Correlia: an ImageJ plug-in to co-register and visualise multimodal correlative micrographs

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Summary

The correlation of different microscopic imaging techniques alongside with microanalytical methods is crucial to better understand biological processes on a subcellular level. For that, micrographs and chemical maps exhibiting both, very different spatial resolution and field-of-view but also a highly multimodal content has to be co-registered. We developed the ImageJ/Fiji plug-in Correlia that provides an environment for handling multimodal correlative microscopy data. Several linear and nonlinear registration methods using either feature or area-based similarity measures can flexibly be cascaded to align and warp 2D microscopy data sets. The registration of data sets containing light- and electron micrographs as well as chemical maps acquired by secondary-ion mass spectroscopy and energy-dispersive X-ray spectroscopy is demonstrated. Correlia is an open-source tool developed particularly for the registration and analysis of highly multimodal 2D correlative microscopy data.

Introduction

The whole is greater than the sum of its parts could serve as a brief description of the philosophy of the rapidly emerging field of correlative microscopy: An object is imaged with different microscopes in combination with imaging micro- and nanoanalytical techniques in order to gain a more comprehensive picture compared to single measurements (Caplan et al., 2011). In cell biology, for instance, correlative light-electron microscopy (CLEM) is well established (de Boer et al., 2015). CLEM in combination with fluorescently tagged proteins, immunolabelling and cryotechniques became a powerful tool to link structure and function of cellular units (van Rijnsoever et al., 2008; McDonald, 2009). Furthermore, CLEM allows for quasi-simultaneous acquisition of dynamic and ultra-structural information of subcellular processes, for instance, for the analysis of cell division (Redemann & Müller-Reichert, 2013). The combination of microscopic and microanalytical techniques was used to reveal the complex morphology and chemical structure of bacterial biofilms (Lawrence et al., 2003). In order to link identity and metabolic activity of microbial cells in environmental microbiology the correlation of fluorescence in situ hybridization, microautoradiography, confocal Raman microscopy and nanosecondary-ion mass spectroscopy (nanoSIMS) in combination with stable isotope labelling techniques can be employed (Musat et al., 2012). The current state of the art in correlating high-resolution transmission electron microscopy with secondary-ion mass spectrometry (SIMS) is presented in Eswara et al. (2019). A recent review on subcellular chemical imaging using correlated workflows is given in Decelle et al. (2020).

During the past years, a number commercial solutions for correlative microscopy workflows – mostly combining electron microscopy with light microscopy – from data acquisition to image processing became available (Perkel, 2014). An integrated CLEM solution for scanning electron microscopes (SEM) for instance is available from Delmic B.V., Delft, Netherlands, named as SECOM platform for integrated fluorescence and electron microscopy (den Hoedt et al., 2014). The aIESM technology (Solomonov et al., 2014) commercialized by B-nano LTD, Rehovot, Israel allows for CLEM on tissues under ambient conditions. An embedded system named ‘RISE microscopy’ for correlative SEM and Raman microscopy is commercialized by B-nano LTD, Rehovot, Israel allows for CLEM on tissues under ambient conditions. An embedded system named ‘RISE microscopy’ for correlative SEM and Raman microscopy is commercialized by B-nano LTD, Rehovot, Israel allows for CLEM on tissues under ambient conditions.
image registration implemented in the WITec Suite Project FIVE software.\textsuperscript{3} Carl Zeiss Microscopy, Oberkochen, Germany offers a comprehensive solution for correlative microscopy using a particular sample holder in combination with the Shuttle & Find module in ZEN and ATLAS software packages.\textsuperscript{4}

While so far the majority of correlative approaches combines merely two techniques, the demand for correlating more techniques is rapidly growing. The motivation for this work was mainly drawn from the correlative workflows at ProVIS – Centre for Chemical Microscopy at Helmholtz Centre for Environmental Research, Leipzig.\textsuperscript{5} At the centre correlative microscopic imaging (e.g. light microscopy, SEM and helium microscopy), and microanalytical techniques (e.g. SIMS) are employed to investigate microbiologically mediated processes and the role of microorganisms in element cycles in natural and polluted environments. Workflows including preparation of samples, imaging and microanalytics have been developed (Decelle et al., 2019; Moreno Osorio et al., 2019), and all demand co-registration of the multimodal micrographs.

