correlation (rFRC) that automatically and rapidly estimates image quality throughout terabyte-sized acquisitions while accounting for common sCMOS camera patterns (**Supplementary Fig. 1,7,8 & Online Methods**).

For downstream analysis datasets can be fused or directly analyzed using BigDataViewer plugins. We implemented a new algorithm for real-time fusion by multithreaded processing of the currently visible plane in virtual images using blockwise multi-resolution loading, which can optionally be performed downsampled and on regions of the sample (**Supplementary Fig. 18**) while supporting brightness-equalization. It enables fusion of terabyte-sized images on machines with little memory (**Supplementary Fig. 19**), while increased memory and compute power enables fast processing (**Supplementary Table 2**).

Deconvolution is an established method to increase contrast and resolution in light microscopy acquisitions and required point spread functions (PSF) are typically estimated using fluorescent beads19. To handle multi-tile, multi-view acquisitions, we extended deconvolution code19 allowing BigStitcher to deconvolve selected regions and improve image quality (**Fig. 3b-e**). To estimate required PSFs in cleared samples we developed a new protocol for embedding fluorescent beads in polymerization solution (**Fig. 3b,e** and **Online Methods**). We furthermore combine ExM with IsoView light-sheet microscopy4 allowing acquisition of multi-view, multi-tile datasets of expanded tissues enabling reconstruction of entire *Drosophila* larval nervous systems with spatially isotropic subcellular resolution (**Fig. 3c,e**).

BigStitcher enables efficient and automatic processing of terabyte-sized datasets and addresses major unsolved issues such as easy import, managing of large images, datasets acquired in a non-regular grid, globally optimal alignment of sparse datasets, illumination selection, rigid and non-rigid multi-view alignment of multi-tile acquisitions, PSF extraction, quality estimation and interactive fusion. The aligned dataset and intermediate steps are interactively displayed, enabling the user to verify and interact with the alignment process to confirm and potentially guide proper alignment of complicated datasets (**Supplementary Fig. 5,16,20,21**). Automatic reconstruction of even large datasets can be achieved within tens of minutes and BigStitcher outperforms existing software in terms of functionality, user-interaction, and performance (**Supplementary Tab. 2**)11,14,15. BigStitcher supports cleared samples (**Fig. 3a,b)**, ExM samples (**Fig. 3c,e** and **Supplementary Fig. 22**), standard 2D and 3D confocal and widefield acquisitions, as well as tiled, multi-view light-sheet acquisitions (**Fig. 3f**). BigStitcher is implemented in ImgLib210, open-source and provided as a Fiji20 plugin with comprehensive documentation (<http://imagej.net/BigStitcher>). It is compatible with the ImageJ Macro language for most of its functionality and can thus easily be automated. These properties make BigStitcher a powerful and scalable tool for the handling and reconstruction of tiled, high resolution image datasets acquired by new light microscopy technologies.

**Online Methods**

*Animals*

For clearing we used a previously generated BsxH2BeGFP mouse line21, 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. *C. elegans* dauer larvae expressing tagRFP fused to a nuclear localizing sequence under the pan-neuronal rab-3 promotor in all neuron nuclei22 were obtained by selecting dauer larvae in 1% SDS for 30 minutes23. Dauer larvae were fixed with 4% PFA for 30 minutes on ice, placed in 70% Ethanol overnight at 4°C and subsequently stained with DAPI. Experiments were conducted according to institutional guidelines of the Max Delbrück 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). *Drosophila* larva used for ExM were obtained from the strain w;;attp2, carrying an empty attp2 landing site24.

*Clearing and Expansion*

Tissue clearing was performed using the CLARITY protocol1. 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 2 more 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 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.

For ExM, the nervous system of a 1st instar *Drosophila* larva of was extracted, fixed in 4% PFA/1xPBS/0.1%Triton for 1 hour and washed 2x10 min in 1xPBS/0.1% Triton. Before each antibody usage, the nervous system and the antibodies were blocked in 5% goat serum/1xPBS/0.1%Triton for one hour. Following the blocking, the nervous system was incubated overnight at 4°C in 1:500 monoclonal Anti-α-Tubulin antibody produced in mouse (Sigma Aldrich T6199 1mg/ml). After 5x10 min washing (1xPBS/0.1%Triton), the secondary antibody 1:250 Anti-Mouse CF™488A antibody produced in goat (Sigma Aldrich AB4600387 2mg/ml) was added overnight at 4°C. The stained brain was washed in 1x PBS and then processed using an Expansion Microscopy (ExM) method in which the gel recipe and procedure was modified to achieve 7,5-fold expansion in each dimension (**Supplementary Note 8**). In summary, the specimen was treated with acryloyl-X as in standard ExM and embedded using a gel recipe modified from the original method2. The modified recipe uses a reduced cross-linker concentration to achieve greater expansion. After digestion with proteinase K as in the original method, a re-embedding step toughens up the gel, which would otherwise have poor mechanical properties.

