Superresolution by single-molecule localization

Introduction

Up to now, we have discussed several light-microscopy techniques that were, however, all limited by a physical restriction as to the size of structures possible to resolve. For a long time, researchers believed that a light microscope could never yield a resolution better than 0.2 µm. This is due to the diffraction of light, that results in an object being represented as a blurred version of itself instead of a sharp image. Two points with a distance smaller than the resolution limit cannot be distinguished but are seen as one (see figure 5-1A). With this inherit limitation, it was possible to observe entire cells and the contour of a few large structures within cells, e.g., mitochondria. However, it was impossible to discern smaller objects, such as viral particles, ribosomes or individual proteins (see figure 5-1B).

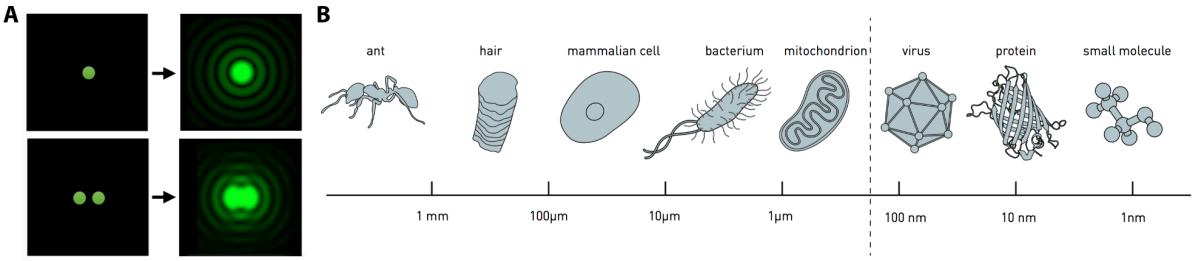

Figure 5-1 The diffraction of light limited the resolution of optical microscopes until the development of superresolution techniques. (A) Due to the diffraction of light, a point is imaged as a blurred disc. Two points that are closer than 0.2µm cannot be distinguished, because their disc-like images overlap. **(B)** While larger objects such as whole cells or large organelles could be observed, smaller structures such as macromolecules or viral particles could not be discerned as single objects. (B: From http://www.nobelprize.org, Nobel Prize in Chemistry, 2014)

During the last two decades, several techniques have been developed that bypass the limitation of diffraction; collectively, they form a new branch of light microscopy called superresolution microscopy[1]. Because this has revolutionized the field of microscopy, three researchers (W.E. Moerner, E. Betzig, and S.W. Hell) who developed superresolution techniques were awarded the Nobel Prize in Chemistry in 2014.

Here, we will introduce the method of photoactivated localization microscopy (PALM)[2], which combines and builds on several concepts of the previous lessons:

First, objects have to be apart farther than 0.2 µm in order to be distinguished by light microscopy. Second, molecules, e.g., proteins, can be tagged by fluorescent markers to highlight them specifically. Fluorescence is achieved by illuminating the specimen at a specific wavelength, thereby exciting the fluorophore to a higher-energy state whose reversal results in emission of light that can be detected. If fluorophores are excited very often, they will be bleached, i.e., be no longer excitable. This effect plays a crucial role in a PALM experiment as we will see later in this lesson.

Photoactivated localization microscopy (PALM)

How is it possible to overcome the diffraction barrier of 0.2 µm with a light microscope? In PALM, this is achieved by an, in principle, simple mechanism: Instead of exciting and detecting all fluorophores

[1] Another term for superresolution microscopy that you may encounter is 'nanoscopy', since it allows visualization of objects on the nanometer scale.

[2] Another name for the same fundamental principle is stochastic optical reconstruction microscopy (STORM). The two methods were published using different fluorescent molecules: While in PALM, fluorescent proteins such as GFP were used, STORM experiments were performed using synthetic fluorescent dyes.

at once, we look at them individually. By doing this for all fluorophores in the sample and combining the images, we can reconstruct a "superresolution image".

In practice, it is, of course, not that simple, because if we were to just superimpose images of individual fluorophores, we would end up with the same blurred image as yielded by detection of the signal from all fluorophores at once. Therefore, we need a step between detecting single fluorophores and combining the images that reduces the blur: Although single fluorophores appear as circular blurry discs when imaged, if sufficient photons have contributed to this image, the precise mathematical center of the disc can be determined accurately, thereby allowing the localization of a fluorophore with nanometer precision. But the problem with a specimen that contains a large number of adjacent fluorophores is that these fluorophores appear as overlapping blurry discs on an image and their individual positions cannot be determined. A way to solve this problem is exciting so few fluorophores at once that is it very unlikely that two fluorophores closer than 0.2 µm are excited. How is this accomplished?

Here, special photoactivatable fluorophores come into play that do not fluoresce until activated by illumination at a certain high-energy wavelength. One example of such a fluorophore is photoactivatable green fluorescent protein (PA-GFP) that, in comparison to wild-type GFP, has a mutation in one amino acid. This mutation causes the protein to show almost no fluorescence at its normal excitation wavelength when no photoactivation at 413 nm has occurred prior to excitation.