Basically, in order to exploit the possibilities of the multi-correlative approach in their entirety, the different data acquired on one sample have to be co-registered. Although each instrument at ProVIS produces image data, major challenges for the registration are (i) data format due to different instrument manufacturers, (ii) resolution and field-of-view of the instruments can vary for the different instruments by up to four orders of magnitude and (iii) the acquired image data are highly multimodal. In particular, the latter cannot easily be handled by the existing software packages, since these were mainly designed for the registration of image data rather than maps acquired by microanalytical methods. A consequence of the latter is that objects which are clearly visible in either light or electron microscopy may be completely invisible in a chemical map because of their chemical composition. In such case, the use of extrinsic markers could enable easy alignment of images but is often impracticable due to incompatible image modalities.

Furthermore, spatial deformations may occur in the correlative data set. On the one hand, these can be caused by microscope-induced aberrations or due to required sample processing, e.g. drying, in between the image acquisitions. On the other hand, data from different destructive techniques that consume or damage the sample may be needed. In such case, the correlative data will have to be acquired on consecutive histological sections which naturally do not match perfectly due to the slightly different depth from inside the respective specimen. This variety of causes for deformations and distortions in particular requires flexible models for correction, i.e. co-registration.

Powerful tools optimized for registration of monomodal (e.g. computed tomography) data such as ITK (Insight Software Consortium, 2001-), elastix (Klein et al., 2010), bUnwarp (Arganda-Carreras et al., 2006), BigWarp (Bogovic et al., 2016) or TrakEM2 (Cardona et al., 2012) do exist. Many concepts for the co-registration of multimodal image data originate from remote sensing problems but can easily be transferred to microscopy data. Image registration methods were systematized by Zitová & Flusser (2003) and earlier by Gottesfeld Brown (1992). Ingla & Giros (2004) adopted several similarity measures for multimodal images, including Kolmogorov distance and mutual information (MI), to mention a few. Among the multimodal image registration software, there is the tool eC-CLEM (Paul-Gilloteaux et al., 2017), a plug-in for ICY (Institut Pasteur & France-BioImaging, 2011-; de Chaumont et al., 2012), which is specifically designed for treating CLEM data. eC-CLEM offers feature-based registration but cannot manage data sets of arbitrary sizes. Different strategies for the registration of highly multimodal CLEM data are described in the PhD thesis of Toledo Acosta (2018). To the best of our knowledge, there are no tools described yet that provide suitable similarity measures for multimodal images, nonlinear registration methods and an integrated solution for management and visualisation of multiple correlative images at the same time.

Therefore, in this work we developed the flexible open-source image registration and visualisation tool Correlia for correlative microscopy data. Correlia is based on the open-source image-processing software ImageJ (Rueden et al., 2017). It allows for co-registering an arbitrary number of micrographs acquired by different microscopes or microanalytical tools in different field-of-views using linear or nonlinear transformation models as well as combinations of them. Both area and feature-based registration algorithms have been implemented. The possibility to co-register images onto any other image in the data set allows for zoom-cascades which makes the software particularly suitable for data sets that bridge large scales. Correlia furthermore provides a platform for the correlation of 2D-2D microscopy data which organizes the data in projects such that different users can view available data, add to the project and in turn benefit from the correlative approach.

Implementation
When formulating the design of Correlia, we addressed the following points:

- platform independence
- open-source code
- compatibility with existing image-processing software
- handling of arbitrary 2D microscopy data independent of the manufacturer of the microscope

\textsuperscript{3} https://www.witec.de/products/accessories/software-witec-suite.

\textsuperscript{4} https://www.zeiss.com/microscopy/int/products/microscope-software/atlas.html.

\textsuperscript{5} https://www.ufz.de/provis.
• organization of correlative data in extendable projects of arbitrary size
• preservation of raw data
• registration of any micrograph within the project onto any other
• possibility to cascade registration steps
• visualisation of arbitrary subsets of micrographs in the project in user-defined false colours
• extraction of user-defined regions-of-interest from a visualisation of the project

All this shall be outlined in the course of this section.

