

# Three-dimensional Reconstruction of Large Tissue Volumes From Scanning Laser Confocal Microscopy

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## ABSTRACT

Phase correlation is applied to the mosaicing of confocal scanning laser microscopy data. A large specimen (i.e., a murine heart) is cut into a number of individual sections with appropriate thickness. The sections are scanned horizontally and vertically to produce tiles of a 3D volume. Image processing based on phase correlation is used to rebuild the 3D volume and stitch the tiles together. Specifically, 2D registration of in-plane tiles and 3D alignment of optical slices within a given physical section are performed. The approach and performance are presented in this paper along with examples.

**Keywords:** Phase Correlation, Mosaic, Confocal Scanning Laser Microscopy

## 1. INTRODUCTION

The ability to analyze the organization of a relatively large (i.e., tens of millimeters) biological sample in three dimensions (3D) is becoming increasingly critical in biomedical research. Very high spatial resolution (sub-micrometer) can be obtained from confocal scanning laser microscopy (CSLM) [1,2]. However, because of light absorption and scatter, typically only 100–200 micrometer thick physical sections can be imaged at a time at low magnifications, and much less than this at higher magnifications. This necessitates cutting a large specimen into a number of individual sections with appropriate thickness. Each physical slice can then be imaged by recording a series (i.e., a stack) of images in parallel optical planes, defined by varying the axial focal depth. Therefore, imaging a sample with a large trans-axial cross-section requires a number of individual, in-plane images, referred to as “tiles,” which form pieces of a “mosaic”. The tiles have to be “stitched” or “glued” together to form a single integrated trans-axial image in a given optical plane. This process is referred to as mosaicing. Building a 3D image from 2D tiles obtained for a number of individual physical sections requires three steps: a) 2D registration of tiles, b) 3D alignment of reconstructed optical slices within a given physical section, and c) 3D alignment of reconstructed physical sections.

In this paper we describe the application of an image-processing procedure, based on the phase-correlation method, to enable the “fully-automatic” 2D registration of in-plane images (*i.e.*, tiles), and 3D alignment of reconstructed optical slices within a given physical section. The procedure has been tested on the whole set of CSLM images of murine heart (3D FOV = ~10 mm x 10 mm x 10mm).

## 2. MATERIALS AND METHODS

### 2.1. Introduction

Murine hearts fixed in 4% paraformaldehyde were sectioned (100-150  $\mu\text{m}$ ) on an Oxford vibratome. Sections were rinsed in PBS containing 0.1M glycine and 1% BSA and stained with a 1:20 dilution of rhodamine phalloidin (Molecular Probes, Eugene, OR) in PBS overnight at 4°C. Rhodamine phalloidin is a specific stain for f-actin, which is a primary component of the cardiac contractile apparatus. Imaging was performed with a 4 $\times$  (NA 0.2/WD 15.7) objective on a BioRad MRC1024 ES confocal microscope equipped with an Argon/Krypton laser. Each image consisted of a 512  $\times$  512 pixel array and each pixel was 4.83  $\mu\text{m}^2$ . Each optical Z-series through the vibratome sections was collected at 5  $\mu\text{m}$  intervals and, for each vibratome section, it was necessary to collect images from several overlapping areas to complete a montaged image of the entire heart. Data sets were converted from the BioRad *pic* format to standard uncompressed *tiff* images in order to be analyzed.

### 2.2. Mosaicing In-plane Tiles Into an Optical Slice

Denote by XY an in-plane coordinate system. Since the microscope stage moves only horizontally in the X and Y directions and does not perform any rotations, the relationship between overlapping tiles consists of a straight-line shift in the XY direction. As a result, an image-processing procedure derived from phase correlation can be designed for the mosaicing task. The **phase correlation** approach is based on frequency analysis of data correlation between two in-plane images, and stems from the property that translation in the space domain corresponds to a phase shift in the frequency domain [3,4].

Taking the inverse Fourier transform (IFT) of the phase shift, and then finding the maximum amplitude yields the XY displacement. Whenever the two images are identical in content and related by pure in-plane translation ( $X_0, Y_0$ ), IFT results in a delta function centered around the displacement ( $X_0, Y_0$ ).