*Imaging*

3D images of cleared mouse brains were imaged using the Zeiss Lightsheet Z.1 microscope. Each sample was attached to the sample holder using a cyanoacrylate-based glue. The mounted sample was placed in the 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 on a Zeiss Light-sheet Z.1. The data was acquired using dual side illumination and from different angles. Images were collected with two 1920 X 1920 pixel sCMOS cameras and stored in the Zeiss CZI file format. Fixed *C. elegans* dauer larvae were embedded in 1.2% agarose containing fluorescent beads and imaged using the same microscope in a water-filled sample chamber. Imaging was performed using the 20x/ NA 1.0 objective with additional 2x zoom. 3D images from a cleared and expanded central nervous system of a *Drosophila* 1st instar larva were acquired using an IsoView light-sheet microscope4 that has been modified for multi-tile acquisition. To prepare the sample for imaging, excess gel surrounding the expanded sample was removed using a scalpel, leaving four flat and smooth gel surfaces for imaging. Some extra gel was left underneath the sample for mounting, and the sample was affixed to a cylindrical post using a cyanoacrylate-based glue. The mounted sample was placed in the imaging chamber filled with deionized water. Orthogonal views for each tile of the sample were acquired sequentially by switching the illumination and detection orders in IsoView. Images were acquired using SpecialOptics 16x/NA 0.71 objectives and recorded using full frames (2048 x 2048 pixels, pixel pitch of 0.4125 μm in sample space) of Orca Flash 4.0 v2 sCMOS cameras. The sample was held stationary during multi-view acquisition of each tile, and depth-sectioned images were acquired every 0.8125 μm by translating the detection piezos over a range of 750 μm. A tile for each view thus covered a field of 832 μm (X), 832 μm (Y), and 750 μm (Z). Automated tiling across the entire sample was achieved by moving the sample in predefined steps of 600 μm in X, Y, and Z until full coverage was achieved. Bi-directional light-sheet illumination was achieved using opposing SpecialOptics objectives and the illumination NA was chosen to be 0.0315 for a confocal parameter of approximately 416 μm. The light-sheets from opposing arms were shifted approximately by their Rayleigh length (208 μm) toward the illumination objectives so that each light-sheet provided uniform coverage of the respective half in the acquired image. A summary of the most important acquisition parameters can be found in **Supplementary Table 1**.

*PSF Measurement and PSF Extraction*

In light-sheet microscopy, measured PSFs often differ significantly from simulated ones due to variable precision of light-sheet alignment in every experiment. Therefore, light-sheet deconvolution usually relies on the extraction of PSFs from the actual experiment19,25. To be able to perform PSF extraction in cleared tissue we developed a new protocol. 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 polymerized 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 Z.1 microscope with the same experimental settings used to acquire previous samples. For *C. elegans* dauer imaging fixed larvae were embedded in 1.2% agarose together with Estapor Fluorescent Microspheres (F-Z 030), diluted 1:2000. For ExM data acquired on the IsoView microscope depth-sectioned images (0.4125 μm step) of fluorescent beads (200nm diameter) embedded in 0.6% low-melting-temperature agarose were imaged using the same experimental settings as for sample imaging. For all samples, PSFs were 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 subsequently converted to the HDF5 format. For Zeiss CZI files, approximate tile positions and rotation angles were imported automatically, for other files they were specified by hand using BigStitcher tools (**Supplementary Fig. 5, 21**). For each tile, the best illumination was selected. Tiles were aligned using the phase correlation method together with two-round global optimization, followed by ICP16 refinement. Interest point detection for each multi-tile view was performed. The fast descriptor-based rotation-invariant algorithm or the descriptor-based translation-invariant algorithm after manual rotation application were used to register the interest points of each angle, followed by another round of ICP16 refinement of all image tiles of the acquisition. Fused and deconvolved images were exported as TIFF files.

*Quality Estimation based on Fourier Ring Correlation*

To estimate image quality across entire terabyte sized acquisitions, we use a derivative of the Fourier Ring Correlation (FRC)26 that is robust as well as insensitive to camera noise. *FRCr1,r2(f)* constitutes the per-spatial-frequency (*f*) correlation between two independent realizations *r1*, *r2* of the same signal. In localization-based super-resolution, point clouds are therefore typically split into two sets of independent pixels. Here, we use consecutive slices instead and take advantage of the fact that they are nearly identical due to the axial extent of the PSF. Since the result *FRCr1,r2(f)* is an entire correlation spectrum for each *z* plane, we compute a single quality value *Q(z)* by integration over all frequencies *f*

A smoother, symmetric result can be obtained by averaging the FRC spectra obtained using z planes above and below the measured plane

For computing the 2d-FRC we adapted methods from the FRC ImageJ plugin27. This results in a precise estimation of image quality, except if patterned noise (e.g. sCMOS camera noise) is the dominant source of signal (**Supplementary Fig. 1**). To overcome this instability, we compute a relative FRC (rFRC), which subtracts a *loess*-smoothed28 (locally estimated scatterplot smoothing) baseline FRC of z-planes spaced by *m* slices that are beyond the axial extent of the PSF.

This effectively measures which additional frequencies the central planes *z*, *z+1* and *z-1*, *z* have in common compared to the planes *z-m*, *z+m* that are beyond the size of the PSF. The resulting values robustly measure image quality in the sample (**Supplementary Fig. 1g**). Since image content can change drastically within a slice, we support computation using a manually defined block-size (e.g. 512x512). To estimate the quality metric for entire acquisitions, we compute for defined points in each image stack. Over all input stacks, these measurements are held as sparse representations using ImgLib2 *RealPointSampleList* that can be rendered virtually and overlaid onto entire fused volumes (**Suppl. Fig. 7,8 and Suppl. Video 8,9**).

*Illumination Selection*

When imaging large samples using sequential dual-sided illumination, typically only illumination from one direction provides good image quality (**Supplementary Fig. 2**). We therefore implemented *illumination selection* functionality in BigStitcher. We first *combine* all (selected) images by their illumination attribute and thereby group all images that share other attributes besides illumination direction. In each of the resulting groups we select the best image. As quality criteria we offer either rFRC on full resolution images or fast approximation using mean intensity or mean gradient magnitude at the lowest resolution level. While rFRC provides highest distinctive power, both fast approximations are typically sufficient for robust estimation of the higher quality illumination direction (**Supplementary Fig 2** and **Fig. 1d**). The image with the highest quality score is kept, while all other images are marked as *missing* in the dataset, which will lead to them being ignored in subsequent processing steps. Optional resaving of the dataset after this step potentially decreases storage requirement two-fold. In any case, prior to applying automatic illumination estimation, the user has the option to verify and potentially change the result.