ImageJ/Fiji

Correlia is based on the well-known open-source image-processing software ImageJ (Rueden et al., 2017). ImageJ does not only provide a variety of built-in tools for the analysis of microscopy data. It can furthermore be extended by macros and external plug-ins which makes ImageJ a highly flexible software for numerous applications. A bundle of ImageJ with commonly used plug-ins is distributed as Fiji\textsuperscript{b} (Schindelin et al., 2012). In particular, it ships with Bio-Formats (Linkert et al., 2010), a library for proprietary life science digital image file formats which in many cases solves problems due to working with microscopes made by different manufacturers. Therefore, we implemented Correlia as a Fiji plug-in benefiting from the reliability and platform independence of the Java (Oracle Corporation, 1998–). Arnold et al., 2005) programming language on the one hand and the sophisticated image-processing tools of ImageJ/Fiji on the other.

Image registration

Registration of micrographs in general requires (i) identification of similarities in source\textsuperscript{7} and target micrographs (feature or area-based), (ii) optimization of a cost function, (iii) setup of a transformation model and (iv) transformation and resampling of the source micrograph. In the case of multimodal data, detection and matching of corresponding features in the images are challenging, and suitable similarity measures which can be used to set up cost functions for automatic registration are needed. We therefore implemented feature as well as area-based similarity measures.

On the one hand, features in source and target image, such as notable small objects, can be matched and their mean distance serves as a cost function that can be minimized during registration. Automatic detection of those corresponding landmarks works well for relatively similar intensities of the features in the images as it is the case for instance when a total-ion-count image acquired by SIMS is registered onto an electron micrograph. Therefore, Correlia supports the usage of other ImageJ plug-ins for landmark detection and matching, e.g. with scale-invariant feature transform descriptors (Lowe, 1999). If features cannot be detected automatically, a manual selection can be carried out by the user. On the other hand, we implemented the well-known area-based similarity measure ‘mutual information’ (Collignon et al., 1995; Viola & Wells, 1997) in addition to the feature-based approach. To our knowledge, MI is not used by any other ImageJ/Fiji-related image registration plug-in presently available. MI is based on the assumption that there is a relationship between the pixel intensities with which certain objects are depicted in two images. If the images are aligned properly their joint intensity distribution is less dispersed than in misregistration cases where the object representations overlap only partially or not at all. Therefore, an algorithm for automatic area-based image registration was implemented. It follows the positive gradient of the MI in registration parameter space while at the same time controlling the step width until a maximum is found. Please note that this approach works particularly well for the registration of highly multimodal micrographs since MI can deal with arbitrary correlations of the pixel intensities.

In Correlia linear (rigid body, i.e. translation, rotation, scaling) as well as nonlinear (warping) transformation models are implemented. Images inside a Correlia project are transformed into continuous functions ($\mathbb{R}^2 \rightarrow \mathbb{R}$) using bilinear interpolation (i.e. a weighted average according to metric distances to the four next-neighbour pixels, obtained as linear interpolation in one direction followed by another in the other). This ensures that transformation operators can act globally without restrictions, like undefined values or discontinuities. For that, naturally, a correct calibration of the microscopes is required. and relative distortions in the micrographs – for instance due to aberrations – must be smaller than the required accuracy. If the micrographs in a correlative data set were acquired by nondestructive techniques in similar areas of the sample, a rigid registration of the images by mere rotation and translation (and possibly slight scaling) may be sufficient for alignment. Linear registration in Correlia is implemented in the common way using 2D transformation matrices acting on the interpolation function of the source image.

In many practical cases, rigid registration does not suffice, and a second registration step is needed in which the overlaid source micrograph requires nonlinear registration, i.e. slight warping to properly align with the underlying target micrograph. This is, for instance, necessary in order to correct for distortions caused by mechanical deformation of the sample introduced during the sequence of the analyses, e.g. shrinkage and/or crack formation during drying or consumption of the sample during a destructive measurement, e.g. SIMS. Warping is also required when multimodal image data are acquired from consecutive histological sections, as depicted on the left of Figure 1. Here, each micrograph exhibits a plane of the object with each plane being acquired at different sample

\textsuperscript{b} Fiji is just ImageJ.

