New insights into information provided by light microscopy: application to fluorescently labelled tissue section

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Abstract

**Background:** Light microscopy is a standard examination tools in cell biology. However, optical microscopy as well as image acquisition and processing suffer from physical and technical prejudices which preclude a correct interpretation of biological observations which reflects in, e.g., medical and pharmacological praxis. Using the examples of a diffracting microbead and fluorescently labelled tissue, this article clarifies some ignored aspects of image build-up in the light microscope and introduce algorithms for maximal extraction of information from the 3D microscopic experiments.

**Results:** We provided a correct set-up of the microscope and, using the approach of information entropy in image processing, we sought a voxel (3D pixel) which localizes the information about the object. In diffraction imaging and light emission, this voxel is an intensity minimum and maximum, respectively. This approach further enabled us to identify z-stack of a DAPI-stained tissue section where at least one object of a relevant fluorescent marker was in focus. The spatial corrections (overlaps) of the DAPI-labelled region with in-focus autofluorescent regions then enabled us to co-localize these three regions in the optimal way when considering physical laws and information theory.

**Conclusions:** We demonstrate that superresolution down to the Nobelish level can be obtained from commonplace widefield bright-field and fluorescence microscopy and bring new perspectives on co-localization in fluorescent microscopy.

**Keywords:** Electromagnetic centroid; Point divergence gain; Superresolution; 3D Structure reconstruction

Introduction

Light microscopy is often the only suitable source of information about an internal structure of (semi-)transparent objects. Here we analyze deviations of the intensity profile of the electromagnetic field (light) due to interactions of light with matter and due to its modification along the optical path of the microscope [1]. In interactions with living cells, there are observed mainly: (i) the diffraction of light on particles and (ii) the emission of fluorescent light after absorption.

In biology and medicine, a microscope is a tool which provides answers to specific questions about the function of cells, tissues, and consequently organs. The boom of specific fluorescent labels gave rise to a high amount of pseudocoloured images, in most cases interpreted from a summary projection of images which were captured
at one “compromise” or “optimal” focal plane. The analytic approach given in our previous papers [2, 3, 4] provides tools for the detection of spatially in-focus objects. This analysis can provide so far hidden information.

The diffraction of light is a process whose complete explanation is complicated and, for the majority of real cases, practically impossible [5]. A common view of light passing behind a scattering object is a picture of a dark “cone” gradually contracting as the Huygens waves enfold the gap. When the object is circular enough, a bright spot on axis, called the Arago spot [6], arises. Biological objects consist of dense matter made of proteins, nucleic acids, lipids, and other molecules. Diffracting elements in the cell interior differ in their refractivity index from that of their surroundings and are internally inhomogeneous, as well. Light scattering can characterize the spectral properties of intracellular objects properly. All we obtain after the light passes through the biological sample and the microscope is ‘information’ from the microscopy experiment. About the origin of this kind of information, we have only limited knowledge.

Fluorescent microscopy of a living cell [1] has numerous advantages over the diffraction observation, namely a very small light source that is a single chemical bond. This enables experimentalists to explore and exploit different limits of the modification of light along the light path of the microscope separately. The breaking of the long-established resolution limit [7] of the light microscope was awarded the Nobel Prize [8]. These so-called superresolution methods are theoretically based on the description of observed phenomena using the Maxwell theory of the electromagnetic field [9]. This theory preceded quantum mechanics with its additional uncertainty limit given by the Heisenberg principle [10], which describes a single photon and is seemingly contradicted by superresolution. The Maxwell theory, in contrast, is a proper description of the behaviour of an ensemble of photons. When the maximum of this ensemble is sought, superresolution is not in conflict with contemporary theories of light.

As we have shown [2, 3], the information reaching the screen of a digital camera can be scrutinized down to the level of a single sensor element of the digital camera, i.e. of a single pixel in a digital image. In other words, the information limit is given by the size of an element which is theoretically projected on a single pixel of the digital camera. The size of this elementary information may be experimentally determined and can have an area of a few squared nanometres. As discussed above, no principles of quantum mechanics are broken if we determine the location of the distribution profile for a sufficiently large ensemble of photons. In such a distribution it is then possible to seek a maximum, a median, skewness, etc.

