3D Imaging and Analysis

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Using entropy for evaluation of colocalization of multimodal and multichannel microscopic images

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Collagen is an important component of the extracellular matrix (ECM) of connective tissue. Therefore, analysis of collagen deposition and remodeling is essential in tissue engineering. Second harmonic generation (SHG) imaging technique allows us to image non-centrosymmetric structures such as collagen in the ECM. The cells of human osteoblast cell line Saos-2 are able to grow extensively, and they can differentiate and produce ECM under specific conditions. They can be used for evaluation of biocompatibility of biomaterials for bone implants coatings, such as nanocrystalline diamond films (NCD) [1]. We have studied the production of type I collagen by Saos-2 after cultivation in differentiation medium. The native type I collagen fibers were visualized by SHG imaging, using a polarization filter with light planes rotated by 0°, 60°, and 120°, together with immunofluorescence staining and fluorescence microscopy, Figure 1. SHG signal provides information about collagen tissue that depends on direction of its fibers. When using the polarization filter under different angles, the excitation signal should interact more especially with collagen fibers that go along direction of the polarization angle. Thus, resulting SHG images are similar, but not the same, and should describe more precisely the morphology and formation of the fibers. However, as a result we get multimodal (fluorescence vs. SHG) and multichannel data (4 channels in this case, but even more can be obtained) that may be difficult to analyze. Colocalization analysis is understood as evaluation whether and how much two fluorescently labeled molecules are associated with one another. In our case we wish to analyze whether and how much amount of collagen mass detected in SHG data is produced by mass of Saos-2 cells detected in fluorescence data. Pearson's coefficient (PC) and Mander's coefficient (MC), e.g. [2], are probably the most popular approaches to colocalization analysis nowadays. They are restricted to a pair of images only; analysis of multichannel data is not easily possible. Moreover, when analyzing multimodal images, results obtained using them may be ambiguous. For example, when taking a look at Table 1-right column, we analyze here monomodal data (SHG 0° vs. SHG 60°) with distinct conclusion that images are well colocalized: PC $\rightarrow$ 1; MC $\rightarrow$ 1; scatter plot values are distributed along a linear function. A different situation is in Table 1-left column where we see the analysis of multimodal data (fluorescence vs. SHG 0°): PC = 0.5; MC $\rightarrow$ 0.5; scatter plot values are spread independently of the linear function. The conclusion about colocalization cannot be drawn here [2]. In the latter case we performed an additional "cross-correlation" analysis based on computing joint entropy of images using the discrete Kozachenko-Leonenko estimator, giving possibility to apply both multimodal and multichannel data [3]. We shifted the fluorescence image with respect to three SHG polarization images (1+3 multichannel analysis), ±10 pixels around the original position, pixel per pixel in both the x- and y-direction, computing the value of the entropy in each position using pixel values of all four images at the same time. The resulting mesh in Figure 3 shows values of entropy in the vicinity of the original position of the images. The mesh and corresponding line profiles demonstrate the maximum value of the entropy is not a random value, but it corresponds to a position of maximum similarity of the images; thus, all four images are truly colocalized. Conclusion: Multimodal and multichannel analysis of the vicinity of original position of images, based on Kozachenko-Leonenko entropy estimation, provides additional information about colocalization of images, and is useful in the real practice.

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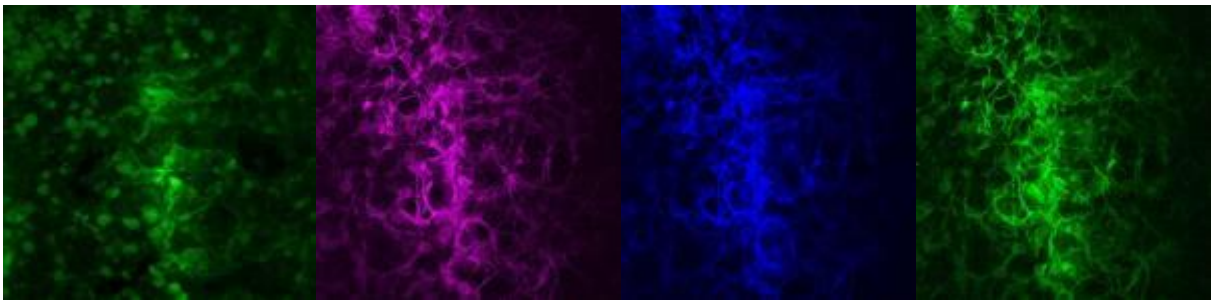


Figure 1. Microscopy images of human osteoblast cell line Saos-2. Left: a fluorescence confocal image (Leica SP2 AOBS); middle left: corresponding second harmonic generation (SHG) signal of collagen structures accompanying the cells using polarization filter with the light plane of 0°; middle right: SHG of 60°; right: SHG of 120° (Leica SP2 with a mode-locked Ti:Sapphire Chameleon Ultra laser (Coherent Inc., Santa Clara, California), tuneable from 690 to 1040 nm for TPE).