\textsuperscript{7} Often “Source” is referred to as “moving image”. 

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Fig. 1. Two-step based registration of multimodal microscopy image data from consecutive histological sections. In the first step, rigid registration is employed to prealign the micrographs by rotation and translation. The subsequent warping corrects for deformations in the micrographs in order to achieve a high-quality registration.

In order to match the coordinate system of the source (image to be co-registered) with that of the target, the coordinates of the source are mapped to a ‘warped space’ by a nonlinear function providing a displacement vector field. Subsequently, the warped source image is calculated by copying the intensities accordingly onto the target image. Figure 1 displays an example of a two-step registration of multiple histological sections acquired by different microscopes.

For warping, a B-spline transformation model inspired by and partially based on the ImageJ plug-in bUnwarpJ (Arganda-Carreras et al., 2006) was implemented in order to treat smooth deformations. The piecewise application of polynomials enables locally variable image corrections. An important parameter for B-spline-based models is the density of the control grid. The finer the grid the more local a deformation can be, e.g. if an image appears distortion-free on a global level but has local artefacts, a fine grid is necessary for correction. In general, in correlative data sets deformations have to be expected on both, local and global level. Hence, a multilevel approach based on the algorithms described in Lee et al. (1997) is used such that all types of deformations can be treated. In particular, the cascaded correction of deformations starting with a coarse grid and successively refining it allows for choosing the appropriate scale for the correction. In other words, by choosing the lower and upper grid density a trade-off between smoothness and accuracy can be chosen.

Four warping methods were implemented, out of which two are using an analytical solution based on landmark correspondences and the others an iterative optimization approach with MI as the area-based similarity measure. These methods vary in both their flexibility and computational expense but share the same B-spline-based transformation model. They can be cascaded to use their respective advantages resulting in a unified model providing even more flexibility in the registration process. This final model can be efficiently applied to the deformed source image with minimized interpolation losses which is then resampled and visualised as described in the next subsection.

Visualisation

A meaningful visualisation of a correlative data set, naturally, will not display all co-registered micrographs overlaid in one image. Rather, different subsets of selected micrographs and false-coloured chemical maps are selected in order to visualise particular aspects of the data. For that, the micrographs in a correlative project can either be excluded from visualisation, overlaid fullyopaquely or overlaid additively in user-defined false colours. A user-selected false colour can be attributed to each micrograph for the visualisation. In Figure 2, an example representation of a correlative data set is displayed as it looks in Correlia. Micrographs in a Correlia project are not pixel-based but are continuous functions. This internal interpolation always allows for an export of the particular representation of the correlative data set at arbitrary field-of-view and resolution. Exported data are being disconnected from Correlia and become mere (calibrated) images in ImageJ/Fiji. Thus, properties are difficult to choose. We therefore believe that our approach is more intuitive to the user.

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the full toolbox available in ImageJ/Fiji can be used to further process and evaluate the data.

User interface

Correlia was designed to be an easy-to-use software that serves as a platform for registration and organization of a correlative microscopy data set compiled by multiple users. After the plug-in is correctly installed and started from within the ImageJ/Fiji environment (plug-ins menu) the Correlia user interface will open. It consists of two frames, the viewer and the control dialogue. The viewer visualised the actual representation of the data as described in subsection ‘Visualisation’. The control dialogue (left side of Fig. 3) lists all micrographs in the project. The panel on the right side of the control dialogue displays the micrograph that the user selected from the list. Furthermore, the user can manually shift, rotate and scale the micrograph via mouse-clicks or by editing the alignment parameters or use the automated methods for rigid registration by matching landmarks or maximizing MI in the source and target image. Project and image-related operations as well as export functions are accessible via the menu bar. Here, image data can be added to or removed from the project and additional information about the data can be attached for colleagues that are going to work on the sample in future.

The bottom right button switches the user interface into the advanced registration mode for the selected micrograph in which different nonlinear registration steps can be cascaded. In this mode, the left side of the dialogue shows a list of applied transformation methods, and the right side shows the resulting deformation as a vector field, and depending on the selected transformation the corresponding parameters. The used parameters for registration cascades can be saved in an XML file for future registration tasks on similar samples.