The location of the maximum or minimum of the intensity profile does not determine the position of the object, which causes the change in the electromagnetic field profile. For instance, in diffractive imaging the darkest and smallest point is located outside the object at a position governed by the diffraction process. The experimentally determined light intensity maximum or minimum is found, in the proper definition (for all types of imaging), as a centroid of the outcome of the electromagnetic process. Later in the text, this point is called the electromagnetic centroid.

The analysis of information from the digital image also includes a description of all non-idealities of the optical path. When a colour digital camera is used, each
camera channel typically detects different information [2, 11, 12]. This difference is typically associated with the differences in chemical composition of the object that gives rise to the signal. This interpretation is ultimately true, but the exact way of the transfer of this difference to the camera chip by the microscope needs to be examined carefully. For instance, modern apochromatic objectives utilize combinations of lenses to project all colours at the same place. This assumption is indeed valid only for idealised samples and with a finite precision. When minute details of the microscopic image are interpreted, the apochromaticity cannot be expected and the properties of the light path should be experimentally examined for each lens separately.

As we have summarized [3], the microscopic observation should answer,
1 where the object giving rise to the response is located,
2 what the shape of the object is, and
3 what the spectral characteristics of the object are.

We have analyzed a response of a standard object – a single microparticle – and a section of fluorescently labelled tissue. We have systematically determined the electromagnetic centroid of the diffracting object as a centroid of the information in 3D space with the precision of a single voxel (3D pixel). We have applied the approach to widefield fluorescence micrographs of a tissue section in which the electromagnetic centroid and the projected position of the light emitting object are at the same place. Specific algorithms were developed to detect different modes of binding of fluorophores, fluorophore densities and intensities of the emission. These analyses demonstrate the power of the approach.

In this article, we also demonstrate that the specificity of the fluorescent labelling can be improved. We demonstrate the enormous increase of the intelligibility of the fluorescence image data. Thus, cell microscopy is dominated by an idea that if, in an image, there are two colours (emitted wavelengths) projected at the same point, then these colours are co-localized. However, when we analyze the image signal in 3D, we can determine that each colour (wavelength) is projected onto a different point of space. The conclusions on co-localization can be made only after alignment of the focal planes of the colours and we provide a tool for such a correction.

**Materials and methods**

**Experiment on latex particle**

A latex particle of the diameter of 2000 nm was placed on a carbon layer on an electron microscopy copper grid covered by amorphous carbon (prepared at the Institute of Parasitology AS CR, České Budějovice, CZ). The sample was scanned under an optical transmission microscope [2, 4] – nanoscope (Institute of Complex Systems, Nové Hrady, CZ) – equipped by a 12-bit colour Kodak KAI-16000 digital camera with a chip of 4872×3248 resolution (Camera Offset 200, Camera Gain 383, Camera Exposure 2950 ms). A Nikon objective (60×/0.8, ∞/0.17, WD 0.3), which gives the resulting size of an image pixel as 46×46 nm², was used. The sample was illuminated by two Luminus 360 LEDs charged by the current of 4000 mA. The standard deviation of the z-step was minimized by the pngparser.exe software [2] which gave a z-stack of 258 images of the average step of 152 nm.
Experiment on prostate cancer tissue
A fixed sample of prostate cancer tissue was immunofluorescently labelled by 4',6-diamidine-2'-phenylindole (DAPI). The treated sample was scanned using a TissueFaxs PLUS fluorescent microscope (TissueGnostics GmbH, Vienna, AT) equipped by a grayscale camera with a chip of 1560×1960 resolution. A 100× oil objective gave the resulting image pixel of the size of 328×328 nm$^2$. The microscope’s step along the z-axis was 100 nm. The full z-stacks (DAPI in the blue channel and autofluorescence in red and green channel) contained 81 14-bit images. The real z-position of each image was read out from the name of the image.

Image processing and visualization
Processing of primary microscopic data was similar to the procedure published previously in [2]. Figures in the article were visualized using the LIL conversion into 8 bits and plotted using Matlab$^\text{R} 2016$b (Mathworks, USA) software. The original and processed image sets and relevant Matlab algorithms are available at [14].