Let  $f_1(x,y)$  and  $f_2(x,y)$  denote functions of pixel values in images 1 and 2, respectively [5]. Further, let

$$f_2(x, y) = f_1(x - x_0, y - y_0) \quad (1)$$

According to the Fourier shift property,

$$F_2(u, v) = F_1(u, v) e^{-j(u x_0 + v y_0)} \quad (2)$$

Where  $F_m(u,v)$  denote the Fourier transform of  $f_m(x,y)$ ,  $m = 1,2$ . The normalized cross power spectrum is given by:

$$\frac{F_2(u, v) F_1^*(u, v)}{|F_2(u, v) F_1^*(u, v)|} = e^{-j(u x_0 + v y_0)} \quad (3)$$

where \* denotes the complex conjugate. Taking the inverse Fourier transform (IFT) of the normalized cross power spectrum in (3) results in a Dirac delta function centered at  $(x_0, y_0)$ :

$$e^{-j(u x_0 + v y_0)} \xrightarrow[IFT]{} \delta(x - x_0, y - y_0) \quad (4)$$

This is shown in Figure 1.

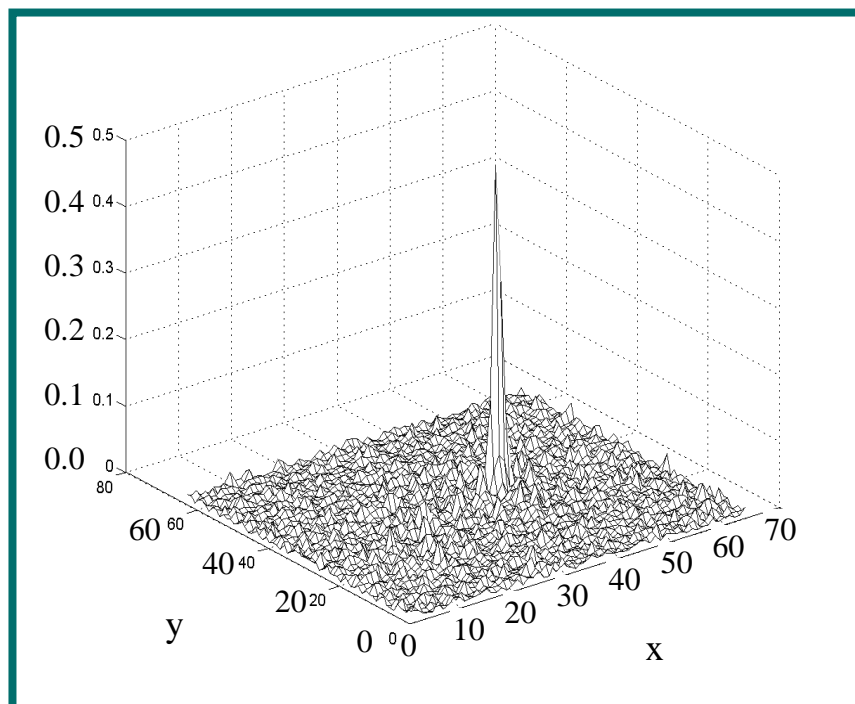


Fig. 1. Example of a Dirac Delta function centered around  $(x_0, y_0) = (40, 30)$  in the XY plane

In the case of confocal image tiles, the difference between the two images is not a simple shift (i.e., translation) but involves disjoint areas that cannot be found in both images at once. Some structures disappear from the image and other structures emerge when going from one tile to the other. This causes the appearance of high-intensity side lobes, while broadening and reducing the delta-function peak, thus making it difficult to find the location of the actual peak. Another major problem is that while the size of a tile and its FFT is limited to  $M \times N$ , the shift in phase can be up to  $2M \times 2N$  because of the extent by which one tile can move relative to another, as shown in the examples of Figure 2. Because of the  $M \times N$  limitation, a higher phase indication is “wrapped” into the smaller values making it difficult to decide whether the value obtained for a shift is a real one or a wrapped one. These factors have been successfully addressed in the present work.