*Simulation of light propagation in tissue using raytracing*

We observed non-rigid deformations occurring in areas where image data from opposing lightsheets is recorded by the same camera (**Fig. 2a**). While it is intuitive and clear from existing simulations of light propagation29 that imaging of the same lightsheet from opposing objectives can lead to non-rigid deformations due to different aberrations in the detection light paths, we wanted to understand how only different lightsheet directions can introduce non-rigid deformations. These effects are clearly visible in large samples like entire mouse brains (**Fig. 2a**), which are still beyond the range of simulation using reasonable efforts. We therefore developed a simple raytracing-based method to simulate light propagation, which aims at recapitulating these observations at a significantly smaller, manageable scale.

To describe the scene we use two phantom images of the same size that separately define the visible light image (corresponding to fluorescent probe distribution) and the refractive indices map (**Fig. 2b,c**). We deliberately embed the spheroid-like object of varying refractive index (Ri=1.1 – 1.21) within a dense, invisible material with high refractive index (Ri=1.1) surrounded by air (Ri=1.0) to recapitulate significant aberrations in the illumination light path using a relatively small simulation volume of 289x289x289px. The object simulations are based on the multiview-simulations package19.

We virtually scan a concave lightsheet (diameter of 1 pixel in the center, and 3 pixels at the edge) in 1-pixel steps and alternating left and right illumination through the sample (**Fig. 2d**), simulating an entire volume for each lightsheet position and direction (**Fig. 2e** and **Supplementary Video 7**). Therefore, we send 200.000 individual rays originating from random positions within the concave lightsheet through the sample for each lightsheet simulation. The initial vector of each ray points approximately along the lightsheet illumination direction and moves in 1-pixel steps through the volume. After each move we locally compute the Eigenvector of the largest Eigenvalueusing the refractive index map, which defines the normal vector of the refractive surface at the current, sub-pixel ray position. Using this estimated refraction surface, we compute the refraction angle using raytracing30 algebra, update the ray vector accordingly, and add a Gaussian distribution with an intensity defined by the visible light image to the simulation volume. For simplicity we ignore total reflection since it is mostly caused by numerical instabilities. We confirmed correct refraction of rays based on our computation of local Eigenvectors in discrete pixel-images by comparing it to refraction of rays in the corresponding continuous, parametric description of the same scene (not shown). The result of these simulations are 578 3d-volumes that recapitulate the principles of dual-sided lightsheet illumination.

Inspired by classical raytracing, we perform a simplified detection simulation and therefore invert the ray path and only modulate signal intensity as a function of distance from the focal plane. Per camera pixel (289x289) we send 500 rays at random positions within each pixel into the scene that are refracted as described above. For detection, we use the same the same refractive index map, and the result of each respective lightsheet illumination simulation serves as image data. However, since we assume an extremely high refractive index mismatch for illumination simulation to recapitulate the behavior in large samples, we assume a lower refractive index mismatch for the embedding material (Ri=1.01) to acquire reasonably distorted images. The relative refractive index mismatch within the spheroid-like object is conserved (Ri=1.01 – 1.11). We assume the focal point of the objective to lie in the center of the currently simulated lightsheet position. The light captured by each ray on its path through the sample is then computed as the sum of all light integrated when traveling through the sample, at each ray location gaussian-weighted () by the distance to the expected lightsheet position . The simulations were performed in parallel on the local compute cluster at the MDC.

Although very simple, this simulation recapitulates the observed images (**Fig. 2a,e**) and thereby illustrates that different refraction of the illumination lightsheets alone can lead to non-rigid deformations in the acquired image stacks as it leads to illumination of different contents of the sample in z (**Fig 2d,e** and **Supplementary Video 7**). Since typically detection objectives with relatively low NA are used for detection, those can still both appear in-focus and these transformations should be corrected for (**Supplementary Table 1**).

*Pairwise Stitching using Fourier-based Phase Correlation*

We calculate pairwise translational shifts using our ImgLib210 implementation of the Fourier-based *phase correlation* algorithm31. In noiseless images, the method produces a phase correlation matrix (PCM) *Q* containing a single δ-impulse at the location corresponding to the shift between the two images. Real images might contain multiple peaks (**Supplementary Fig. 11**) and we localize the *n* highest peaks in *Q* by detecting peaks with subpixel accuracy using a *n*-dimensional implementation of a quadratic fit32.Aside from allowing subpixel-accurate registration, we can use the precision obtained from the subpixel accuracy of the phase correlation to counteract the effects of downsampling, allowing us to achieve registration of similar quality to full-resolution with significant performance gains (**Supplementary Fig. 13-15**). Due to the periodic nature of the Fourier shift theorem, each peak in the PCM actually correspond to 2n possible shifts in *n* dimensions. We therefore test each of these candidate shifts by calculating the cross-correlation between the images *I1* and *I2*, optionally with interpolation in the case of sub-pixel shifts. We choose the shift vector *t* corresponding to the highest cross correlation as the final result after applying downsampling correction, if necessary.

It is often necessary to not only align two single images but groups of images, e.g. all channels of a tile. We therefore implemented a flexible framework for the registration of grouped images (**Supplementary Note 3**). The two images *I1* and *I2* can have arbitrary affine pre-registrations such as sample rotation, correction of axial scaling or already performed registration steps. If pre-registrations of *I1* and *I2* are identical or are only based on different translations or axis-aligned scalings we run the phase correlation on (downsampled) raw input images, otherwise on virtually fused images (**Supplementary Note 3**).