Latex bead
Processing of the 12-bit raw files (258 images; Fig. 1) of the point spread function (PSF) of the 2-µm bead started with its segmentation (Algorithm 1), when for each pair of two consecutive images, we found all green pixels (in a GBRG Bayer mask) of unchanged intensities darker (lower) than the intensity mode of the surroundings of the PSF. It created a cumulative binary mask which was applied to the whole series. So-segmented images of PSF underwent the 8-bit least information loss (LIL) conversion [11] which enables the systematic merging of the intensity bins with the preservation of the maximum amount of information and to compare intensities of images throughout the whole stack. The images of reduced bit-depth were further characterized by $\alpha$-dependent spectra of point divergence gain entropy

$$I_{\alpha} = \frac{1}{1-\alpha} \sum_{i=1}^{n} \log_2 \frac{\sum_{j=1}^{m} p_{k/l}^\alpha}{\sum_{j=1}^{n} p_{i}^\alpha}$$

and point divergence gain entropy density

$$P_{\alpha} = \frac{1}{1-\alpha} \sum_{i=1}^{m} \log_2 \frac{\sum_{j=1}^{j} p_{k/l}^\alpha}{\sum_{i=1}^{m} p_{i}^\alpha}$$

for the set of Rényi coefficient $\alpha = \{0.1, 0.3, 0.5, 0.7, 0.99, 1.3, 1.5, 1.7, 2.0, 2.5, 3.0, 3.5, 4.0\}$ and for each colour of pixels separately. In Eqs. 1–2, the $p_{k/l}$ is a probability of the occurrence of the pixel of intensity $k$ after exchanging for the pixel of intensity $l$ at the same position in the following image and the $p_{i}$ is a probability of the occurrence of each intensity in the image with the intensity $k$. Variable $i = \{1,2, ..., k, ..., l, ..., j\}$ is the label of a bin in the intensity histogram; the $n$ corresponds to the number of pixels in the image channel and the $m$ corresponds to the number of pixels of unique values of the point divergence gain (see Eq. 4). For the red and blue pixels of the Bayer mask, Eqs. 1–2 were used as written, while in case of the green pixels, two relevant pixels of the Bayer mask were exchanged.
This type of calculation was performed using an Image Info Extractor Professional software (IIEP; Institute of Complex Systems USB, Czech Republic) while omitting black (zero) pixels, i.e. while omitting the uninformative background from the image intensity histograms. All resulted α-dependent spectra of the $I_\alpha$ and $P_\alpha$ (for all three colour channels of each image) gave a matrix of the size of 2588x78. Each row of this matrix (vector) was normalized by calculation of the z-score (SNV)

$$z = \frac{x - \mu}{\sigma},$$

where $x$ is a value of $I_\alpha$ or $P_\alpha$ in the vector and $\mu$ and $\sigma$ are the mean and the standard deviation of the relevant vector, and clustered by the standard k-means++ algorithm (with squared Euclidian metrics [13]) into two (in-focus and out-of-focus) groups. The middle – in-focus – image subseries was separated. In this region, the pixels demarcated by the darkest green isocontour (intensity 1000) were segmented from each z-level and the obtained images were further processed into a 3D model of the bead’s PSF.

In order to create the model, we firstly computed the point (set-up of the IIEP software: a GBRG mask, ignore black pixels) divergence gains

$$\Omega_{\alpha,k/l} = \frac{1}{1 - \alpha} \log_2 \frac{\sum_{i=1}^{j} p^\alpha_{k/l}}{\sum_{i=1}^{j} p^\alpha_i},$$

which are parts (a summand) of Eqs. 1–2 and define information changes after an exchange of one pixel for another one at the same position in the next image. The best results of the computation were achieved for $\alpha = 4$. For each pair of the in-focus series, the positions of all zero $\Omega_{\alpha,k/l}$ were found to give a binary mask for extraction of relevant points of the PSF (lines 1–14 in Algorithm 2). In this way, we modelled a theoretical interlayer of the bead’s PSF. After stacking these layers into a 3D matrix, intensity minima and maxima as the responses of the diffraction phenomena were localized for each colour channel separately.