The procedure was coded in MATLAB (The MathWorks, Inc., Natick, MA) and applied to each pair of adjacent tiles in each optical plane. Then, using one tile as a reference, an optical slice was built by mosaicing all the tiles of the optical plane. The approach was tested on all data and proved to be 99% successful. The flowchart of the algorithm is shown in Fig. 3.

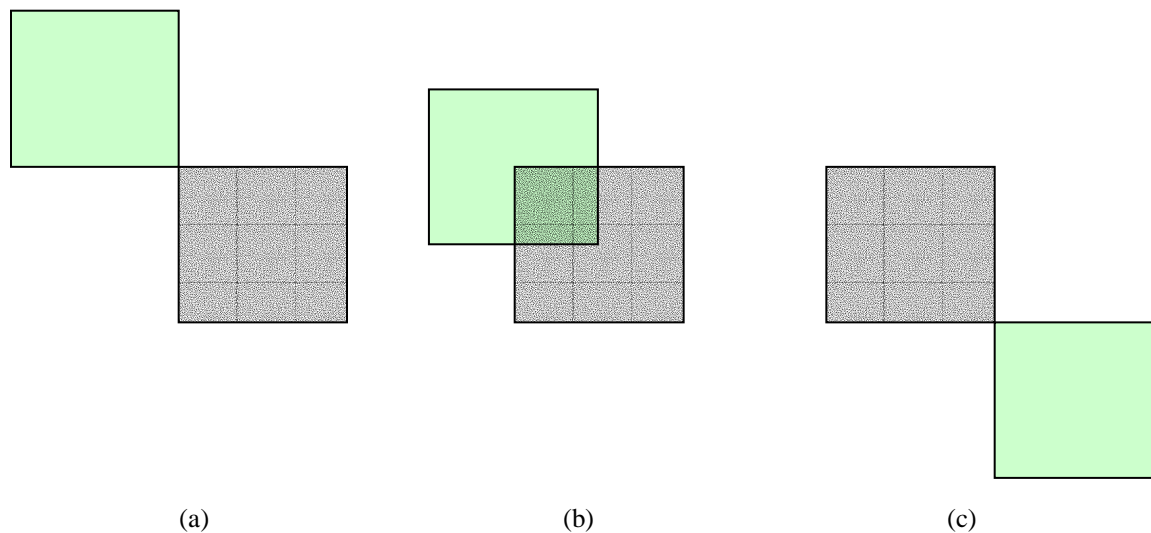


Fig. 2. Possible shifts between consecutive  $M \times N$  tiles: (a)  $x_0 = 0$  and  $y_0 = 0$ , (b)  $x_0 = M/2$  and  $y_0 = N/2$ , and (c)  $x_0 = 2M$  and  $y_0 = 2N$

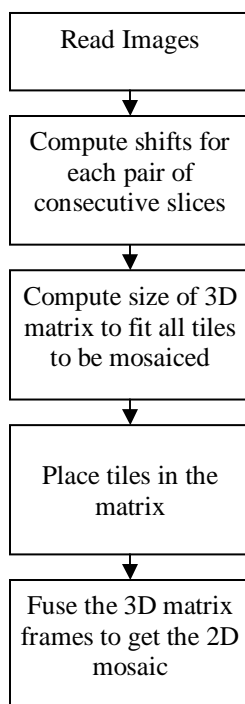


Fig. 3. Flow chart of the algorithm

### 3. RESULTS

Our test LSCM set obtained for a murine heart consists of 10,800 images (i.e., individual tiles) each of size  $512 \times 512$  pixels (262 ), forming a set of data with total size of 2.8 GB. The set contains 72 physical (vibratome) sections, each 150  $\mu\text{m}$  thick. Each vibratome section was divided into approximately 31 parallel optical planes (Z-series) 5  $\mu\text{m}$  apart. A 1.5 GHz PC computer with 1 GB of RAM was used to process all tiles in all optical planes. A total of 1,459 mosaic images were constructed. The total time elapsed was a) 22 hours to build individual-optical-plane images (2D mosaicing), and b) 28 hours to align all individual-optical-plane images within each vibratome section (3D mosaicing).