In total there are now three characteristic wavelengths of the fluorophores: The activation wavelength (413 nm for PA-GFP), the excitation wavelength (488 nm) and the emission wavelength (515 nm).

In a PALM experiment, the specimen is first illuminated by a laser at the activation wavelength. If the intensity is low enough, only very few random fluorophores are activated while the remaining ones stay inactive. In a next step, activated fluorophores are excited by another laser at the excitation wavelength and their fluorescence (at the emission wavelength) detected. After every excitation of a fluorophore, it emits a photon. Frequently used fluorophores are bleached after a few hundred to a few thousand excitation cycles. In figure 5-2 you see that the amount of photons that are detected, i.e., the number of excitation cycles, determine the accuracy with which a fluorophore can be localized. Generally, a few thousand photons are required for the position of the fluorophore to be determined computationally to a precision of a few nanometers. After a fluorophore has been bleached, it can no longer be excited or activated. This means that in a next activation step, a subset of only the remaining fluorophores will be activated.

In a PALM experiment, photoactivation, excitation and single-molecule localization, and bleaching are repeated until all fluorophores have been imaged. The exact coordinates of the full set can be combined and digitally displayed as an image in which the computed location of each individual molecule is marked.

The resolution limit of other light-microscopy methods was determined by the diffraction of light; but what limits the resolution of this technique? The natural limit of PALM is the size of the fluorophore and the distance to the tagged molecule. A fluorescent marker only reports information about the location of a molecule, but not its structure or position relative to the fluorophore. The resolution limit for PALM is at approximately 10 nm, which is the average distance between macromolecules within cells.