**Fluorescently labelled tissue section**

The z-stacks of the fluorescing prostate cancer tissue (Fig. 2) were processed in a simpler way. It began with the selection of an in-focus region via computation of the $I_\alpha$- and $P_\alpha$-spectra for a set of $\alpha = \{0.1, 0.3, 0.5, 0.7, 0.99, 1.3, 1.5, 1.7, 2.0, 2.5, 3.0, 3.5, 4.0, 5.0, 6.0\}$ and continued with the clustering as described for the bead’s PSF, only without normalization of the spectra via z-score. With this procedure, we found the in-focus range from img. 26 to 77 for red autofluorescence and DAPI and from img. 1 to 66 for green autofluorescence. The localization of the zero $\Omega_{\alpha,k/l}$ at $\alpha = 6$ for red and green autofluorescence and at $\alpha = 7$ for DAPI then helped to reconstruct the fluorescent objects which were further selected on the basis of either Otsu’s thresholding or by a combination of the image morphological operations (Algorithm 2).

Since the in-focus series of red autofluorescence and DAPI had the same number of images and red and green autofluorescence are expected to mark the same (similar) intracellular structures, the in-focus images of fluorescent labels were co-localized.
via overlapping of the relevant images of the red autofluorescence and DAPI followed by the alignment of the information foci (the image with the lowest value of the \( I_{0.99} \) in the green channel) of green autofluorescence with red autofluorescence.

### Results

**Electromagnetic centroid of a diffractive object**

In each colour (red, green, and blue) channel, the intensity distributions (PSFs) which are a consequence of the interaction of light with the object have different shapes and positions. Fig. 3c shows a common microscopic image of the simplest object – a latex particle of the diameter of 2 \( \mu \text{m} \) – at the position of information focus.
Input: \( N \) raw files from bright-field microscope
Output: BM as a binary mask for export of the PSF

\[
\begin{array}{l}
\text{for } i = 1 \text{ to } (N - 1) \text{ do} \\
\text{raw1} = \text{readIm}(i); \\
\text{raw2} = \text{readIm}(i+1); \\
\text{G1} = \text{debayerG}(	ext{raw1}); \\
\text{G2} = \text{debayerG}(	ext{raw2}); \\
\text{difG} = \text{double(G2)} - \text{double(G1)}; \\
\text{zeros} = \text{uint(difG} == 0); \\
\text{constG} = \text{G2} \ast \text{zeros}; \\
\text{mode} = \text{BgIntMode(G2)}; \\
\text{darkConstG} = (0 < \text{constG} < 0.95 \ast \text{mode}); \\
\text{cumG} = \text{cumG} + \text{darkConstG}; \\
\text{BM} = (\text{cumG} > 0); \\
\text{BM} = \text{erodeIm(BM}, \text{disk3}); \\
\text{BM} = \text{fillHoles(BM)}; \\
\end{array}
\]

Algorithm 1: Creation of a binary mask for segmentation of a point spread function of a microbead from a z-stack of optical bright-field transmission micrographs

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Input: a focused region of \( N_f \) grayscale images (of the size of \( x \times y \) pixels) from fluorescent microscope
\( r-1 \) matrices of the \( \Omega_{\alpha,k/l} \) (in .mat files)
M as a zero \( x \times y \times N_f \) matrix

Output: distM as a 3D matrix of large fluorescently labelled objects
otsuM as a 3D matrix of strongly fluorescing objects

\[
\begin{array}{l}
\text{for } i = 2 \text{ to } N_f \text{ do} \\
\text{PDG1} = \text{readMat}(i); \\
\text{PDG2} = \text{readMat}(i-1); \\
\text{zPDG1} = (\text{PDG1} == 0); \\
\text{zPDG2} = (\text{PDG2} == 0); \\
\text{join} = (\text{PDG1} + \text{PDG2}); \\
\text{BI} = \text{uint(join} > 0); \\
\text{img} = \text{readIm}(i); \\
\text{M}(i-1) = \text{img} \ast \text{BI}; \\
\text{distM} = \text{remSmallObj(M)}; \\
\text{otsuM} = \text{otsu(M)}; \\
\end{array}
\]

Algorithm 2: Algorithm for 3D reconstructions using zero \( \Omega_{\alpha,k/l} \)
In each colour channel, we can experimentally determine two smallest objects along the diffracting object’s (bead’s) PSF: (1) A dark spot is an outcome of the shading, non-idealities of light behaviour and destructive light diffraction, and (2) a bright spot, which is surrounded by dark intensities and is a real-life manifestation of the Arago spot [6] (Fig. 3c).