Figure 4 shows an example of consecutive tiles and the resulting mosaic.

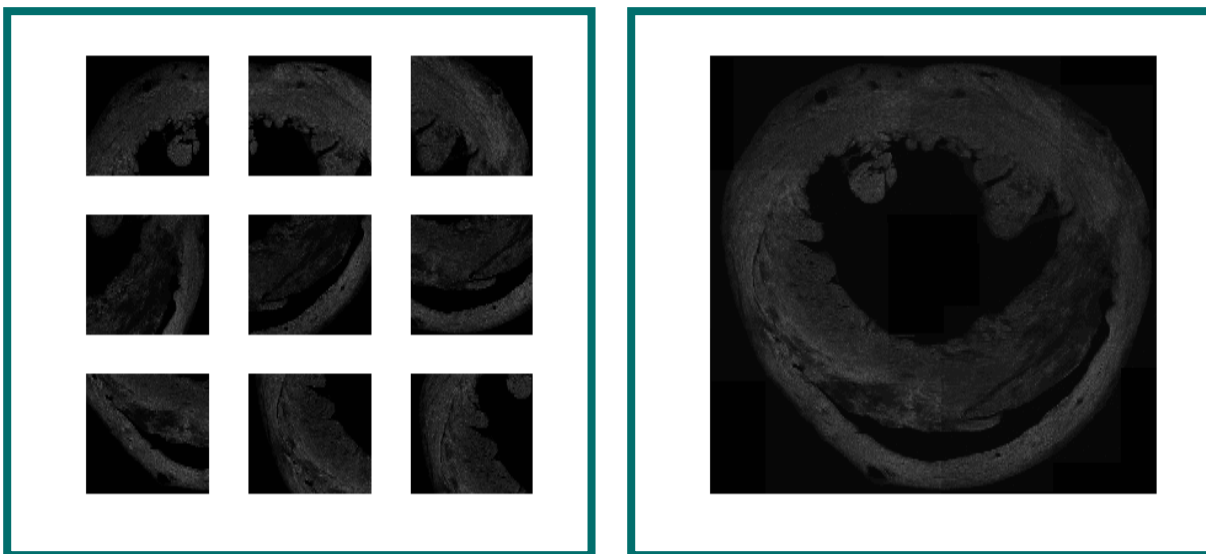


Fig. 4. Example of mosaicing: tiles (left), and resulting mosaic (right)

The quality of 2D image integration can be evaluated by inspecting line profiles through the images (e.g., as shown in Figure 5). Very similar bias, noise, and signal levels are evident in both profiles.

The only cases for which the phase correlation method failed were those that had very small or no overlapping regions between two consecutive tiles (Fig. 6-a). Even in those cases, however, the procedure worked successfully, just by rearranging the order of the consecutive tiles and choosing a different reference tile (i.e., starting tile). The corrected version of Fig. 6-a is shown in Fig. 6-b.

Because of noise in the images, the centroids of the local minima and maxima could be aligned only on average, resulting in possible small local misalignment. However, we estimate that the resolution loss attributable to this phenomenon is below 2 pixels.

Contrast variation among tiles corresponding to the same optical plane has not created any problems in the phase-correlation mosaicing procedure.