*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 light-sheet microscopy including optical sectioning, 3-fold anisotropy, light attenuation, convolution, and pixel intensity generation using Poisson processes19. 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. 12**). We simulate 500 pairwise overlaps, each at SNRs ranging from 1 to 32, and lateral downsamplings ranging from 1x to 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 exceeds that of registration at full resolution for low SNRs. This is achieved through a combination of the smoothing effect during downsampling (**Supplementary Fig. 12**) and precise subpixel-localization (**Supplementary Fig. 13-15**). Due to the smoothing effect registration quality therefore initially increases at 2-fold and 4-fold downsampling (**Supplementary Fig. 13-15**), while when using larger downsampling, the loss of pixel resolution outweighs the effect of smoothing hence the quality drops. Registrations with a constant quality of an average error of below one pixel can be computed at a fraction of the computing time compared to full resolution, typically 4 - 120 times faster. Existing outliers are filtered during global optimization and overall registration quality can further be improved during the ICP16 refinement step.

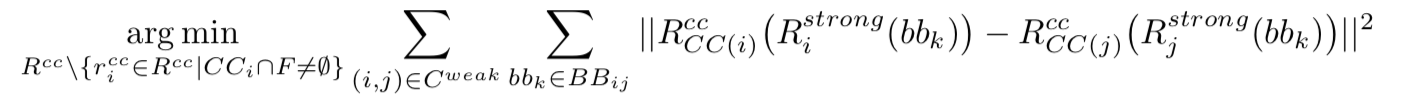
*Global Optimization*

To calculate the final transformations of each image tile we extend the concept of globally optimal registration by iterative minimization of square displacement of point correspondences (**Supplementary Note 4**)11,12. 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* in all connected components *CC* of the link graph of images *V* and (strong) links *C*, given point matches (corresponding points) *PM* and fixed images *F* by minimizing:



In some cases, erroneous pairwise links might not have been filtered out, e.g. due to medium 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 distance error from the link graph and repeating the global optimization leads to convergence to user-defined thresholds11. We extend this concept from Preibisch et al.11 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 empty tiles or multiple disconnected objects with image tiles that do not have links between them, the final transformations will not be propagated between them (**Supplementary Fig. 10**). We therefore developed a novel two-round global optimization that is capable of aligning independent connected components of the link graph using weak links defined by the current transformations of each tile (e.g. approximate locations from metadata or manual alignments). For that purpose, we 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. 10**).

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 ICP16 refinement or geometric descriptor matching.

*Iterative Closest Point Refinement*  
Although the Phase Correlation-based image stitching produces relatively high-quality alignments, smaller errors can remain (**Supplementary Fig. 13-15**). Furthermore, it is not able to correct for non-translational effects such as chromatic and spherical aberration or sample-induced light refraction (**Fig. 2** and **Supplementary Fig. 3,4,9,17**). These effects can be better approximated using affine transformations. We therefore automatically detect interest points and run an Iterative Closest Point algorithm16 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 transformation12. The resulting alignment usually improves the alignment quality and the same strategy can be applied to multichannel alignment if the same autofluorescent structures are visible in multiple channels (**Supplementary Fig. 3**). However, only small chromatic aberrations within a few pixels can be corrected by approximations based on affine or split-affine transformations (Supplementary **Fig. 3**), while non-rigid transformations are unstable since correspondences in between different channels would have to be distributed over the entire image. Therefore, it is in those cases a better choice to employ specific software such as the work by Matsuda et al.33 for chromatic aberration prior to importing the data into BigSticher.

*Geometric Local Descriptor Matching*

To identify corresponding interest points in between two point clouds, geometric local descriptor matching combined with random sample consensus (RANSAC)34 has been proven to be a powerful technique35,36. The basic idea to express each interest point as a geometric constellation using its *n* (typically three) nearest neighboring interest points. The 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 if it is at least *m* (typically one to ten) times more similar than the second most similar GLD32. True corresponding interest points between two point clouds are finally identified using RANSAC on a regularized affine transformation model. However, fast GLD matching using the rotation-invariant technique based on geometric hashing17 requires relatively randomly distributed points to robustly identify correspondences, while the non-accelerated, redundant, translation-invariant counterpart36 identifies correspondences reliably in non-rotated point clouds of only up to a few thousand points in reasonable time. Here, we developed a new matching procedure by extending both techniques to better suit the requirements when attempting to identify corresponding interest point in between point clouds of prior unknow size 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 mis-detected interest points36. We therefore extend the fast rotation-invariant technique based on geometric hashing35 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 are available in BigStitcher to enable robust multi-view alignment.

*Virtual Re-blocking*

To allow piece-wise affine transformations or refined illumination selection we developed a virtual re-blocking of all 3d image stacks of an acquisition (**Fig. 2k,l**). The implementation distributes 3d blocks onto each input image stack using a defined overlap and thereby defined a new set of input image stacks for the acquisition. The new, virtual image stacks are computed on the fly for all resolution levels from the original image data. Any ImgLoader is supported, including multi-resolution image stacks. Optional resaving of the dataset as HDF5 or TIFF transforms the virtual image stacks into physical representations.

*Non-Rigid Transformation*

To be able compensate for potentially strong aberrations introduced by light refraction due to the sample we implemented a non-rigid alignment method that we based on the concept of moving least squares37, which has been show to perform well in biomedical applications38. The underlying principle is to non-rigidly transform images using a set of corresponding points. Therefore, a local transformation is computed for each pixel using a distance-weighted fit of all corresponding points ensuring smoothness. In BigStitcher, corresponding points are a direct result of all interest point-based registration algorithms. To provide a sufficient amount of corresponding interest points, it is yet most useful to derive corresponding interest points using ICP. Regularization is achieved since corresponding interest points are identified on a regularized affine transformation model either using RANSAC34 or ICP16, which both specify a maximum error. This ensures that corresponding points cannot diverge more than this specified error from the regularized affine transformation of each image tile. In combination with virtual re-blocking, this error can be limited to smaller regions than the acquired, physical tiles.

