

# Super-resolution 3D Reconstruction of thick biological samples: a computer vision perspective

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#### Abstract

In this paper we present a case-study about recent breakthroughs of three-dimensional (3D) super-resolution livecell imaging through thick specimens (50 – 150  $\mu$ m). This technology is enabling the deep understanding of cellular mechanism by obtaining very detailed 3D descriptions of cells. In particular, we discuss the image analysis problems related to the accurate localization of single molecules. This problem is hard because of the extreme noise conditions, the high and heterogeneous density of the cell molecules and the distortions induced by light-sample interactions on the imaging capabilities. For this reason, robust computational tools are required to obtain the localization of the photo-activated molecules and to enable the super-resolution accuracy. In such context, we show that a novel set of challenges exists and novel Computer Vision approaches are needed for delivering high-performing imaging systems for life science.

### 1. Super-resolution imaging and its challenges

In the last years particular attention has been addressed to the spatial resolution improvement of fluorescence optical microscopy techniques. The advent of novel fluorescent probes, with suitable spectral capabilities, lead to the development of an innovative family of far field super-resolution microscopy techniques able to push the resolution limit beyond the diffraction barrier. In particular, in the past few years emerging techniques, based on the localization process of single molecule, allowed to image biological structures at the molecular level since they enabled the investigation of sub-cellular structures with a resolution never attempted before (10-30nm). All these techniques are well established at the cellular level and they performs optimally when imaging of thin samples is required. Despite this fact, imaging of thicker samples still represents a challenge since the effects induced by the interactions of the light with the sample, such as the crowded molecular environment, scattering and absorption effects, prevent a strong accuracy in the single molecule localization process. In particular, one of the main limitations is represented both by the required acquisition time and by the decreased localization accuracy which induce a consequent worsening of the system performance in dense live samples. To address this issue, the development of effective tools able to improve localization performances, in particular when the required molecule sparseness regime is disrupt, represents a key point.

Emerging techniques, based on temporal focusing [21], selective plane illumination microscopy (SPIM) [4] [3] and array tomography [15] extended the imaging capabilities of localization based methods to thicker and thicker samples thus representing a key point towards super-resolution imaging in vivo (such as embryos and tissues). However it is clear that such evolving techniques require an increasing interaction with advanced computational tools that can provide the needed boost in order to extend further the state of the art in super-resolution microscopy. The aim of this paper is to provide an overall view of the mechanism underlying the super-resolution approaches with a focus on single molecule localization methods. In particular, we will present a setup developed in [4] that enables the 3D reconstruction of thick biological samples called IML-SPIM. Our focus is to unveil the underlying computational problems that can be of most interest to the Signal Processing and, in particular, Computer Vision communities. Furthermore, we will discuss different aspect of this challenging problem and provide possible directions to increase the performance of current live cell imaging systems and the related applications for Bioscience.

The rest of the paper is organized as follows. Section 2 introduces the single molecule localization concept at the basis of the setup for super-resolution microscopy. Section 3 describes the IML-SPIM setup developed in [4] with more emphasis on the most important computational issues to solve. Finally, in Section 4 we discuss further improvements and possible future challenges for the computer vi-

sion community.



Figure 1. Concept behind IML: A small subset of sparse molecules are imaged, localized and turned off by photobleaching. The cycle is repeated as long as a sufficient number of molecules have been localized (from a) to g)). The plotted positions of all the localized molecules build up the superresolved image (i). The IML image shows details below the diffraction limit that would not be visible in conventional optical microscopy (h)

# 2. Super-resolution based on individual molecule localization (IML)

The concept behind individual localization techniques (IML) relies on the image acquisition of separated single molecules and the subsequent localization of their center. In the last few years several photoactivation localization based techniques have been developed, such as PALM [2], FPALM [8], STORM [16] and PALMIRA [6]. All these techniques exploit the same principle and they differ for the optical system used or for the fluorophore employed.

The rapid growth of far field optical super-resolution techniques brought to a fast development of individual molecule localization (IML) based techniques and their extension to three-dimensional imaging [10, 9] and multicolor applications [1] [17]. Furthermore IML techniques can be exploited to identify single molecules trajectories [12] and to perform orientation studies of the molecular orientation [7] [19].