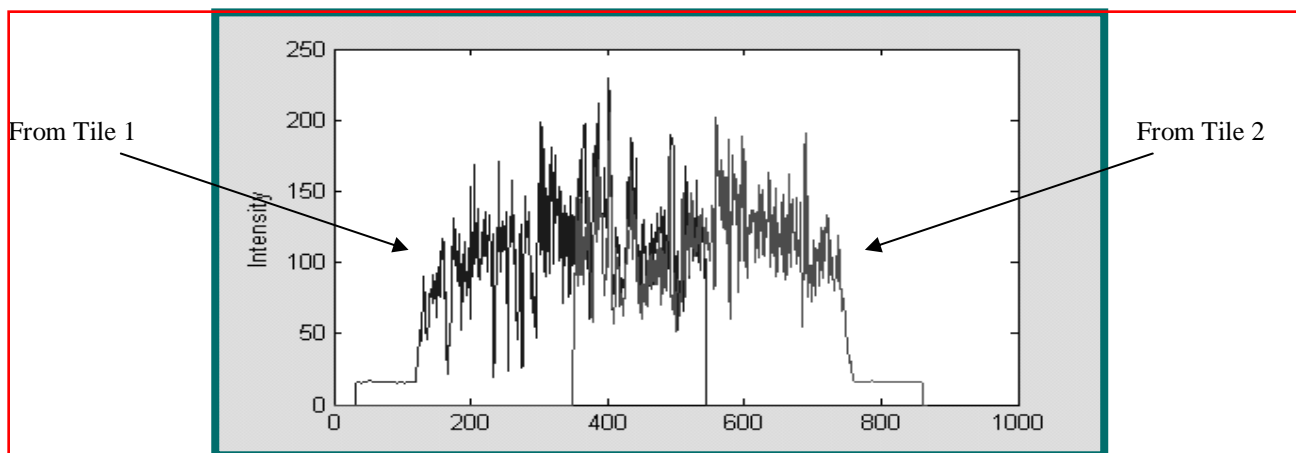


Fig. 5. Line profiles (row 200) through aligned tiles 1 and 2, physical section 43, optical slice 00 of tiles shown in Fig. 3

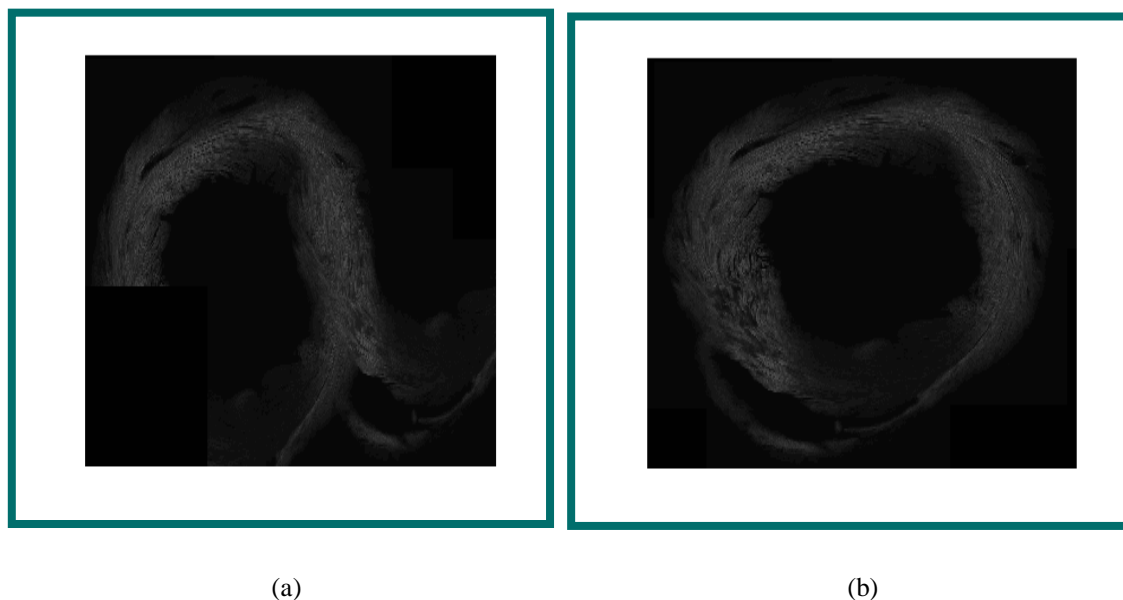


Fig. 6. (a) Incorrect optical-plane image attributable to insufficient or non-overlapping regions between consecutive tiles; (b) Correct optical-plane image 6, obtained after choosing a different reference tile

### Alignment of Individual Optical Planes within a Physical Section

Consecutive optical planes within a given physical section are acquired via vertical translation of the sample stage. Again, because no rotations are involved, this alignment can be achieved by finding the straight xy phase shift between consecutive optical planes. The same procedure, based on phase correlation, was used for this task with complete success. Figure 7 shows an example of two consecutive slices after alignment.