When computing local transformations for each image, it is necessary to ensure smoothness across *n* overlapping images by defining appropriate point correspondences. The registration identifies pairwise correspondences in between pairs of images, from which we first identify all unique interest points across all images (**Supplementary Fig. 23**). For each unique point we determine its location by averaging the locations of all contributing interest points after applying their respective affine transformations. Thereby, the non-rigid transformation only accounts for the remaining error after affine alignment. For each image, corresponding points for moving least squares are subsequently defined between the unique point and the corresponding interest point of the transformed image.

Moving least squares requires to compute a transformation for each pixel, which is computationally expensive and usually cannot be cached due to the image sizes. We therefore implemented a virtual, cached layer that only computes transformation every *m*-th pixel and interpolates affine transformations in between. Since BigDataViewer currently only supports affine transformations, we additionally implemented a multi-resolution preview based on virtually fused non-rigid volumes that can be interactively displayed in an extra BigDataViewer window. We support hybrid fusion of non-rigidly and affine transformed image tiles as non-rigid registration requires significantly increased computational effort.

*Quantification of Registration Results*

To quantify final registration quality, we used a small cleared section of an adult mouse brain imaged at low magnification from two angles (0° and 180°) in 2x3 tiles and dual-sided illumination for each angle (**Fig. 2k,l**, **Supplementary Fig. 2,17** and **Supplementary Table 1**).

We identified a ground-truth set of corresponding interest points in directly adjacent images by manually selecting bright spots in the overlapping volume and mapping them to the closest point in a set of interest points automatically detected using Difference-of-Gaussian filtering and subpixel-accurate local maxima determination. For each image pair, we selected between 19 and 52 corresponding points, in total 692.

We then registered the dataset in BigStitcher, grouping the images either by angle and illumination direction, just by angle, or not at all. For the groupings by angle or by angle and illumination direction, we performed translational alignment by stitching the images using phase correlation. For an all-to-all registration with a translation model, the images of angle 2 were manually rotated by exactly 180° and then all images were aligned using interest points by fast translation-invariant GLD matching followed by RANSAC and global optimization using a translation model. All the translation-model alignments were refined using ICP as described above. The point correspondences determined during ICP were further used for non-rigid refinement.

For virtual re-blocking, each original image was split into 2x2x2 sub-blocks (with 120px overlap in x,y and 100px overlap in z). After the blocking, 4-28 manually selected point correspondences remained between each set of directly adjacent blocks.

For each of the image groupings and registration models used, we calculated an average error of the manually selected point correspondences:

With *I* being the set of images, *C(i)* the adjacent images of an image *i* (ignoring diagonal pairs for which no corresponding interest points were manually selected as well as pairs that are not in the same group, e.g. when grouping by angle and illumination direction, and pairs from the same original image in the virtually blocked dataset), *PM(i,j)* the corresponding manually selected interest points of images *i,j* and *Ti* the transformation of image *i*.

To estimate the lowest theoretically achievable errors given a certain transformation, we use only the manually selected point correspondences to calculate a globally optimal registration (and optionally the non-rigid refinement thereof) of the images and then calculate the average error from the same point correspondences as described above. In the virtually blocked case, we also use manually selected point correspondences (826 in total) between adjacent blocks within the same original image for the registration (but ignore them for the final error calculation).

Despite relatively small aberrations in this sample as compared to entire mouse brains (compare with **Fig. 2f,g,h** and **Fig. 2m**) we illustrate that using only translation as transformation model is only reasonable for tiled acquisitions that do not include multiple illuminations or multiple acquisition angles, yet even there spherical aberrations persist that question the standard use of translation models in general (**Supplementary Fig. 4,9**). The alignment errors increase when aligning across illumination directions and greatly increase when aligning different acquisition angles. Importantly, please note that the alignment quality across different illumination directions is significantly reduced on larger samples when using only translation models (compare **Fig. 2f,g,h** and **Fig. 2m**). Using the affine, split-affine or non-rigid registration functionality, BigStitcher can sharply reduce the registration errors in large cleared and expanded samples. As a trade-off between speed and quality we usually choose affine or split-affine registrations.

*Virtual Image Fusion*

A set of overlapping, transformed image tiles are 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**)35. To correct for unequal brightness and contrast in adjacent images, we optionally perform adjustment of the pixel intensities using a linear transformation per image. An optimal adjustment can be estimated using the same optimization framework used for image registration39 (**Supplementary Note 7**). 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 novel framework based on ImgLib2 *RandomAcessibleIntervals*10, 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 image8. 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 (**Supplementary Table 2** and **Supplementary Fig. 17**), but even machines with very low RAM will be able to fuse terabyte-sized volumes (**Supplementary Fig. 19**). Fused 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 real-time image fusion, we offer deconvolution of bounding-box-defined volumes using a multi-view formulation of the iterative Richardson-Lucy deconvolution algorithm40,41 with Tikhonov regularization42 and various optimizations19. 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 with odd dimensions by the user. BigStitcher offers GPU acceleration of the deconvolution on CUDA-capable Nvidia GPUs.

To allow deconvolution of multi-tile views, we extended the original deconvolution19 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 multi-view deconvolution. Proper multi-view deconvolution of partly overlapping samples requires sophisticated weight normalization in between views19, which we implemented to be computed virtually. Since also the input views are also virtually loaded, the memory requirement of the deconvolution solely depends on the output image size and 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 standalone Fiji plugins for most of the individual steps, such as data import, illumination selection, pairwise shift calculation, link filtering, multi-view alignment, global optimization and image fusion/deconvolution. In macro mode results will not be displayed interactively but are instead 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 processing43.

