**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** (1796 words)

Sample clearing1 and expansion microscopy (ExM)2 are powerful protocols that create large, transparent volumes of whole tissues and organisms (**Fig. 1a,b** and **Online Methods** and **Supplementary Note 1-3**). 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** and **Supplementary Fig. 1**). Additionally, overlapping images suffer from spherical and chromatic aberrations (**Supplementary Fig. 3,4**). For reconstruction, and to 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 based on BioFormats9 and accesses image data through memory-cached, virtual loading10, optionally combined with virtual flatfield correction (**Supplementary Fig. 5,6** and **Supplementary Note 4,5**). 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**).

Firstly, overlapping 3d image tiles are acquired to cover the entire sample for 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) method11-14 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 Fig. 11** and **Online Methods** and **Supplementary Note 6**). 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** and **Online Methods** and **Supplementary Note 7**). 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** and **Supplementary Video 1**). Our new global optimization method extends the concept of identifying tile positions by minimizing the distance between all links12,15, which, compared to computing a minimum spanning tree16, 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 result12 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 them12,13, 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 **Online Methods**). 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)17 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**).

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 proposed18, 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 points19 as well as piece-wise, ICP-based affine alignment through virtual splitting of image tiles into smaller blocks (**Fig. 2f-m** and **Online Methods**). Depending on the magnitude of refraction, affine, split-affine or non-rigid is the right choice for precise alignment (**Fig. 2g,h,m**), which is possible as long as the lightsheet remains within focus. 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 process20.

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 (**Supplementary Note 8**). 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. 2g,h,m**, **Supplementary Fig. 3,4,17** and **Supplementary Note 9**), which illustrates 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. Therefore, we developed the relative Fourier Ring Correlation (rFRC), which is based on the Fourier Ring Correlation (FRC)21. The rFRC is able to automatically and rapidly estimates image quality throughout terabyte-sized lightsheet acquisitions while accounting for common sCMOS camera patterns (**Supplementary Fig. 1,7,8** and **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 (**Online Methods**), 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 beads22,23. To handle multi-tile, multi-view acquisitions, we extended deconvolution code23 allowing BigStitcher to deconvolve selected regions and improve image quality (**Fig. 3b-e** and **Supplementary Note 10**). To estimate required PSFs in cleared samples we developed a new protocol for embedding fluorescent beads in polymerization solution (**Fig. 3b,e** and **Supplementary Note 11**). 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**)12,13,16. 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 Fiji24 plugin with comprehensive documentation (<http://imagej.net/BigStitcher>). It is compatible with the ImageJ Macro language (**Online Methods**) 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.

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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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**Figure Legends**

**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 acquisitions3 **(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. (**b,d-n**) Clearing and alignment (**Online Methods**) with similar results was performed on 6 independent samples (**Supplementary Table 1**).

**Figure 2**: *Optical Aberrations, Light Simulations, and Alignment Quantification* **(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 non-rigid deformations shown in (a), compare red box to (ii) in (a). **Supplementary Video 2** shows a corresponding 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 an as-small-as-possible 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, about 10-times larger errors are compensated in the much larger sample in (g,h). (**a-j**) Non-rigid deformations of varying degree were observed for all dual-illumination datasets (**Supplementary Table 1**), a single simulated dataset was created, and a single dataset for precise quantification was acquired. Different affine and non-rigid alignments were applied to those three datasets.

**Figure 3**: Reconstructed samples. **(a)** One slice through an acquisition of an adult mouse BsxH2BGFP coronal slice encompassing the hypothalamus (**Supplementary Video 3**). 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 (**Supplementary Video 4,5**), boxes are magnified in (e). (**d**) One slice through a whole multi-tile, multi-view reconstructed adult BsxH2B-GFP/+ mouse brain (**Supplementary Video 6**), 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 (**Supplementary Video 7**). Boxes highlight the quality of axial and lateral raw input data, multi-view fusion, and multi-view deconvolution. (**a-f**) In total 8 different datasets were acquired for this publication (**Supplementary Table 1**), which were all reconstructed as described in the **Online Methods**. Additionally, the BigStitcher reconstruction pipeline has been applied to >50 samples in our lab (not shown).

**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 the tagRFP gene 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 (**Supplementary Note 1**). Before imaging, the tissue sample were placed overnight in FocusClear for refractive index matching. For ExM, the nervous system of a 1st instar *Drosophila* larva of was extracted, fixed, and antibody-stained for tubulin (**Supplementary Note 2**). The stained sample was washed in 1x PBS and then processed using a modified ExM method to achieve 7,5-fold expansion in each dimension (**Supplementary Note 2**). In summary, the specimen was treated with acryloyl-X25 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, a new re-embedding step toughens up the gel, which would otherwise have poor mechanical properties.