Figure 3 3D intensity maps of the 2000-nm latex bead in the red, green, and blue image channels. The minimal/maximal intensities are 623/2706 (R), 759/4095 (G), 430/3752 (B). Voxel size is 46 nm (horizontally) and 152 nm (vertically). a) Sections in the xz- and yz-plane, respectively. b) Positions of electromagnetic centroids (colour-coded). The bigger points and the smaller points correspond to negative and positive light interferences, respectively. The bright spot in the green channel is not sufficiently resolved due to the saturation of the signal. The positions of xz- and yz-planes relevant to a are highlighted by bold lines. c) Image 127 from a z-stack of microscopic images of a 2000-nm latex particle whose 3D PSF and electromagnetic centroids are depicted in a–b.

Fig. 3a depicts the xz- and yz-planes of the 3D microscopic image of the latex particle in Fig. 3c. It is a combination of real-life diffraction behaviour, the mixed response of different wavelengths, which are summed in each colour channel, and of all non-idealities of the microscope optics. In other words, the detected intensities are combinations of an interaction of the light electromagnetic field with the sample followed by a transformation of the intensities by the microscope optics. Fig. 3b shows the positions of the dark and bright electromagnetic centroids in each colour channel. The bright green maximum (Fig. 3b, left) consists of several points of
identical intensities due to the saturation of the 12-bit intensity signal at high intensities.

Fluorescence microscopy of tissue sections

Fig. 6 shows a typical microscopic image of a fluorescently labelled tissue. The observed intensities arise as a result of the fluorescence emission of fluorophores at particular positions in the sample. The fluorophores change their spectral properties and quantum efficiency in response to the environment. Better information about the positions of the fluorophores can be obtained via information analysis of the given z-stack using the point divergence gain [2, 4].

The z-stacks were acquired in a way that for each fluorophore the microscope found a focus automatically and the microscope was then set-up to scan 40 images symmetrically below and above this focus. In these ranges of 81 images, the information-entropic analysis (Sect. ) identified in-focus images where at least one fluorophore should be in the focus. Similar to the standard autofocusing of the microscope, this method will determine the so-called information focus, which is an image where the majority of the fluorophores are localized. These positions correspond to the image of the minimal (non-moving objects autofluorescing in red and green) or maximal (DAPI-labelled objects) value of the $I_{0.99}$ (a sum of all point divergence gains in an image). Generally, in the case of non-moving (stable in time) objects, the number of zero $\Omega_{0.99,k/l}$ gradually decrease up to the focus (read in detail below). In contrast, in the information focus, moving objects change their spectral properties along the z-axis substantially more than they do out of the focus which increases the average value of the $\Omega_{0.99,k/l}$ in the image. The unique value of $\alpha = 0.99$ (approximation of the Rényi entropy to the Shannon entropy) was chosen, since the intensity histogram of the given images exhibit normal-like distributions.

The real positions and ranges of the z-stacks for the microscope’s autofocusing and those obtained via the information-entropic analysis are compared in Fig. 5a. The focal region of the green autofluorescence was broader than the focal regions of the red autofluorescence and DAPI. This can be attributed to the broader green part of the light spectrum that is projected along the broader optical path due to chromatic aberration. Both DAPI foci correspond to the same image (img. 41). The standard autofocus of the red and green autofluorescence are shifted about 434 nm and 1,000 nm, respectively, above the DAPI focus. Whereas the information focus of the red autofluorescence lies about 1,100 nm (about 11 images) above its standard autofocus, the green autofluorescence has its information focus about 200 nm (about 2 images) lower than its standard focus. After the information-entropic clustering, the ranges of all z-stacks were narrowed (see Sect. ) so that they were limited by the images with the points of the highest emission and their images were asymmetrically distributed around their information foci.

The next step in the analysis was to find points of zero point divergence gain, i.e. points whose intensity minimally changes over two z-levels, in a series of images [2, 4]. This analysis provides spatial locations of all in-focus objects that are giving rise to the fluorescence emission. The amount of non-zero points at each of the levels is rather low and decreases the complexity of the information dramatically (Fig. 5).