*Limitations of the Framework and other Software Solutions*

BigStitcher is designed for the reconstruction of large multi-tile, dual-illumination, multi-view datasets. To the best of our knowledge it is the only software that supports such type of acquisitions, which includes affine, split-affine and non-rigid registrations to solve the alignment process for terabyte-sized image data. Several solutions based on image correlation support multi-tile-only data, such as Terastitcher15, XUVTools13, ImageJ Stitching11, which however, only support translation models making them only somewhat applicable to these kind of datasets (**Fig. 2** and **Supplementary Fig. 3,4,9,17**). A recent stitching solution developed by the Saalfeld lab can handle even larger datasets (100s of terabytes) and supports affine transformations based on local cross correlation. It is, however, limited to multi-tile acquisitions, is designed to run on a compute cluster and has no user interface to access its functionality44. Currently, BigStitcher scales well until about 1000 large 3d image tiles per timepoint and sizes of about 10s of terabytes per timepoint. This is, however, mostly due to a limit in rendering capacity of BigDataViewer. Future optimizations of BDV and/or BigStitcher will further increase this limit. BigStitcher can correct for chromatic and spherical aberrations by approximation with affine transformations if errors are within a few pixels and, specifically for chromatic aberrations, if enough autofluorescent structure is visible across channels (**Supp. Fig. 3,4,9**). While BigStitcher can correct for geometric transformations introduced by the acquisition process (**Fig. 2** and **Supplementary Fig. 17**), it is not possible to correct for out-of-focus acquisitions.

*Example Data*

Example datasets are explained and linked for anonymous download in Supplementary Note 10.

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**Author contributions**

S.P. conceived the idea in discussions with H.H., H.L, and M.T. D.H. and S.P. developed the algorithms and implemented the software. F.R.R. performed all clearing experiments, reconstructions and benchmarks. F.P. imaged and reconstructed the C. elegans. P.T. and N.R performed ExM sample preparation. R.K.C. and P.J.K. developed the ExM-optimized IsoView microscope and imaged the sample. S.P. reconstructed the ExM sample. S.P., M.T., H.L, H.H., P.J.K, and A.C. supported and supervised the project. S.P., D.H., and F.R.R. wrote the manuscript with input from the coauthors.

**Competing Financial Interests**

The authors declare no competing financial interests.

