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Super-resolved 3-D imaging of live cells' organelles from bright-field photon transmission micrographs

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ABSTRACT

Current biological and medical research is aimed at obtaining a detailed spatiotemporal map of a live cell's interior to describe and predict cell's physiological state. We present here an algorithm for complete 3-D modelling of cellular structures from a z-stack of images obtained using label-free wide-field bright-field light-transmitted microscopy. The method visualizes 3-D objects with a volume equivalent to the area of a camera pixel multiplied by the z-height. The computation is based on finding pixels of unchanged intensities between two consecutive images of an object spread function. These pixels represent strongly light-diffracting, light-absorbing, or light-emitting objects. To accomplish this, variables derived from Rényi entropy are used to suppress camera noise. Using this algorithm, the detection limit of objects is only limited by the technical specifications of the microscope setup–we achieve the detection of objects of the size of one camera pixel. This method allows us to obtain 3-D reconstructions of cells from bright-field microscopy images that are comparable in quality to those from electron microscopy images.

1. Introduction

Bright-field microscopy is a classical method, favored for its convenience and ability to observe the physiology and morphology of unlabelled living cells and tissues. It avoids potentially complicated sample preparation procedures and visual artifacts due to complex optical paths and, in addition, is non-destructive. However, the main issue that hinders the segmentation and analysis of bright-field microscopy images [1–8] is the low contrast of structures in the focal plane caused by distortions from an object spread function (OSF), which is unknown for most objects. These distortions are particularly relevant in a biological context, as biological specimens are significantly thicker than the depth-of-field of typical bright-field microscope lenses [9] and also have particular physicochemical properties that lead to optical inhomogeneities and further complicate the OSF. Its analysis is in addition complicated by the dynamic nature of living cells, which causes spatiotemporal changes in the image. Finally, the discretization performed during image capture may also produce inaccuracies. The resulting standard bright-field microscopy image represents multiple processes and exhibits a multifractal character.

These issues impose several constraints on the type of algorithm and microscope appropriate for this task:

1. It is necessary to obtain the most real and natural images possible in order to discover the spectral properties of a cell's spread function. This can be carried out using a high-resolution camera equipped with an image sensor overlaid with a Bayer filter, capturing RAW files in a higher-bit colour depth and processing them using an non-interpolating algorithm [10,11]. Precise microscope mechanics should ensure the smallest possible movement along the z-axis.
2. The analytical method must be able to recognize spontaneous and random processes that underlie self-organization and multifractality [12]. Extracting the information from an image using Rényi entropy [13] parametrized by α ($\alpha \geq 0$ and $\alpha \neq 1$) serves as an appropriate basis for this task.
3. The method must be sensitive to diffraction, which is the main interactive process between light and cellular structures. Properties of the light wavefront that arises from diffraction and is projected at the objective lenses are described in full by Mie scattering theory [14]. Under the condition that the size of a particle is much larger than the wavelength of light, ray tracing techniques (geometry optics) provide a sufficient model for the characterization of the shape of the particle. Then, the behaviour of light at the

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Table 1
Microscope setup.

Cell	Series			Camera			Piezo[a]
	Number of img.[b]	Step (nm)	Time (min:s)	Offset	Gain	Exposure (ms)	
MG63-**a**	93 (155)	119	03:35.4	0	268	3327	Yes
MG63-**b**	128 (201)	150	10:22.7	266	347	2466	No
L929	173 (358)	158	11:09.0	221	336	2632	No

[a] If yes, the image series underwent image alignment (registration).
[b] The original number of image in the series before z-step selection is parenthesized.

interface of the strong diffracting object can be described by the phenomenon of total external light reflection and diffraction (**Supplementary Fig. 1b**).

4. The method must recognize the focus of the cell in its spread function. According to the Extended Nijboer-Zernike (ENZ) theory [15–17], the focus of a fluorescent and light-diffracting object is located at the position of the highest and lowest energy density, respectively (**Supplementary Fig. 1a**).

Here, we demonstrate a novel mathematical approach to reach superresolution in bright-field microscopy. This method, validated using atomic force microscopy, was applied to 3-D reconstructions and spectral and dynamic analysis of organelles and OSFs from z-stacks of bright-field microscopy images of live mammalian cells.

2. Results

The method is demonstrated on two cells of MG-63 human osteosarcoma (labelled **a** and **b**) from different cultivation batches and a cell of L929 mouse adipose tissue; the z-stacks of 12-bit bright-field microscopic RAW files were collected with an average z-step of 119, 150, and 158 nm, respectively. The detailed scanning conditions are described in Table 1. The z-stacks underwent image pre-processing such as vertical image registration (the MG63-**a** cell) and the removal of defective (dead and hot) camera pixels (the MG63-**b** and L929 cells) to avoid image defects, which, in addition, demonstrates the robustness of the method.

The overall preview of the image processing of the z-stack of the input data—12-bit RAW files with a cell of interest and background—with respect to the items mentioned above is shown in Fig. 1a and discussed in detail in the following sections.