Specific fluorescent labelling is often a basis of interpretation of biological data at the cell or tissue level. In case that labelling is not specific enough, i.e. fluorescing
Figure 4 Procedure of colocalization of red and green autofluorescence with DAPI in prostate tissue section based on the information-entropic analysis. The real z-positions are normalized on the beginning of the original z-stack of the DAPI-labelled images (zero z-position). a) Ranges of the scanned z-stacks (gray) and subseries (focal regions) which were acquired by clustering of the \( \alpha \)-dependent \( I_\alpha \) and \( P_\alpha \) spectra (black). The positions of the standard and information foci are marked by gray circles and black squares, respectively. The blue arrows depict the direction and size of the shift of the focal regions to obtain the 3D co-localized maps. b) Paths of the co-localized (corrected) focal regions characterized by the \( I_{0.99} \).

Figure 5 Images (number-coded) of the series from which Fig. 7a was constructed. Pixel size is \( 328 \times 328 \) nm\(^2\).

points are distributed in the whole image, we can define rules according to which the examined points differ from the others. This is explained in Figs. 7–8. In Fig. 7c–d, left, Fig. 8a and Video S1, on the basis of existence of a voxel with in-focus emission in the close proximity of another voxel with in-focus emission (dense areas), we identified nuclei. This statement is further strengthened because we deal with ellipsoidal objects labelled by DAPI. However, we identified another class of DAPI-labelled objects which dominate in Fig. 7c–d, right, Fig. 8b and Video S2. These objects were selected on the basis of the different intensities of the DAPI emission.
in the in-focus voxels, i.e. there was specific binding but it was more scattered. However, as seen in Fig. 7a–b, any simple rule for the selection of points is not often available (or necessary) and the image has to (or can be) analyzed from the complete dataset. In this case, the 3D model was reconstructed from images in which the points of unchanged intensity occurred only very sparsely. This indicates that we deal with autofluorescence.

Figure 6 Microscopic image of the section of prostate cancer tissue autofluorescing in red (red) and green (green) region of visible spectrum and DAPI targeted to nuclei (blue). Pixel size is 328×328 nm². The images were obtained by standard autofocusing.

DAPI with the autofluorescence in the red and green regions of the light spectrum were co-localized by the procedure which is visualized in Fig. 4a. The whole z-stack of images of red autofluorescence was, in agreement with the focus determination calculation, shifted down by 434 nm to overlap the z-stack with DAPI-labelled objects. The green fluorescing z-stack was shifted by 300 nm up to reach the red focus. This treatment gave the resulting RGB stack where the course of the \( I_{0.99} \) for green autofluorescence copies this course for red autofluorescence. The focus of DAPI was brought even closer to the others (Fig. 4b) with the shift of 1,200 nm.

With the example of autofluorescence in red and green regions, we shall further explain technical aspects of the focal plane location and co-localization. Object O1 (Fig. 8a and Video S1) is autofluorescing in green and red in images 1–50 and 40–51, respectively. This phenomena can be only explained by the different projection of each emitted light wavelength along the light path of the microscope. In many other objects, e.g. O2 in Fig. 8b and Video S2, both colour channels co-localize at all levels where the green and red autofluoresce is in focus. We probably deal with autofluorescing filaments which span the whole sample (cf. Fig. 7a,b).

Regarding DAPI, its co-localization with red and green autofluorescence in nuclei is marginal and occurs only at the surface of the nuclei (Fig. 8a and Video S1). In contrast, inside the sparsely DAPI-labelled objects in Fig. 8b and Video S2, a
significant co-localization of this type was found. This further confirms the above-mentioned claim that these regions are not nuclei but other organelles which are
spanned by the autofluorescing filaments. Without the 3D analysis, these labelled cell compartments would be mixed up with “true” nuclei.