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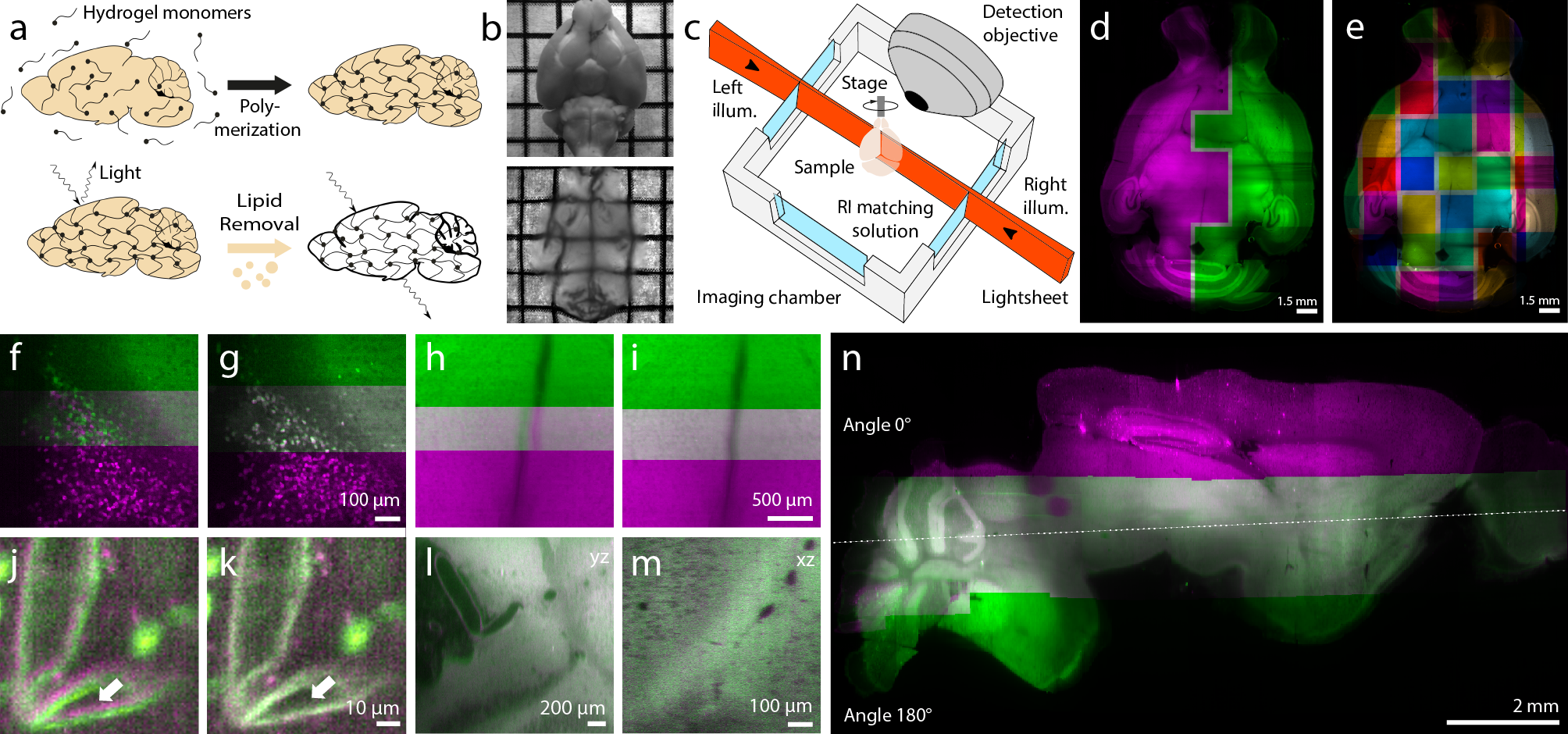
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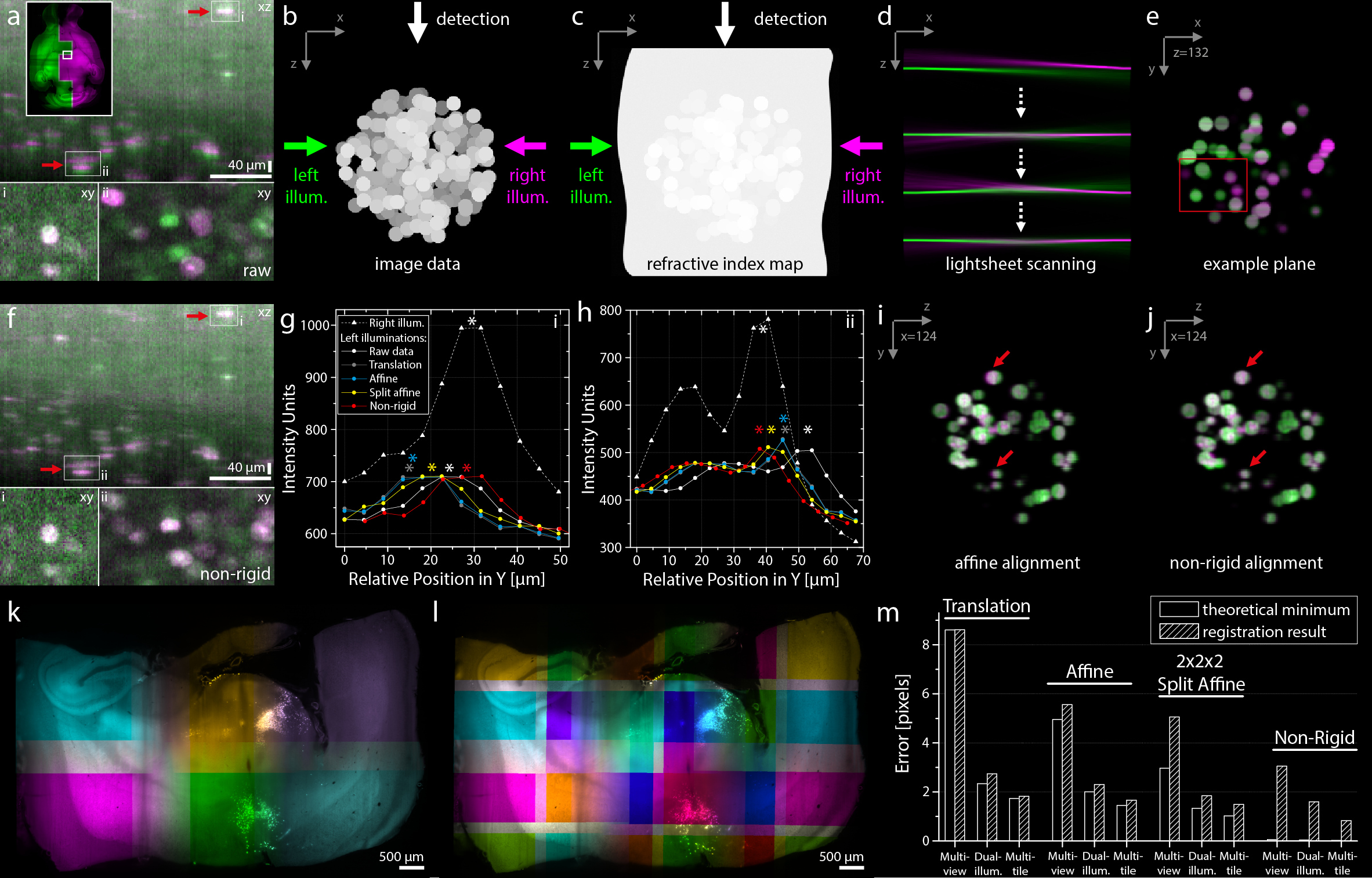
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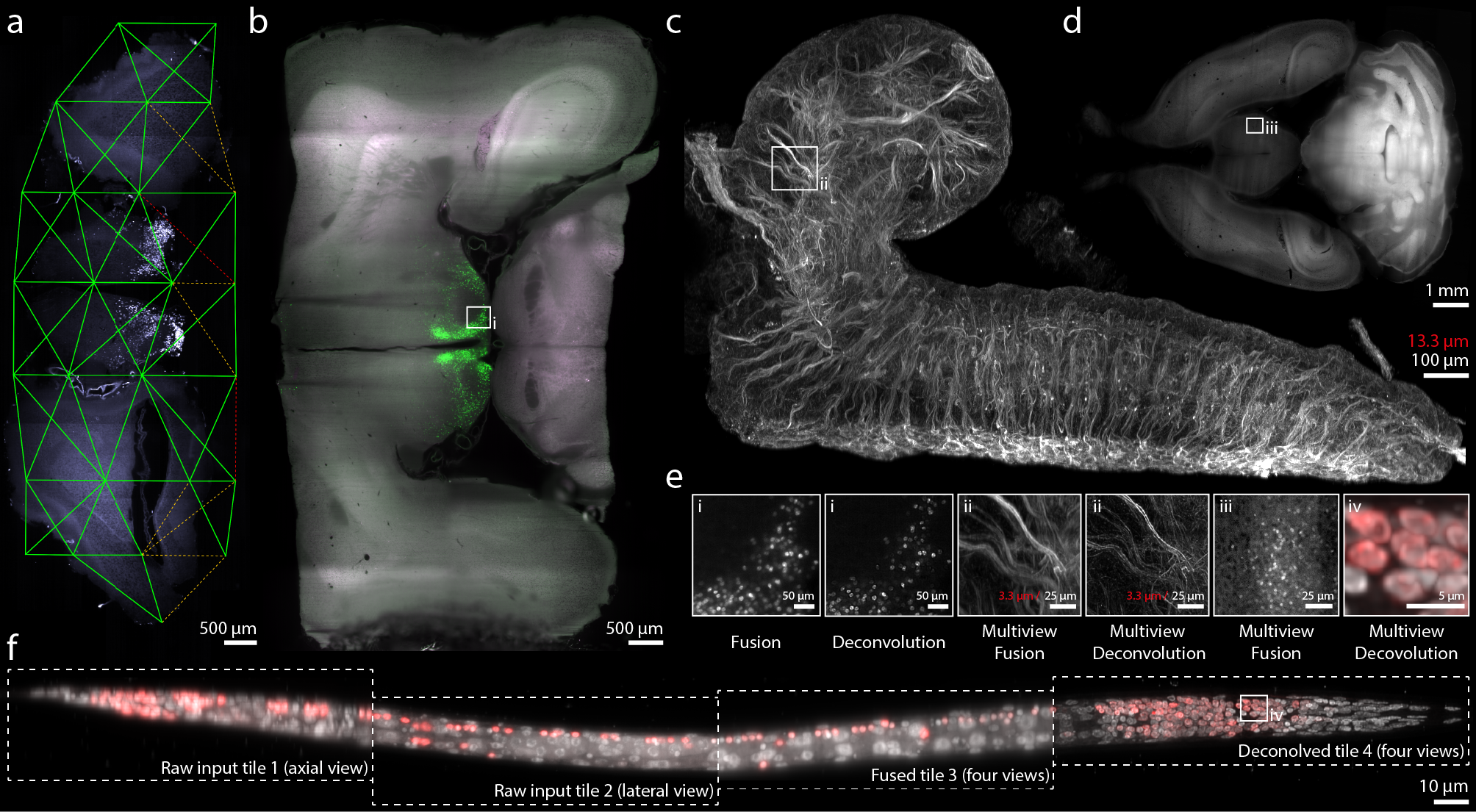
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**Figures**