Results and discussion

In the following, typical applications for Correlia shall be illustrated using example data sets acquired at ProVIS – Centre for Chemical Microscopy.

The first example is a data set of a biofilm grown by microalgae onto a polyvinyl chloride carrier which was part of the work published in Moreno Osorio et al. (2019). In order

Fig. 2. A correlative data set measured on an algal biofilm visualised by Correlia: The light microscopy image (A) serves as background and is overlaid with a scanning electron micrograph (B, opaque) and two images of the red fluorescence of the algae (C, opaque and D, red). The chemical map of the phosphorous distribution measured by energy-dispersive X-ray spectroscopy (E, yellow) is co-registered on top (Moreno Osorio et al.,.)
Fig. 3. Control dialogue of Correlia for managing micrographs and rigid registration (left) and for cascading of multiple nonlinear registration steps (right).

to visualise the distribution of phosphorous in the early-stage biofilm, a combination of bright-field light microscopy, fluorescence microscopy, SEM and energy-dispersive X-ray spectroscopy was employed. Figure 2 displays a visualisation of a data set that illustrates its multimodal content as well as the different fields-of-view of the micrographs. In order to recognize the field of analysis conveniently in every microscope laser marks were burned into the sample. The bright-field light micrograph (A) serves as canvas (base image) for the registration and defines the coordinate system with respect to which an SEM micrograph (B), two fluorescence microscopy images (C) and (D) and the distribution of phosphorus in the sample as measured by energy-dispersive X-ray spectroscopy (E) were co-registered. The SEM was operated in secondary electron detection mode which allowed for surface sensitive analysis of the biofilm imaging mainly the extra-cellular polymer substances under which the algal cells are covered. The micrograph could easily be aligned using the line of the laser mark (Fig. 2B). The exact position of the cells under the extra-cellular polymer substances layer was determined in the fluorescence microscope. Due to the fluorescence of the chlorophyll, the algal cells are visible as red dots (Figs. 2C,D). Rigid alignment of the fluorescence maps was achieved using landmarks defined by cells that could be recognized in both the fluorescence maps and the SEM micrograph. For illustration of the different display modes available in Correlia, one map is displayed fully opaquely (Fig. 2C), the other in a semitransparent overlay (Fig. 2D). The energy-dispersive X-ray spectroscopy map of the phosphorous distribution (Fig. 2E, displayed in yellow) together with the fluorescence of the algal cells reveals, that in this stage of the biofilm, phosphorous is in the biofilm rather than in the cells. This example illustrates how a multimodal data set can be handled in Correlia and that the programme can cope with the different resolutions of the instruments as well as with different fields-of-view.

Figure 4 displays the correlation of SEM and nanoSIMS data acquired from consecutive histological sections with similar fields of view. The purpose of the study was to investigate the degradation of leaf-litter by microbes and study the flux of carbon from leaf to microbes. For that, the stable isotope (\(^{13}\)C) labelled sample of leaf-litter was dewatered and embedded in resin. By ultra-microtomy consecutive sections of 400-nm thickness were cut and put on silicon wafers. The study is going to be described in detail in Fabian et al. (2020).