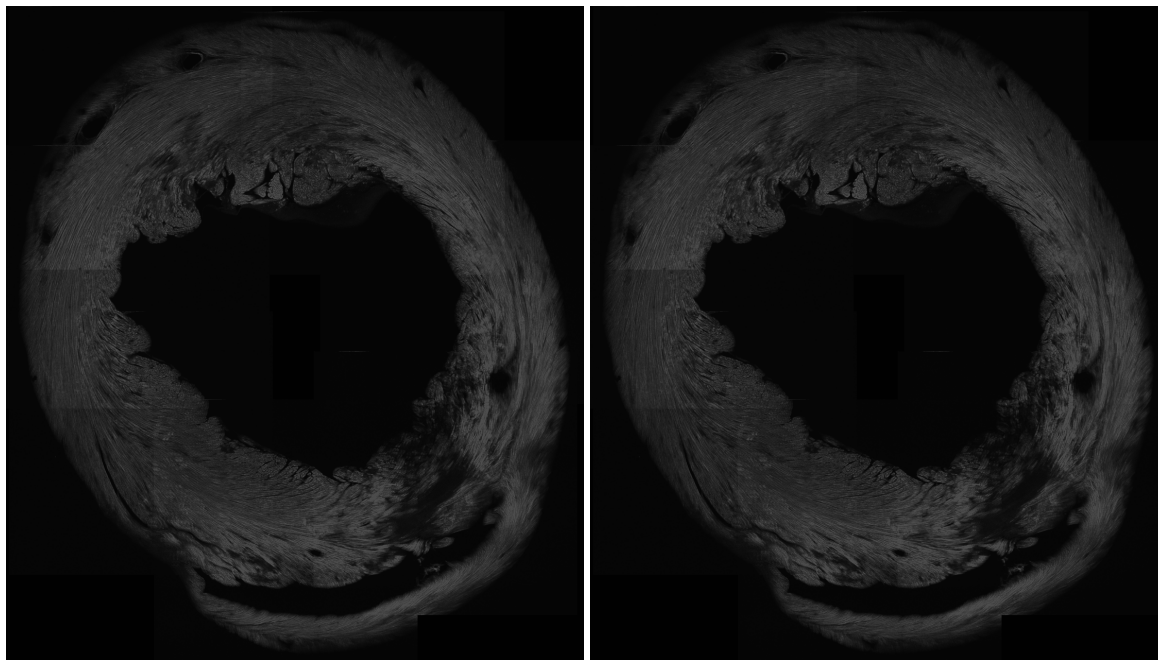


Fig. 7. Example of two consecutive optical planes within the same physical section aligned using the phase-correlation procedure

#### 4. CONCLUSION

The phase-correlation procedure was 99% successful in 2D mosaicing. The only cases for which the method failed were those involving optical planes that had very small or no overlapping regions between consecutive tiles (Fig. 6-a). However, even in those cases, the procedure worked successfully after rearranging the order of the tiles (Fig. 6-b). The 3D mosaicing using phase correlation was 100% successful in the case of optical planes within a given physical section.

The phase-correlation-based procedure offers a very efficient and robust approach to building a large 3D image from 2D tiles obtained by CSLM in a series of optical planes in the same vibratome section of the sample. The 1% failure rate in 2D mosaicing can easily be remedied by implementation of CSLM software that would a) perform error checking to detect inadequate overlap between consecutive tiles, and b) check whether all tiles in all optical planes have been acquired. In cases where there are such problems, rescanning would then be done automatically.

More work and a different approach are required to implement 3D registration of consecutive vibratome sections, since the process of sectioning is done manually. Manual sectioning results in loss of material ( $\sim 5\mu\text{m}$  thick), inadvertent relative rotation, tilt, and distortion of consecutive physical sections.

## 5. REFERENCES

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