**Figure 1**: BigStitcher Principles. **(a)** schematic of the CLARITY sample clearing process. **(b)** adult mouse brain before (up) and after (down) clearing. **(c)** general layout of the type of light-sheet microscope used for most acquisitions17 **(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)** 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)** illustrates the high quality of the multi-view 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)** 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.

**Figure 2**: *Optical Aberrations, Light Simulations, and Alignment* **(a)** Zoom into an area that shows misalignment between the two different illumination directions (green left, magenta right illumination) of the original acquired images. An overview of the entire mouse brain is shown for orientation. Two selected areas (red arrows) in the *xz* view are shown in the *xy* view below, highlighting the misalignment. **(b-e)** Simulation of a spheroid-like object using raytracing that recapitulates effects shown in (a) (compare red box to ii in a) and thereby illustrates that sample-induced light refraction in the illumination light paths can cause non-rigid deformations since different parts of the sample are illuminated, see **Supplementary Video 7** for an animation. **(b)** shows simulated image data, **(c)** shows the refractive index map, **(d)** illustrates the effects on the lightsheet while scanning, and **(e)** shows an example slice of the simulated object. **(f)** shows the same views as (a), but corrected using non-rigid alignment. **(g,h)** show plots of cross-sections of the selected areas in (a,f) comparing different BigStitcher alignment methods, asterisks mark the approximate center of the nuclei that should ideally overlap in between different illumination directions **(i,j)** compare the alignment quality on the simulated data between affine and non-rigid. **(k,l)** show a small 166GB multi-view, dual-illumination, multi-tile dataset specifically acquired for error quantification in original configuration **(k)** and after the virtual 2x2x2 split **(l)**. **(m)** compares the best theoretically achievable registration quality based on manually selected corresponding points to a typical registration result when using different alignment modes in BigStitcher. Note that the amount of increase in registration quality depends on the dataset, for example in (g,h) errors are reduced by about 10 times the amount as the dataset is bigger and aberrations are larger.

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**Figure 3**: 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 unconcise. **(b)** One slice through an adult mouse brain expressing an H2B-eGFP lineage tracing marker in BSX-expressing neurons, the box is highlighted in (e). **(c)** Maximum intensity projection of a central part of a 7.5-times expanded central nervous system of a *Drosophila* 1st instar larva with immunostaining for tubulin (Alexa-488) and imaged with multi-tile IsoView light-sheet microscopy, boxes are magnified in (e). (**d**) One slice through a whole multi-tile, multi-view reconstructed adult BsxH2B-GFP/+ mouse brain, the inset is highlighted in (e). **(e)** Zoom-ins to specific areas of (b,c,d,f) illustrating   
(sub-)cellular resolution and the advantage of (multi-view) deconvolution over (multi-view) fusion. **(f)** Fixed *C. elegans* dauer acquired in four tiles with four views each expressing tagRFP in all neuron nuclei, co-stained with DAPI. Boxes highlight the quality of axial and lateral raw input data, multi-view fusion, and multi-view deconvolution.