On the first section, SEM analysis was employed to view the structure of plant cells as well as to identify the positions of microbes in the leaf-litter such that a suitable field-of-view for nanoSIMS analysis could be found. This field-of-view was then retrieved on the following section and a nanoSIMS measurement was conducted. The organic carbon was measured as \(\text{CN}^-\). Regions containing high amounts of the \(^{13}\)C label were identified using the calculated intensity map of the relative intensity of \(^{13}\)C\(^{14}\)N\(^-\), namely, \(\frac{I(13\text{C}^{14}\text{N}^-)}{I(12\text{C}^{14}\text{N}^-)}+I(13\text{C}^{14}\text{N}^-)\). In the first step, the nanoSIMS data were co-registered onto the SEM micrograph by rigid registration using the landmark-based approach, left side of Figure 4. However, since the measurements were done on consecutive sections, naturally, the shapes of the cells differ due to the different depths in which they were cut. In the second registration step, additional landmarks were distributed over the micrographs serving as a similarity measure to warp the nanoSIMS map in order to better match the shapes of the plant cells measured by SEM, Figure 4 right. In doing so in particular the central area as well as the ‘northern’ part of the micrographs were co-registered satisfactorily well such that the contours in the nanoSIMS map
match with the walls of the plant cells. In other words, in this experiment the $^{13}$C label mainly accumulated in the cell walls of the plant cells. Please note that the nanoSIMS signal is broadened compared to the SEM micrograph not only because of the lower lateral resolution of the nanoSIMS but rather because a crater is being sputtered (several layers) during the SIMS measurement over which the signal is averaged. Even though being beyond the scope of this paper it is worth mentioning that co-registered and warped nanoSIMS data can potentially gain lateral resolution from the SEM micrograph using image-fusion techniques. Vollnhals et al. (2017) demonstrated this possibility for transmission electron micrographs and nanoSIMS maps using Laplacian pyramids as well as intensity-hue-saturation techniques.

Finally, two handy features for the registration of chemical maps shall be mentioned: First, microanalytical techniques often collect many chemical or isotope maps at a time; in a nanoSIMS experiment, for example, up to seven isotope maps are obtained. Obviously, the parameters gained by the successful registration of one map will immediately co-register the others. Hence, connected micrographs can be linked in Correlia such that co-registering the most convenient one suffices to register the whole set. The second feature is some minimum support implemented for depth-resolved measurements with data organized in $z$-stacks. This is, for instance, the case in a SIMS measurement where layer by layer is sputtered away and analysed separately. Such data can be imported to a Correlia project, and an arbitrary slice of the stack can be selected for visualisation and registration.

Conclusions

We developed the ImageJ/Fiji plug-in Correlia for registration of correlative 2D microscopy data. Correlia is open-source software and can be downloaded from https://www.ufz.de/correlia. Unlike existing image registration tools, Correlia was designed specifically for the registration of highly multimodal data, in particular to correlate light, electron or ion micrographs with chemical maps obtained by different microscopic or microanalytical techniques. Correlia benefits from the flexibility of the Java programming language on the one hand and the full image-processing framework provided by ImageJ/Fiji on the other hand. There are no limitations for the data, e.g. support only for particular types of microscopes or manufacturers, as any calibrated micrograph that can be imported to ImageJ/Fiji can be imported to Correlia as well. With regard to the vast variety of different types of microscopy data that the user potentially wants to correlate and co-register, Correlia supports different registration methods that can be cascaded. For that, in every step of the registration process the user may decide upon rigid or nonlinear B-spline-based transformation models and choose from either feature or area-based similarity measures. In particular, MI, which to our knowledge is not available in ImageJ/Fiji so far, was implemented and is used as similarity measure for automatic area-based registration.

Correlia not only aims to be an image registration tool but additionally serves as a software platform for correlative 2D microscopy data. The data are organized in projects in which the raw data are preserved, whereas micrographs can be added or removed at any time and all registration steps are documented. The possibility to add annotations and vector drawings to the data in a Correlia project opens up an elegant way to exchange data between correlative working microscopists: regions-of-interest which are worth being analysed

9 From the tagged image file format, or from other formats using the ImageJ plug-in Bio-Formats provided by the Open Microscopy Environment: https://www.openmicroscopy.org/bio-formats.

10 This is a feature inherited from the ImageJ/Fiji environment.
can be indicated and annotations for further analysis can be shared with the data. The great advantage of the Correlia project as a means to exchange correlative microscopy data is that the whole data analysis is documented and can be tracked back. Correlia allows for extracting arbitrary combinations of correlated microscopy data and thus is ready to provide input to other image-processing tools, like image fusion software or automatic correlation finders such that information that is not available from the single measurements can be gained from correlative microscopy.

In future, Correlia may be extended with further registration methods or components such as a specialized transformation model for radial distortions. Also the integration of tools for interactive landmark-based registration, such as described by Bogovic et al. (2016), can be perceived as an alternative possibility to co-register micrographs in cases where the MI approach fails due to unsuit image properties.

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