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Image processing for precise three-dimensional registration and stitching of thick high-resolution laser-scanning microscopy image stacks

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ABSTRACT

The possible depth of imaging of laser-scanning microscopy is limited not only by the working distances of objective lenses but also by image degradation caused by attenuation and diffraction of light passing through the specimen. To tackle this problem, one can either flip the sample to record images from both sides of the specimen or consecutively cut off shallow parts of the sample after taking serial images of certain thickness. Multiple image substacks acquired in these ways should be combined afterwards to generate a single stack. However, subtle movements of samples during image acquisition cause mismatch not only in the translation along x-, y-, and z-axes and rotation around z-axis but also tilting around x- and y-axes, making it difficult to register the substacks precisely. In this work, we developed a novel approach called 2D-SIFT-in-3D-Space using Scale Invariant Feature Transform (SIFT) to achieve robust three-dimensional matching of image substacks. Our method registers the substacks by separately fixing translation and rotation along x-, y-, and z-axes, through extraction and matching of stable features across two-dimensional sections of the 3D stacks. To validate the quality of registration, we developed a simulator of laser-scanning microscopy images to generate a virtual stack in which noise levels and rotation angles are controlled with known parameters. We illustrate quantitatively the performance of our approach by registering an entire brain of Drosophila melanogaster consisting of 800 sections. Our approach is also demonstrated to be extendable to other types of data that share large dimensions and need of fine registration of multiple image substacks. This method is implemented in Java and distributed as ImageJ/Fiji plugin. The source code is available via Github (http://www.creatis.insa-lyon.fr/site7/fr/MicroTools).

1. Introduction

Laser-scanning fluorescent microscopy is a powerful tool for analyzing three-dimensional (3D) complex structures found in life sciences such as neuronal structures, which can be visualized using fluorophore-conjugated antibody labeling or targeted-expression of fluorescent proteins [1]. Combinations of multiple fluorescent markers and excitation filters can be used to highlight various neuronal objects, e.g., axons as well as pre- and postsynaptic sites, in the same specimen [2]. Such imaging techniques promise to give access, for instance in neurology, to connectomics which aims to produce a comprehensive and systematic analysis of the connections between brain regions and between numerous neurons within them [3]. The success of such analysis depends on the capacity to acquire in great detail, i.e., at the scale of the synapsis, the entire volume of the brain specimen.

Although laser-scanning microscopy can acquire images of thin optical sections from thick tissues [4], the possible depth of imaging is limited by three factors. First, the working distance of the microscope objective limits the depth for which images can be recorded. Second, signals become darker in deeper regions of the samples, because both excitation laser beam and emitted fluorescence are attenuated by the sample tissue that is optically not completely translucent [5]. This causes diminution of signal intensity and signal-to-noise ratio. And third, image quality degrades in deeper regions even when it is still within the working distance of the lens. Although new techniques such as clearing agents (e.g., CLARITY and Scale) can make sample tissues transparent [6,7], light rays are deflected and scattered when they pass through tissues. Deep objects therefore appear blurry and lose contrast.

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Thus, spatial resolution decreases with the depth from the sample surface. Even with the objectives with high numerical aperture and long working distance (more than 200 μm), image quality decreases considerably when the focal plane becomes as deep as 100 μm [8]. Two work around techniques have been employed to overcome this problem. For the specimen that is thinner than 300 μm along the optical axis (z-axis), it is possible to record the image from both sides of the sample that is embedded between thin cover slips. The first image substack covering the frontal half of the sample is taken from one side of the cover slip. The sample is then flipped, and the second image substack is recorded from the other side. For a thicker specimen, the sample can be embedded in a soft medium and using tissue sectioning (e.g., vibratome), a sample section is cut off from the top of the sample block after taking image stacks of this section. By repeating this procedure, called two-photon tomography, in principle any thick specimen can be imaged [9,10].

In both approaches, image substacks should be acquired in an overlapping manner: the overlapping sections will serve as a guide indicating how neighboring substacks can be concatenated, or stitched. However, such concatenation is not straightforward, because small rotation and translation can occur when the sample is flipped or when the block surface is cut off. Rotation can occur not only around the z-axis of the specimen but also around x- and y-axes (tilting). Moreover, because of the photobleaching that occurs during image stack acquisition and because of the different depths from the sample surface, the intensity of the corresponding optical sections in two overlapping stacks often appear different.

This is why registration is crucial for properly stitching image substacks of the same sample. Each neuronal fiber from neighboring stacks must be perfectly connected to each other after stitching. A discontinuity in the final image stack would strongly affect further analysis such as neuron tracing [2,11,12]. Registration is one of the most important general problems of image processing [13]. Therefore, there exists a wide offer of commercial or free, manual or automated, 2D or 3D, software platforms that address this issue. Application specific solutions, however, that are relevant for common types of samples and acquisition protocols, are often difficult to access for life scientist. Such a perspective could be judged as relatively narrow in a general computer vision context. However, as recently illustrated in this journal [14–17], it is actually specifically meaningful in biomedical imaging where important communities of life scientists work on the same types of samples.