The localization analysis of single particles is based on the fact that, although the size of the observed object is limited by the resolution of the microscope, the center of the object can be determined with high precision accuracy if a sufficient number of photons (N) are collected (Fig. 1 shows a graphical representation). In individual molecule localization the images consist of a collection of sparse single molecules signal and it is therefore possible to determine the relative positions of the objects to a precision much better than the diffraction limited spot. Notwithstanding the accuracy of these measurements is dependent from the counting statistics of the detected signal and from the noise introduced by the detection device and processing electronics. Additional factors, like background noise and optical system characteristics affect the maximum precision achievable in the single molecule localization especially when imaging of thick and dense samples is performed. For this reason more robust algorithms and suitable optical arrangements are required to increase the imaging depth capability of localization based techniques.

The analysis is based on the idea of least-squares fitting [20] [8] with a Gaussian restricted to the one dimensional case that can be easily extended to the two-dimensional case. If the noise in each pixel is dominated by photons produced by the localized particle, the photon shot noise starts to play a relevant role. In this case the estimate of the molecule position is given by the mean of the positions of the individual detected photons and the error in the localization can be provided by the standard statistical error associated with [20]:

$$\sigma_x^2 = \frac{s^2}{N},\tag{1}$$

where N is the number of photon collected and s is the standard deviation of the Gaussian intensity Point Spread Function (PSF).

The background noise comprises both dark current noise and out-of-focus fluorescence and the error in the localization in the case of pure background noise can be described by:

$$\sigma_x^2 = \frac{4 \cdot \sqrt{\pi} \cdot s^3 \cdot b^2}{a \cdot N^2}.$$
 (2)

The total localization precision in bi-dimensional case be obtained summing the contribution due to photon-counting noise, pixelation noise and background noise [20]:

$$\sigma_{xy}^2 = \frac{s^2 + \frac{a^2}{12}}{N} + \frac{8 \cdot \pi \cdot s^4 \cdot b^2}{a^2 \cdot N^2},\tag{3}$$

where *b* is the background noise and *a* is the pixel size. This relation shows that the uncertainty falls as the inverse of the number of photons *N* for the background noise and as  $\frac{1}{\sqrt{N}}$  for the photon counting noise. Therefore the maximization of the number of photons collected for each molecule is a crucial aspect in IML imaging and it allows to increase the localization precision. Furthermore, the effective resolution in IML techniques is affected by the molecular density and also the distance between contiguous molecules has to be taken into account. For this reason the overall resolution of the system takes into account both the localization precision and the molecules sparseness.



Figure 2. A set of images of a nanocapsule collected by the IML-SPIM setup

# 3. IML-SPIM, towards thick samples

Investigation of 3D structures of living biological specimens represents a key point since it provides fundamental knowledge of the relation between function and structure of biological molecules. Within this framework, the possibility to perform imaging at the molecular level of biological structures directly within entire animals or tissues became a crucial point. In this context light-sheet based microscopy (LSM) played a relevant role to overcome the limitations of conventional IML techniques. In particular, it was recently proved [4, 11] that individual molecule localization coupled with selective plane illumination microscopy (IML-SPIM) can be successfully implemented to perform superresolution deep within mammary cell spheroids. Light sheet based microscopy combines the advantages of confocal approaches in terms of optical sectioning capabilities and the benefits of wide field microscopy, such as fast acquisition and high dynamic range. The sample is mounted in agarose gel and moved within the focal plane in order to perform 3D imaging. Light sheet based techniques combines the advantages of wide-field and confocal approaches: images can be acquired with high signal to noise ratio (SNR) and optical sectioning properties similar to confocal schemes and low photobleaching and fast acquisition time can be reached as in wide-field microscopy.

Additionally, SPIM allows to reduce the energy delivered on the sample providing lower photobleaching and photodamage of the sample since the use of a cylindrical lens to collimate the light in order to create the light sheet brings to a lower photon density in the focal plane. The intrinsic confinement of the excitation volume obtained with the light sheet illumination provides an higher signal to noise ratio inducing a consequent improvement of the localization performances.

#### 3.1. IML-SPIM optical system

A single plane illumination set up has been designed and realized in order to perform 3D imaging of differently sized biological samples. The set up is composed by three basic units as shown in fig. 3: the illumination unit which creates the light sheet from the side of the detection focal plane, the detection unit, which provides high sensitivity signal detec-



Figure 3. Schematic representation of the SPIM setup: illumination and detection path are orthogonally oriented.

tion, and the sample movement unit.

A light sheet is created in the focal plane and is used to excite fluorescence while the emitted photons are orthogonally detected by a orthogonally placed objective. Three dimensional super resolution imaging can be performed using the astigmatism approach, where a cylindrical lens is placed before the camera providing ellipticity in the PSF shape depending on the axial coordinate. For more details regarding the optical setup please refer to [4].