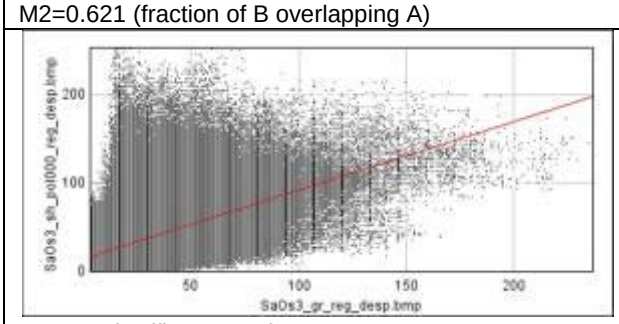
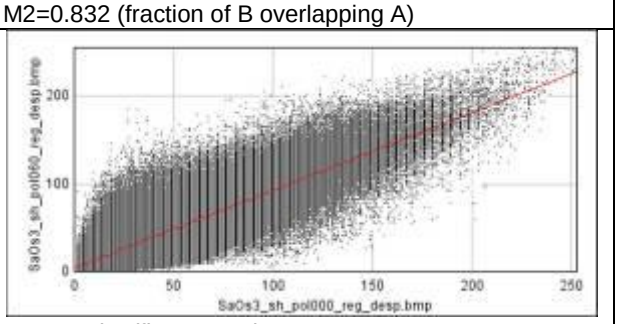
Fluorescence confocal vs. SHG (0°) image	SHG (0°) vs. SHG (60°) image
Pearson's Coefficient: r=0.5	Pearson's Coefficient: r=0.866
Manders' Coefficients (using threshold value of 32 for imgA and 43 for imgB): M1=0.641 (fraction of A overlapping B) M2=0.621 (fraction of B overlapping A)	Manders' Coefficients (using threshold value of 47 for imgA and 48 for imgB): M1=0.830 (fraction of A overlapping B) M2=0.832 (fraction of B overlapping A)
	
Scatter plot (fluorogram)	Scatter plot (fluorogram)

Table 1. Standard colocalization analysis of the images using JACoP plugin [2] of ImageJ software.

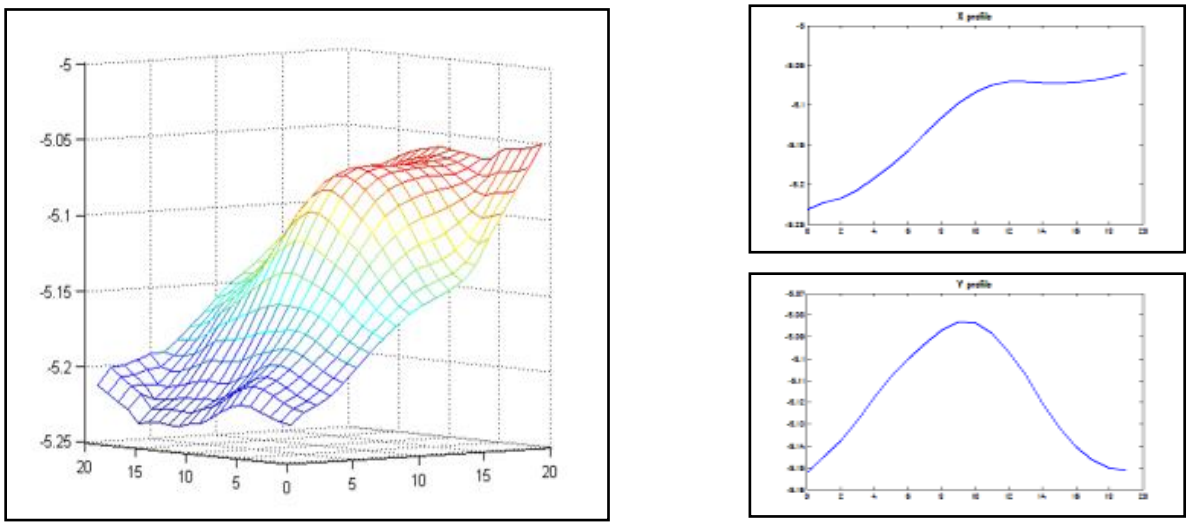


Figure 3. Left: mesh showing values of joint entropy of images in the neighbourhood of original position of the fluorescence image with respect to three SHG images (1+3 multichannel analysis) using Kozachenko-Leonenko entropy estimation; shifting ±10 pixels around the original position, pixel per pixel in both the x- and y-direction.