*Imaging*

3D images of cleared mouse brains placed in a FocusClear filled imaging chamber were imaged using the Zeiss Lightsheet Z.1 microscope. Fixed *C. elegans* dauer larvae were embedded in agarose containing fluorescent beads and imaged using the same microscope in a water-filled sample chamber. 3D images of 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. The ability of the IsoView microscope to rapidly record large specimens allowed us to image the entire nervous system in 10 min with 100 nm isotropic resolution. Details of the imaging strategies are described in **Supplementary Note 3** and a summary of the most important acquisition parameters can be found in **Supplementary Table 1**.

*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 HDF5. 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**). If not stated otherwise reconstruction was performed using the following steps. For each tile, the best illumination was selected. Tiles were aligned using the phase correlation method using two-round global optimization, followed by ICP17 refinement on an affine model. Interest point detection for each multi-tile view was performed. Our extension of the fast descriptor-based rotation-invariant algorithm26 or the descriptor-based translation-invariant algorithm27 after manual rotation application were 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.

*Pairwise Stitching using Fourier-based Phase Correlation*

We calculate pairwise translational shifts using our ImgLib210 implementation of the Fourier-based *phase correlation* algorithm11. The processing time is significantly increased, while our simulations show that at the same time registration errors below 1 pixel are achieved by computing the phase correlation on downsampled images and sub-pixel localization of the shift vector28 (**Supplementary Note 6, 7** and **Supplementary Figure 11-15**).

*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 12**)12,15,26,27. Erroneous pairwise links that might not have been filtered out prior to global optimization, e.g. due to average cross-correlation, repetitive patterns, or a low number of correspondences in the ICP refinement lead to high registration errors after global optimization. This 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 thresholds12. We extend this concept from Preibisch et al.12 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 12**). If the dataset contains empty tiles or even consists of multiple disconnected objects, 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), which preserves distances between neighboring objects as-good-as-possible (**Supplementary Note 12** 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 ICP 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 (ICP) algorithm17 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 transformation15. 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**). At the same time, non-rigid transformations can easily be unstable for this purpose since correspondences in between different channels would have to be distributed over the entire image. Therefore, it is in those cases better to employ specialized software such as the work by Matsuda et al.29 for chromatic aberration prior to importing data into BigSticher.

*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 relative Fourier Ring Correlation (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. However, prior to applying automatic illumination estimation, the user has the option to verify and potentially change the result. Optional resaving of the dataset after this step potentially decreases storage requirement two-fold.

*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 propagation30 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 (**Supplementary Note 13**).

Although quite simple, this simulation recapitulates the effects observed in cleared images (**Fig. 2a,e**) and 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 2**). Since typically detection objectives with relatively low NA and therefore large depth-of-field are used for detection (**Supplementary Table 1**), both lightsheets can still appear in-focus although being tens of microns away from each other. Therefore, these transformations need be corrected for.

*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. It is based on the concept of moving least squares19 that has been shown to perform well in biomedical applications15, which we implicitly regularize using ICP17 or random sample consensus31 (**Supplementary Note 14**). Moving least squares requires to compute a transformation for each pixel, which is computationally expensive. We therefore implemented a virtual, cached layer that only computes transformation every *m*-th pixel and linearly 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. BigStitcher also supports “hybrid” fusion of non-rigid and affine transformed image tiles since non-rigid registration requires significantly increased computational effort (**Supplementary Fig. 17**).

*Virtual Re-blocking*

To allow piece-wise affine transformations or a more 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 defines 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.

*Quality Estimation based on Fourier Ring Correlation*

To estimate image quality across entire terabyte sized acquisitions, we developed an extension of the Fourier Ring Correlation (FRC)21 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 plugin (**Supplementary Note 15**). 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*-smoothed32 (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,7,8**). Since image content can change drastically within a slice, we support computation using a manually defined block-size (e.g. 512x512) and with a z-stepping (e.g. every 20 planes). 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 (**Supplementary Fig. 7,8 and Supplementary Video 8,9**).

*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 16**)33. 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 registration34 (**Supplementary Note 17**). 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 the image stacks in arbitrary orientations do not require loading 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.

*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 processing35.

*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 Terastitcher16, 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 currently no user interface to access its functionality36. 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. For chromatic aberration correction enough autofluorescent structure must be 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 images.

*Data Availability*

Small example datasets are available for download on the Open Science Foundation: https://osf.io/bufza/. Larger datasets are available on request. Additional datasets uploaded at a later stage will be linked from the documentation page: https://imagej.net/BigStitcher#Example\_Datasets. Example datasets are explained in detail in **Supplementary Note 18**.

*Code Availability*

All source code used in this publication (BigStitcher, phase correlation simulation & benchmark, and the simulation of light propagation in tissue using raytracing) is open-source and published under GPL(v2). The latest stable releases used in this publication are attached as **Supplementary Software**, current versions that include bugfixes and updates can be downloaded from GitHub (**Supplementary Note 19,20**). Details on how to use the software are described in **Supplementary Note 21.**

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