In this study, we have developed an optimized automatic registration and stitching algorithm, 2D-SIFT-in-3D-Space, specifically adapted for thick high-resolution laser-scanning microscopy image stacks. A visual flow chart of the proposed algorithm is given in Fig. 1. We applied our method to stitch large 3D image stacks of Drosophila melanogaster brain samples that can be mutually tilted by up to 20°. Our algorithm, made available under the open source Fiji software that is widely used through the international bioimaging community, combines several existing approaches into a new strategy based on reliably detecting features in images using scale invariant feature transform (SIFT). In addition, to validate quantitatively the registration quality, we developed an original simulator that generates artificial 3D image stacks that mimic the properties of noise in laser scanning microscopy. We have used 2D-SIFT-in-3D-Space algorithm to assemble 3D image stacks of neurons of the Drosophila brain at a voxel resolution of 0.2 × 0.2 × 0.2 μm (1600 × 1600 voxels and 800 sections). The stitched dataset serves as a starting point for characterizing fine architecture of such large entire brain at unprecedented resolution but the 2D-SIFT-in-3D-Space is also shown to be useful to other types of datasets and other fluorescent microscopy systems (see supplementary data).

2. Related work

The primary purpose of the proposed registration method, 2D-SIFT-in-3D-Space, is to stitch two image substacks. The registration is achieved by comparing the signals of two overlapping image stacks. In this context,

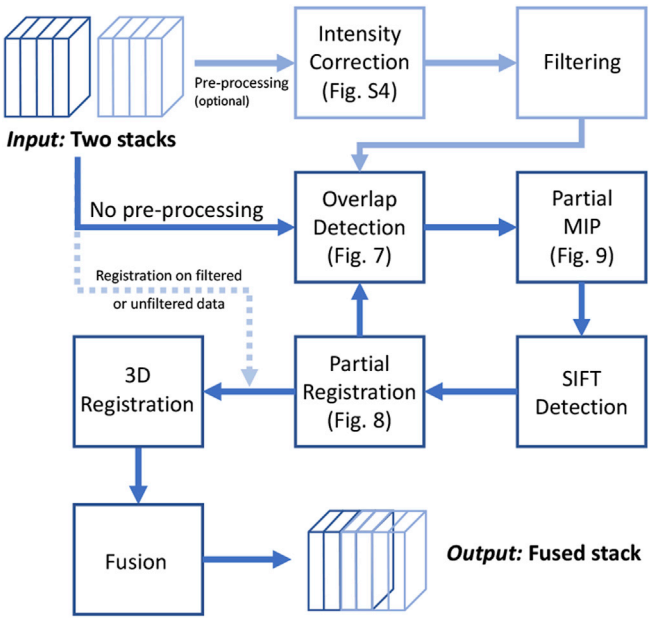

Fig. 1. Flow chart of the proposed automatic registration and stiching algorithm 2D-SIFT-in-3D Space.

it shares some similarities with existing registration algorithms known to be adapted to the bioimaging community interested in the registration of 3D images. A possible classification for image registration approaches is whether the registration is based on intensity or on features [18].

Intensity-based approaches confront, with a correlation metric, the intensity patterns in images to be registered. This includes for instance software solutions such as CMTK (Computational Morphometry Toolkit), elastiX, ANTS, AMIRA. Intensity-based approaches are specifically suited when the homologous structures to be registered in the 3D stacks are well represented by the intensities through the spatial statistics of the gray levels seen as a random variable (see for instance in spatial intensity [19], or with Fourier transform of the intensities [20], or also as recently used in Ref. [21]).

Feature-based approaches, as chosen and developed in this article, realize a correspondence between homologous landmarks in the images to be registered. Feature-based approaches are specifically suited when the images are characterized by spurious small structures (e.g., vessels and blobs) such as the one highlighted in the samples of neuronal fiber images considered in this article. Feature-based approaches works in two steps: First the detection of landmarks and second the match of the homologous landmarks in the images. In some software solutions the landmarks have to be detected with another software or selected manually. Manual positioning of such landmarks can be very time consuming and can also be perturbed by human errors. To circumvent this difficulty automatic detection of homologous landmarks is preferred. This can be achieved by detecting fiducial structures of known shape and size such as fluorescence beads that are purposely added to the sample [22] or endogenous granules in the tissue [23]. Registration error, however, would occur if samples and added beads were put in fluid mounting medium such as glycerol used in our study. Whereas [23] utilized endogenous aging-related pigment granules that are distributed across the mouse neural tissue, similar approach was not applicable to our samples because such granules do not seem to be distributed ubiquitously in young fly brains. Automatic detection of homologous landmarks can also be done purely numerically based on the extraction of local image features (See Ref. [24] for a review). The great advantage of feature-based registration is that instead of using all image intensities, it is possible to register two stacks using only corresponding salient points as a statistic of the image content. Reducing the problem from full resolution image content to a relatively small number of corresponding