#### 3.2. Image analysis and rendering

The setup previously presented collects images that have to be processed to localise each single molecule activation in time. Figure 2 shows an example of a set of images collected in our setup. It is noticeable by visual inspection some photo-activated molecules at some time instances in the sequence. The noise level of the image is also quite remarkable because of fluorescence signal from inactive molecules, from out of focus molecules or by scattered light. For this reason it i necessary to model such noise and possibly remove its effect from the image without corrupting the PSF shape of the photo-activate molecule. Moreover, in order to provide exact metric measurements in the Z-axis, we have to find a mapping between the PSF distortion and the molecule depth.



Figure 4. A set of images showing a probe at different focus

System calibration from images. In order to achieve an accurate localization in depth, a probe molecule (fluorescent beads with a diameter of 40nm) at different planes is used to associate the astigmatic distortion of the PSF to an absolute distance in the Z-axis. Figure 4 shows a set of images of the probe at different distances. Given this set of images, we fit for all image pixel at position (x, y) an elliptical Gaussian function given by :

$$I(x,y) = I_o \cdot e^{-2\left(\frac{(x-x_o)^2}{r_x^2} + \frac{(y-y_o)^2}{r_y^2}\right)} + t$$
(4)

where the parameters to estimate given the image I are the brightness  $I_o$  proportional to the overall molecule intensity, the elliptical Gaussian center  $(x_o, y_o)$ , the variance pairs  $(r_x^2, r_y^2)$  and a general image brightness offset t. Given a set of n images  $I_1, \dots, I_n$  we can fit using robust non-linear regression a set of variances pairs  $(r_{x1}^2, r_{y1}^2), \dots, (r_{xn}^2, r_{yn}^2)$ . Given these discrete quantities we further fit two polynomial curves to the set of variances in x and y. These two curves represent the relational between the PSF distortion and the depth of the molecule (check Fig. 5). Such calibration stage will be used for the molecule detection stage using the images of the biological samples.



Figure 5. The red and blue curves represent the calibration of the microscopy setup that maps the elliptical Gaussian variances to the molecule depth.

**Background subtraction** After the acquisition of the entire image series (e.g. Fig. 2) a noise background subtraction is performed. Background noise can be given either by fluorescence signal from inactive molecules, by out of focus molecules or by scattered light. A spatially-invariant background subtraction can be performed, but a non uniform baseline subtraction is in general preferred. In order to subtract the background in each frame independently, the subtraction is performed using the roll-ball algorithm [18].

Single molecule localization algorithm. In order to discriminate single molecules from background signal a threshold on the number of photons collected for each molecule has to be set. As a rule of the thumb the number of photons detected *per* molecule should be approximately 10 times the photons due to the background noise. The coordinates of the center of mass of each identified molecule is used as initial parameter for the Gaussian fitting routine using Eq. (4) for the PSF model. Several criteria, based on the number of photons collected or on the width of the intensity distribution generated by each single molecule, can be applied in order to discard events due to the acquisition of multiple molecules. In particular, the width of the intensity spot produced by single molecule imaging should be equal to the width of the system PSF. Eventually, larger or smaller spots in the image can be rejected since they are not probably associated to single molecules events. The localization precision for each identified molecule is calculated following Eq. (3).

**Final visualisation.** Once position, intensity and localization accuracy has been calculated, all these information can be used to render the final super-resolution image. A weighted plot can be obtained tracing the localized molecules as spots with a Gaussian profile in which the amplitude is proportional to the number of photons detected and the radius is determined by the localization precision.

#### 3.3. Localisation results

As an example of the capabilities of the system, we show the localization performance of IML-SPIM on nanocapsules, a micrometer sized model system made by layer-bylayer technique with positively and negatively charged polyelectrolytes. A layer of the nanocapsules is stained with the photoactivatable caged-FITC in order to perform single molecule detection. Figure 6 shows the improvement in the resolution for the mean localization precision reached is 35nm (E) and the comparison between the IML-SPIM image (A), reconstructed after localization, and the conventional SPIM image (B). Notice that the borders of the nanocapsules are now clearly visible.

Additionally the axial coordinate z of each single molecule can be determined by the shape of the elliptic Point Spread Function (PSF) obtained introducing a cylindrical lens along the detection path. Calibration curves are obtained with the procedure presented in Sec. 3.2. The 3D super resolution image containing the axial information can be shown as a color coded map showing depth changes according to the calibration procedure previously described.



