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Super-resolution microscopy approaches to nuclear nanostructure imaging

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ABSTRACT

The human genome has been decoded, but we are still far from understanding the regulation of all gene activities. A largely unexplained role in these regulatory mechanisms is played by the spatial organization of the genome in the cell nucleus which has far-reaching functional consequences for gene regulation. Until recently, it appeared to be impossible to study this problem on the nanoscale by light microscopy. However, novel developments in optical imaging technology have radically surpassed the limited resolution of conventional far-field fluorescence microscopy (ca. 200 nm). After a brief review of available super-resolution microscopy (SRM) methods, we focus on a specific SRM approach to study nuclear genome structure at the single cell/single molecule level, Spectral Precision Distance/Position Determination Microscopy (SPDM). SPDM, a variant of localization microscopy, makes use of conventional fluorescent proteins or single standard organic fluorophores in combination with standard (or only slightly modified) specimen preparation conditions; in its actual realization mode, the same laser frequency can be used for both photoswitching and fluorescence read out. Presently, the SPDM method allows us to image nuclear genome organization in individual cells down to few tens of nanometer (nm) of structural resolution, and to perform quantitative analyses of individual small chromatin domains; of the nanoscale distribution of histones, chromatin remodeling proteins, and transcription, splicing and repair related factors. As a biomedical research application, using dual-color SPDM, it became possible to monitor in mouse cardiomyocyte cells quantitatively the effects of ischemia conditions on the chromatin nanostructure (DNA). These novel "molecular optics" approaches open an avenue to study the nuclear landscape directly in individual cells down to the single molecule level and thus to test models of functional genome architecture at unprecedented resolution.

1. Introduction

1.1. The limits of conventional microscopy

For more than a century it was a generally accepted truth that structural details inside cells could only be detected if they were larger than about 0.2 μm (200 nm). Below this optical resolution limit of 200 nm (object plane) and about 600 nm along the optical axis obtained at the maximum numerical aperture (NA) available, the microscopic images were blurred to the observer like the letters of a newspaper seen from a large distance: Only the principal headlines could be read but not the decisive details. The example in Fig. 1 shows a conventional wide field image of a human cell nucleus where two types of nuclear proteins had been labeled with two different colors. The image suggests that the nuclear distribution of these two proteins might be somewhat different but a quantitative detailed analysis is extremely difficult. All attempts to substantially extend these resolution limits of "conventional" light microscopy failed. However, fast growing evidence indicates that the genome in mammalian cell nuclei has a highly complex spatial organization; for reviews see [38,39,48,123,27]. Hence, it will be highly desirable to obtain more information about its nanostructure.

About 140 years ago, Ernst Abbe, the pioneer of modern high resolution microscopy, has dealt extensively with this amazing and disturbing phenomenon and found the solution: It was no longer a technical issue of making better lenses, but a fundamental limit of knowledge using imaging; the limitation was a direct

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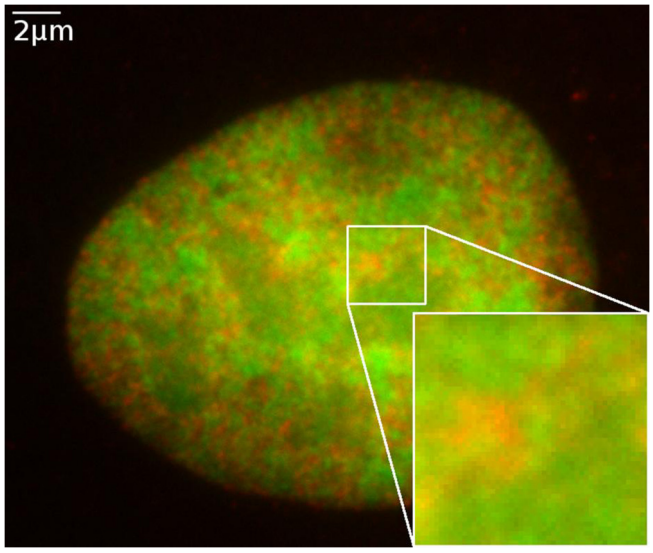

Fig. 1. A human cell nucleus imaged at conventional resolution. Conventional wide field epifluorescence microscope image (NA = 1.4) of a nucleus from a human Osteosarcoma cell line (U2OS). Histone proteins H2A were tagged with a standard Red Fluorescent Protein (mRFP1, red fluorescence), and Snf2H (a chromosome remodeling protein) was tagged with a standard Green Fluorescent Protein (GFP, green fluorescence). For a super-resolution image of the same cell using two-color localization microscopy, see section on localization microscopy. Reprinted (modified) from [53] by permission from Wiley. Copyright 2009 Wiley-VCH Verlag GmbH & Co. KGaA.

consequence of the nature of light waves; thus it was intimately connected with the fundamental laws of Physics. In 1873 Abbe summarized his findings in one of the most famous texts of optical Physics. Surprisingly, it did not contain one single mathematical formula. Nonetheless, the physical content was so fundamental that since then its main results have been presented in every basic Physics and Science course all over the world. Abbe postulated [1]