**Discussion**

This paper describes how to objectively localize unchanged information between two images which are shifted along the optical axis (optical cuts) in light microscopy.
Using this procedure it is possible to find the most localized information about the position of an object. The analysis is obstructed primarily technically:

1. First of all, the comparison of the optical cuts is limited by the analog-to-digital (AD) conversion. The AD converters in standard digital cameras provide 12- or 16-bit conversion (4096 or 65536 intensity levels). Experimenters usually work with 8-bit images with 256 intensity levels. By the standard 12/16-bit to 8-bit conversion, images are heavily distorted [11]. In this paper, we report the analysis of original 12/16-bit datasets.

2. The second main technical determinant is the size of the pixel or voxel (3D pixel) which matches the theoretical size of the observed object. In this article we do not discuss limits of the discriminability of objects (Fig. 9). We assume that the discriminability can be determined experimentally.

3. The third determinant is an exposure time and a linearity of the response of the camera chip. The linear responses of the chip elements to the exposure time and light intensity are never guaranteed and the calibration of the chip is, according to our experience, never correct. Moreover, there always exists a concern that the camera sends out a modified signal.

4. The acquisition time of cameras is also an important aspect. In the case of the observation of moving objects, e.g., living cells, the acquisition time can be so long that the object changes its position and we obtain a false negative signal.

5. An unavoidable objective determinant of the precision of finding the most localized information about the position is the noise from various sources. In the examples presented here, the samples that were observed at high light intensities (properly described when the information about observed differences was carried out by large ensemble of photons), it is unlikely that the key limit that prevents finding the intensity maximum or minimum was the quantum noise [15]. The distortions of the signal were generated along the optical path and in the instrument electronics. The noise changes the signal by the multiplication by a function. No precise characteristics of the noise are known. The most general assumption about the characteristics of the noise is that they have a multifractal character. This multifractal distribution is then discretized in space and in time. A method of calculation of point divergence gain [2, 4] enables us to analyse the whole spectrum of these distortions. The points (pixels) of almost unchanged intensities are grouped according to different multifractal properties. In the diffractive imaging, we used this method [2, 4] for transforming the information in z-stacks of images of living cells into elementary information contributions. In the green image channel, we have observed a lower number of identical points between two consecutive z-levels. It is assigned to a broader intensity spectrum, i.e., a broader wavelength range of the green filters of the Bayer mask [16]. This illustrates a paradox of the information analysis: The broader the information spectrum is, the more the differences are found.

A 2-µm latex bead is an object of a size similar to that of intracellular objects such as mitochondria or other oval organelles. The size of this bead in the visible light spectrum ranges between 5 light waves (shortwave blue, 400 nm) and less
than 3 light waves (far red, 720 nm). This size is between macroscopic behaviour and nanoscopic behaviour at which the quantum dot effect is observed. (Quantum dots are metal nanobeads of a size that is a fraction of the light wavelength which exhibits fluorescence.) In the macroscopic description, we interpret our observation such that the Arago spot [6] is located behind the object and is directly adjacent to the dark area of diffraction.

The image analysis of a single bead/organelle was performed under the scrutiny of two fundamental concepts of the signal analysis [17]: the resolution and the distinguishability (Fig. 9). The microscopic resolution is defined as a distance at which two first-order valleys of the Airy waves [18] exactly merge. As seen in Fig. 3, the Airy pattern is sometimes not observable in a real point (object) spread function. In contrast, the positions of the electromagnetic centroids of particle’s response (i.e., intensity maximum/minimum) can always be found. Thus, searching for electromagnetic centroids is a more realistic approach than the theoretical concept of resolution. In this article, we describe the information resolution concept of microscopic image analysis [2, 4].

The distinguishability can be also defined by the absence or presence of points which do not have an identical intensity at the same position in the next z-level. In other words, a distinguished object has to be enveloped by non-objects. The results are critically dependent on the technical set-up [2] and the approach described in this article can be best applied to images obtained by a simple microscope. However, such a microscope must be equipped with a fine mechanics moving in the z-axis, with a camera with high number of pixels and with control software that does not distort the data.

The interpretation of the fluorescence image is much simpler. Fluorescent molecules are of an infinitely small size (relative to a pixel) and we search for their distribution in a cell. Fig. 7 shows objects of unchanged intensities between two z-levels which were selected upon three simple assumptions:
1 Each voxel contains at least one or none fluorophore which represents one intensity level.

2 When signals are present in numerous neighbouring pixels, these pixels can be assigned to an object. Signals in regions of lower density, which have the same intensity as the majority, belong to the background.