Figure 6. a) A comparison between the super-resolution IML-SPIM (A) and conventional SPIM (B) imaging of nanocapsules. The value of the number of photons/molecule collected (C), the distribution of the molecule radius (in pixels) and the localization precision calculated according to Eq. (3).

Figure 7 shows 3D super resolution imaging both for polyelectrolyte nanocapsules and for a selected cells of interest within mammary cellular spheroids. This shows the imaging performances of the system in thin and thick samples.



Figure 7. 3D imaging of polyelectrolyte nanocapsules and 3D imaging of cells expressing histone H2B fused to photactivatable monomeric Cherry (H2B-PAmCherry).

### 4. Conclusions and discussion

Super-resolution techniques based on single molecule localization opened a path towards the study of biological structures within cells at the molecular scale. Despite several biological questions can be addressed at the cellular level, the possibility to access information about subcellular structures in vivo represents a key step towards a better understanding of the molecular mechanisms involved in several oncological and neurodegenerative diseases. Within this framework, the possibility provided by IML-SPIM to extend the application range of superresolution techniques to thicker samples represents an important point, since it provides a powerful tool to detect and localize single molecules directly within tissues (such as brain slices or tumoral tissues) and entire organisms (such as living embryos). Such understanding of evolving complex biological processes, especially for in-vivo analysis, requires the design of accurate instruments able to reveal the most subtle details of cellular dynamics. In such regard much efforts have been put into designing custom optical setups that can break the barrier of standard resolution. On the other hand, the computational aspects have been only briefly addressed in the literature and there are relevant opportunities for the signal processing and computer vision communities to produce a relevant impact in this field. At this end, the IML-SPIM setup proposed in [4] is a stepforward towards the creation of a reliable and robust system for super-resolution. However, in the following we will discuss a set of possible improvements to current systems that can be addressed by researchers in the Signal Processing and Computer Vision communities.

Super-resolution accuracy and robustness. There are two main trends for single molecule localization that are overcoming the classical, but still precise, robust regression methods. First, compressive sensing algorithms [22] have been used because the implicit structure of the data. Being the single molecule activations sparse, it is possible to enforce a sparse PSF fitting on the data. Drawbacks are related to the needed discretization of the image grid that is necessary for increasing the super-resolution accuracy. Finer the grid, more accuracy is achieved. However this is paid in terms of computational power since sparse optimization requires, in general, iterative algorithms if the problem is large scale. This effect is lessened by decimating the image in parts and solving independently a sparse problem for each image part. Moreover, in the relative optimization problem there is a sparsity parameter ruling how much sparse is the signal i.e. how many molecules might be active in a given image area. This parameter is in general constant but it might be varying given the (unknown) density of the sample. Another interesting strand of research is to adopt a generative approach by modelling the dynamic tri-states of photo-activate molecules [5]. Also such framework it has been applied in deconvolution using a sparsity-promoting Bayesian framework [14].

**Live-cell motion and single molecule tracking.** Another major issue in live-cell imaging scenario is given by the mo-

tion (drift) of the samples during the acquisition. The photoactivation of several single molecules require a short interval of time, nevertheless we might have an overall translation or even rotation of the sample. This introduces a molecule tracking/matching problem [13] that is crucial for obtaining reliable 2D/3D information. This problem has similarities with the classical feature matching problem in multi-view geometry. Given an unknown geometric transformation we have to match the same feature in different images. The problem here is worsened by the fact we might have very sparse points to match in time thus providing a stronger challenge to the community.

**Super-resolution images clustering.** Another important issue is mainly related to the post-processing of super-resolution images in order to detect particular clusters of molecules. This clustering problem is rather important for Biologists in order to understand if a sample has a particular aggregation of molecules that might be a sign of a specific cellular process. Given the sparse image generation procedure of super-resolution, these clustering methods have to deal with a large number of 2D/3D points. This may be not a problem for a batch processing alike pipeline. However, in a live analysis regime, time might be a computational issue in order to understand important dynamical processes of the sample in real-time.

**Conclusions.** The high accuracy provided by the superresolution system have given a new tool for biologists enabling the inspection of biological processes that were previously not accessible. At the same time, new computational challenges have been discovered in such field and the community is actively searching for answers to these hard questions. However, one of the main obstacles between the two communities is the accessibility to the data obtained by each super-resolution setup. In such regard, the creation of public datasets for single molecule localization, tracking and clustering (to name a few) problems might be the most direct path to promote a stronger interaction between these two fields.

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