> *"... that the limit of discrimination will never pass significantly beyond half the wavelength of blue light"*[1]

"Half the wavelength of blue light" is about 0.2 µm or 200 nm ($200{\cdot}10^{-9}$ m). In general terms, Abbe stated a relationship for the smallest distance $d_{\min}$ that two point-like object details (or more correctly thin lines) can have so that they could still be discriminated from each other ("resolved") by microscopy. 'Point-like' means that the object dimensions (or the thickness of the lines) are much smaller than the wavelength used for imaging. According to his relationship expressed in formula (1), the smallest distance $d_{\min}$ is essentially determined by the (vacuum) wavelength λ of the light used for imaging and the factor , the numerical $n \sin\alpha$ aperture NA (n is the refractive index of the sample, and α is the half- aperture angle of the objective lens).

$$d_{min} = \frac{\lambda}{2n \sin\alpha} \quad (1)$$

This means that the optical resolution $d_{\min}$ gets better if a shorter wavelength is used for imaging, and if the microscope objective lens has a larger numerical aperture. While the shortest wavelength in visible light microscopy is about 400 nm, the numerical aperture $n \sin\alpha$ cannot be larger than n (the largest half aperture angle possible for any single objective lens is 90°, and $\sin 90° = 1$). The refractive index n is an optical material "constant"; in cell biology, the typical maximum is around 1.5; useful values for the numerical aperture in advanced light microscopes are around 1.4 (in some cases up to 1.6). Altogether, even assuming the best optical conditions, this limited the best optical resolution of any microscope to a value of about 1/3 to 1/2 of the (vacuum) wavelength of light used, or $d_{min} \sim 150$ to 200 nm in the object plane.

Abbe was concerned with the limits of resolution imposed for transmitted light; this means the object contrast was given by local differences in absorption; for example, if a cell is stained with a DNA binding dye, the nucleus absorbs UV-light strongly, while the cytoplasm does not; this method is still widely used in cellular pathology for chromatin texture analyses. However, in many fields of modern biology and medicine, fluorescence microscopy has become more and more important. In fluorescence microscopy, fluorescent molecules can be specifically attached to the cellular structures to be imaged (or molecules can emit fluorescent light by themselves, such as various types of Fluorescent Proteins); 'labelling' presently allows to visualize in a specific way almost any type of macromolecules in the cell; thus fluorescence microscopy has emerged as one of the most important tools of modern biology. The labelling required can be done by a large variety of methods. If the labeled cells (or other biostructures, such as viruses or bacteria) are illuminated with light of an appropriate wavelength, fluorescence is excited at given wavelengths different from the excitation light; the fluorescence emission is separated from the excitation, and a fluorescence image is obtained. Due to the underlying molecular Physics, the light emitted by these 'fluorochromes' is 'incoherent', i.e. from the optical point of view the individual fluorescent molecules emit light independently from each other, like the stars in the sky. Consequently, the same limits of resolution are valid for all these 'self-luminous' sources as for the telescopes used in Astronomy.

These limits were stated by a famous contemporary of Ernst Abbe in England, Lord Rayleigh. In 1896 [100] he came to the conclusion that the smallest distance $d_{\min}$ between two self-luminous 'point sources' is proportional to the image producing wavelength, and inversely proportional to the numerical aperture NA $= n \sin\alpha$, with a factor 0.61:

$$d_{min} = 0.61\lambda/\mathrm{NA} \quad (2)$$

In fluorescence microscopy, for a numerical aperture NA = 1.4, this amounts to 43% of the imaging wavelength, i.e. again roughly to about half the vacuum wavelength, as stated by Ernst Abbe in 1873.

As the only possibility to further enhance the resolution, Rayleigh suggested the use of shorter wavelengths, such as ultraviolet light. This has also been implemented; however, glass becomes quickly opaque in the ultraviolet region, i.e. with shorter wavelengths, so that the resolution cannot easily be enhanced in this way by a large amount.

For many decades to come, the resolution enhancement needed in biology to image the cellular nanostructures underlying the extremely complex spatial organization of life was achieved by shortening the wavelength not using light but using electrons. Electron microscopy played a major role in the discovery of essential elements of chromatin nanostructure, such as the nucleosomes [92,93] and other important features (for review see [104]).

Nevertheless, it remained of the greatest importance to find ways to enhance the optical resolution also in light microscopy. Notwithstanding the immense contributions of electron microscopy, there are a large number of practical reasons for the use of visible light microscopy at enhanced resolution (if possible). For example, observations of nuclear nanostructure using electron microscopes are very complex and time consuming; typically, they require long and sophisticated fixation protocols to avoid artefacts; they are limited to the imaging of surfaces, i.e. to look into the interior of a nucleus, the cell has to be cut into very thin slices, each

[1] translated into English.