3 The detailed analysis showed that there are both (i) high intensity points in the centre of the dense areas – the nuclei – which most likely represent several fluorophores per voxel and (ii) voxels of different intensities, mostly higher, outside dense regions.

Often, when an image is full of labelled objects of different intensities (Fig. 7a–b), no simple rule of data analysis can be used. However, the selection of zero point divergence gain objects significantly clarifies the dataset and enables a realistic 3D analysis of the observed structures.

Most analyses of co-localisation of fluorescent labels are impaired by misalignment caused by different positions of the focuses of individual colour channels or fluorophores. The objective determination of the extent of the focal region (not plane) and proper objective alignment of images overcome this problem. The determination of the in-focus superresolved – i.e., down to pixel-sized – objects at each z-level in an ordinary dataset (e.g., Figs. 7–8) further informs us about the co-localization and provides a firm ground for addressing biological questions. We believe that this type of analysis is essential for the proper interpretation of biological data.

Conclusion
In this article we have highlighted a few important technical aspects which limit the complete yield of information from a microscopic image in all known cases:

1 Usage of 12-bit colour depth of an image must not be sufficient; usage of a higher-bit depth can be necessary. But to work with standard 8-bit images is not correct in any known existing set-ups.

2 The bright spots in diffractive images are not necessarily (auto)fluorescent objects.

3 The extent of objects in focus has to be determined for each colour channel (i.e. for each wavelength) separately. Focal regions have to be aligned for proper analysis.

4 The information can be localized with an infinite precision. The method requires microscopic cameras with a high number of pixels and the usage of a higher magnification, a problem which did not have to be encountered when photographic films were used for image recording. A low number of pixels on the camera leads to misinterpretations in many aspects.

5 Analysis of a dataset not only in planar projection but in the spatial distribution provides near-complete information about specificity and co-localization of fluorescent labels and, eventually, an ultimately correct interpretation.

Samples in biology are usually expensive and often irreplaceable. It is a rather bad idea to image them in a way by which the maximum information from an optical microscopic experiment cannot be acquired and analyzed. The breakdown provided in this paper gives a recipe for how to collect the maximum information and how to interpret it with a negligible loss of intelligibility.
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Authors’ contributions
RR developed the image-processing algorithm, processed the data, and significantly participated in preparation of the manuscript. GK provided samples of cancer tissue sections. GS provided image data of fluorescently labelled tissue. MBF networked the co-authors. DŠ is an intellectual co-author of the image processing algorithm and wrote the first version of the manuscript which was further edited by other co-authors.

Competing interests
The authors declare that they have no competing interests.

Consent for publication
Not applicable.

Ethics approval and consent to participate
Cancer tissue sections were provided in agreement with ethic rules of the Medical University Vienna and the study was performed according to guidelines of Good medical practice.

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Additional Files
Video S1
The run through a z-stack of \( \Omega_{\mu,l} = 0 \) selected (number-coded) from the 3D stack of the section of prostate cancer tissue. Upper left corner of Fig. 6. Colour coding of localized and co-localized fluorescent labels: red – red autofluorescence, green – green autofluorescence, blue – DAPI, magenta – DAPI + red autofluorescence, yellow – red autofluorescence + green autofluorescence, cyan – DAPI + green autofluorescence, black – all three colour
channels. The imaging of individual colours was constrained to the regions in which in-focus points were identified by the PDGE analysis ($\alpha = 6$ for red and green autofluorescence and $\alpha = 7$ for DAPI).

Video S2
The run through a z-stack of $\Omega_{\alpha,k/l} = 0$ selected (number-coded) from the 3D stack of the section of prostate cancer tissue. Lower left corner of Fig. 6. Colour coding of localized and co-localized fluorescent labels: red – red autofluorescence, green – green autofluorescence, blue – DAPI, magenta – DAPI + red autofluorescence, yellow – red autofluorescence + green autofluorescence, cyan – DAPI + green autofluorescence, black – all three colour channels. The imaging of individual colours was constrained to the regions in which in-focus points were identified by the PDGE analysis ($\alpha = 6$ for red and green autofluorescence and $\alpha = 7$ for DAPI).