2.1. Segmentation of a cell's focal region

In the first step, a cell of interest was segmented from its background by identifying green pixels whose intensities remain unchanged for each two consecutive RAW files (Algorithm 1, Fig. 1b). The intensities of the green pixels in each Bayer mask quadruplet were averaged to give quarter-resolved grayscale images [10,11], which were then subtracted. The unchanged intensities (i.e. zero values in the differential image) concurrently higher than 0 and lower than a 0.95-fold intensity mode of the cell-free second image contributed to the cumulative binary mask. In the focal region, these unchanged dark green pixels are the primary contributors to the cumulative binary mask (**Supplementary video 1**).

This binary mask was further processed by standard morphological operations—dilating the image (a 3 pixels disk-shaped structuring element), filling image holes (corresponding, in the original image, to the fluorescent objects and positive light interferences in the Airy diffraction pattern [18]), and filtering the cell of interest according to its specific features (in our case, as an object of the maximal size)—resulting in a final binary mask. The final binary mask of the cell was rescaled by a factor of two and applied to the whole z-stack of the original RAW files in order to distinguish a sum of point spread functions of the cell.

Computation of the binary mask from RAW files' red and blue pixels did not give the desired results. Due to the high frequency of consecutive pixels with constant intensities, the image of the cell merged with its background. The reason for this may be found either in light absorption in the infra-red and ultra-violet regions [19] or in lower photon quantum efficiency of the respective camera filters [20]. Therefore, in all segmentations, the green intensity wide range histogram was used.

The next step consists of selecting the focal sub-stack of the cell and assessing cell topography. The focal region of the z-stack was determined via clustering point information gain entropy density (Ξ_α) spectra [21] obtained for all RAW files of the separated cell. The variable Ξ_α [bit] was derived from the Rényi entropy as

$$\Xi_{\alpha,l} = \frac{1}{1-\alpha}\sum_{j=1}^{k}\log_2\frac{\sum_{j=1}^{k} p_{j,i,l}^{\alpha}}{\sum_{j=1}^{k} p_{j,l}^{\alpha}}, \tag{1}$$

where p_j and $p_{j,\,i}$ are the probabilities of occurrence of intensity j in an intensity histogram of the lth image in the z-stack with and without an element of the intensity i, respectively. The additive term $\frac{1}{1-\alpha}\log_2\frac{\sum_{j=1}^{k} p_{j,i,l}^{\alpha}}{\sum_{j=1}^{k} p_{j,l}^{\alpha}}$ is called a point information gain ($\Gamma_{\alpha,j}$, bit) and can determine an information contribution of intensity j to the intensity histogram obtained from either the whole image (a global measure $\Xi_{\alpha,Wh}$) or its part (local measures). For image processing of the presented cells, we used local values evaluated from pixels either on the vertical-horizontal cross ($\Xi_{\alpha,Cr}$) or on a 9 pixels circle around the examined pixel ($\Xi_{\alpha,Circle}$). The kind of local information was chosen according to the distribution of intensities in the image. Whereas the z-stacks of the MG63-**a** and L929 cells suffered from cross camera noise, the images of the MG63-**b** cell did not (**Supplementary videos 2 and 3**). In the latter case, the 9 pixels circular type of surroundings approximately traced the borders of intracellular structures.

For the overall multifractal characterization of the images, Ξ_α-spectra were calculated for a set of $\alpha = \{0.1, 0.3, 0.5, 0.7, 0.99, 1.3, 1.5, 1.7, 2.0, 2.5, 3.0, 3.5, 4.0\}$, for each colour channel separately. While the values $\Gamma_{\alpha,j}$, and consequently $\Xi_{\alpha,j}$, for the red and blue channels (indexed $_R$ and $_B$, respectively) were computed by eliminating one element of intensity j from the respective intensity histogram, these values for the green pixels (indexed $_G$) were obtained via eliminating two elements that were relevant to the intensities of the Bayer mask quadruplet.

Matrices composed of vectors that specify each image l in the z-stack via α-dependent subvectors of the respective information context in the respective colour channel, i.e.

$$\Xi_{(l)} = [\Xi_{\alpha,Wh,R}, \Xi_{\alpha,Wh,G}, \Xi_{\alpha,Wh,B}, \Xi_{\alpha,Cr,R}, \Xi_{\alpha,Cr,G}, \Xi_{\alpha,Cr,B}] \tag{2}$$

for series of the MG63-**a** and L929 cells and

$$\Xi_{(l)} = [\Xi_{\alpha,Wh,R}, \Xi_{\alpha,Wh,G}, \Xi_{\alpha,Wh,B}, \Xi_{\alpha,Cr,R}, \Xi_{\alpha,Cr,G}, \Xi_{\alpha,Cr,B}, \Xi_{\alpha,Circle,R}, \Xi_{\alpha,Circle,G}, \Xi_{\alpha,Circle,B}] \tag{3}$$

for the series of the MG63-**b** cell, were standardized with z-scores and underwent k-means clustering (squared Euclidean distance metric, 50 iterations) into two groups (Algorithm 2). Due to the