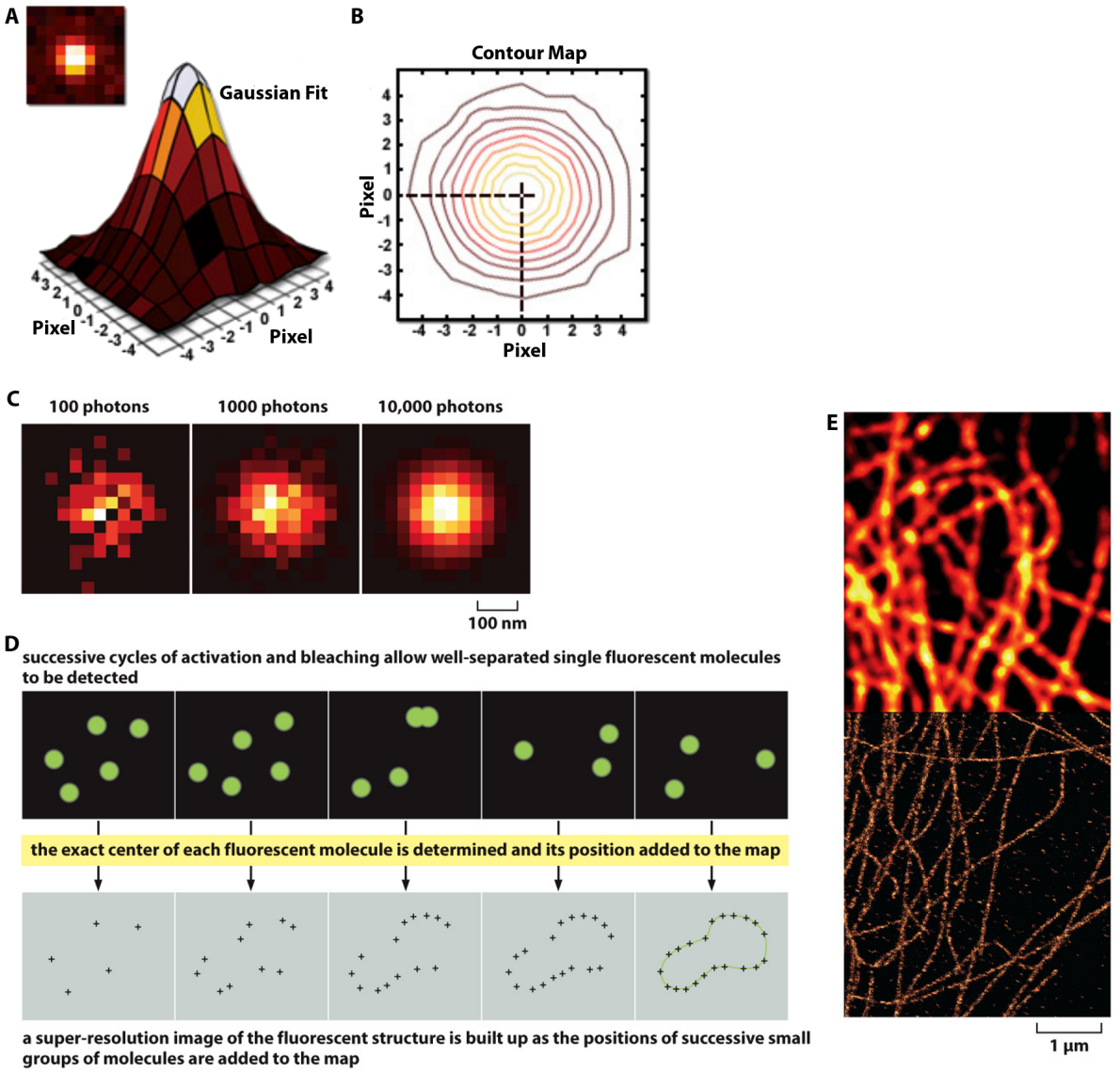

Figure 5-2 Single fluorescent molecules can be located with great accuracy. (A) The pixelated point-spread function of a single fluorophore is shown in the upper left. It can be modeled by a three-dimensional Gaussion function, with the intensity for each pixel color-mapped. **(B)** A contour map of the intensities. The centroid for each fluorophore can be individually localized. **(C)** Determining the exact mathematical center of the blurred image becomes more accurate the more photons contribute to the final image. Although the image is about 200nm across, the position of its center can be pinpointed to within a nanometer. **(D)** In this imaginary specimen, sparse subsets of fluorescent molecules are individually switched on briefly and then bleached. The exact positions of all these well-spaced molecules can be gradually built up into an image at superresolution. **(E)** In this portion of a cell, the microtubules have been fluorescently labeled and imaged at the top in a TIRF microscope and below, at superresolution, in a PALM microscope. The diameter of the microtubules in the lower panel now resembles their true size, about 25 nm, rather than the 250 nm in the blurred image at the top. (A and B: From zeiss-campus.magnet.fse.edu, C - E: Figure 9-38, Molecular Biology of the Cell, Alberts *et al.*, 6th edition, Garland Science)

A second limitation is that the bleaching of fluorophores in the specimen is a stochastic process. This means that the accuracy of the position of the fluorophores differs as well. If we only take into account the fluorophores whose position is determined with very high confidence, there remain only very few molecules, which corresponds to the first image in figure 5-3. On the other hand, if we set the confidence threshold too low, incorrect information may be included in the final image. Therefore, a trade-off between excluding fluorophores whose exact position cannot be determined with high confidence and a high density of molecules has to be made. With respect to figure 5-3, this would mean choosing between having all the spots at the correct position of the boy's face and having as many spots as possible. A lack of either results in a decrease of resolution.

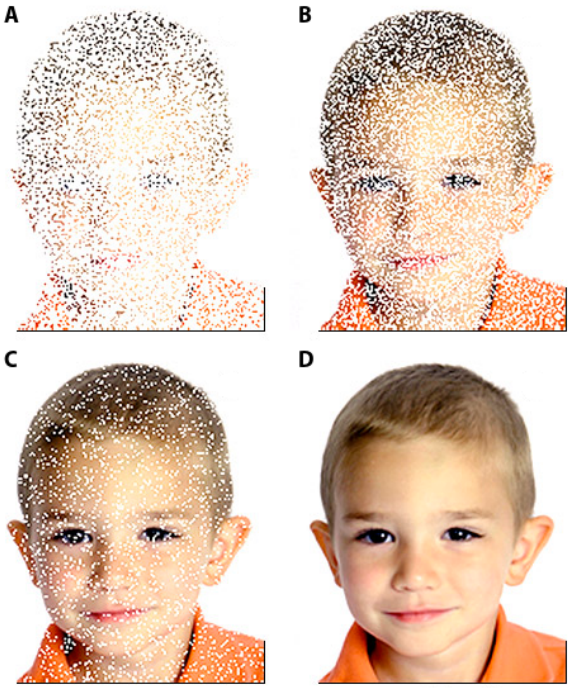

Figure 5-3 Abstract illustration of the concept of localization precision, resolution, and molecular density in single-molecule localization. The boy's image in (A) shows many localized points, but the relatively low density does not enable recognition of the image. As more points are added to the images in (B) and (C), the boy becomes more recognizable, but distinguishing (resolving) the fine features associated with his face requires a higher density of data points, as shown in (D). Precisely localized data points as well as a high density of points contribute to a high-resolution image. (From zeiss-campus.magnet.fsu.edu)

Using this method, components of the cytoskeleton, such as microtubules and actin filaments, the endoplasmic reticulum, and clathrin-coated vesicles have been imaged, to list only a few. PALM and other superresolution techniques are currently being extended to incorporate multicolor, three-dimensional, and live-cell imaging.

Summary

In this lesson, we have introduced the superresolution technique PALM, where repeated cycles of fluorophore activation, localization, and bleaching result in nanometer-resolution images of cellular structures. Here, special photoactivatable fluorophores play a crucial role, because each cycle is only performed on a small subset of molecules that is activated by a high-energy laser. PALM and other superresolution techniques have revolutionized the field of light microscopy, yielding their inventors the Nobel prize in 2014.