High Resolution Imaging at Video Rate with UV Light

Abstract

Imaging integral parts or finest structures of cells is of high relevance for the better understanding of vital processes. Modern light microscopes are limited by the Abbe diffraction limit in resolving structures smaller than 250 nm. However, there are several techniques to overcome this barrier. In addition to super-resolution microscopy, which requires a lot of preparation and computational effort, ultraviolet (UV) microscopy utilizes short wavelengths to decrease the Abbe limitation. This article describes the performance of a UV imaging system. Fine structures of filaments or membranes (e.g. the Hechtian reticulum [1]) are resolved. A crucial part of such a system is the highly sensitive UV camera for optimum imaging performance such as the observation of the movement of white blood cells at video rates.

1. Introduction

In biological research, imaging of the finest cell structures is crucial for an advanced understanding of function, ontogenetic and physiologic processes, as well as phylogenetic relationships. In 1873, Ernst Abbe [2] found that the minimum diameter of an imaged spot is limited by the diffraction of light by the optical parts of a microscope. In modern light microscopes this is typically around 250 nm. Usually such a resolution is sufficient for observing the outlines of cells (100 to 10 μm) or bacteria (10 to 1 μm). Image quality is further improved by use of specific, monochromatic wavelengths for illumination as chromatic aberration is reduced. However, finer structures or smaller objects like viruses of sizes ≤100 nm will appear blurred because their size is smaller than the typical resolution of 250 nm.

A number of different approaches, referred to as super-resolution microscopy [3], have been developed to overcome this limitation. For instance, in Stimulated Emission Depletion (STED) microscopy, which was honored by a Nobel Prize Award in 2014, two laser beams scan over the sample. One of them stimulates fluorescent molecules to emit light and another one cancels out all fluorescence except in a nanometer-sized volume. However, most super-resolution techniques require fluorescent samples, computational image reconstruction and sophisticated setups.

Using ultraviolet (UV) light is another reliable approach to image structures with a resolution better than Abbe's stipulated limit for standard light microscopes. The diffraction barrier is shifted to around 100 nm. In this article, we demonstrate the power of a UV imaging system by recording small biological structures such as cortical membranes of the Hechtian reticulum or granular structures of white blood cells. We will show that a back-thinned CCD-camera, which has very high quantum efficiency in the UV range (e.g. more than 60% at 200 nm), enables fast and sensitive image acquisition thereby minimizing the exposure of cells to otherwise deadly UV light dosages. In addition to improving resolution by lowering the diffraction barrier, the use of UV light also increases the contrast by the absorption of native proteins.
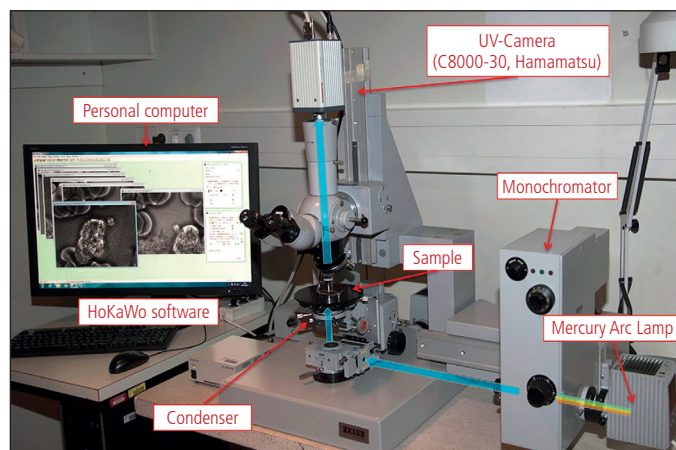

Figure 1: Photograph of the UV microscope setup including UV sensitive camera, personal computer and imaging software. The microscope lenses are all made from quartz to ensure maximum transmission of the UV light.

2. UV Microscopy

To distinguish the maximum of the two point spread functions (PSF) of two light sources, the Rayleigh criterion below

$$d = \frac{1.22\,\lambda}{NA_{obj} + NA_{con}}$$

has to be fulfilled (d: spatial resolution, NA: numerical aperture of the objective and condenser, λ: wavelength). Monochromatic light at short wavelengths decreases the diffraction barrier and enables imaging of small structures down to 50 nm.

In the system used (figure 1), a mercury arc lamp together with a monochromator is used to generate UV light in the range of 280 to 360 nm. This allows for the precise selection of the correct wavelength where particular proteins absorb. All optical parts of the bright-field microscope are made from quartz to allow maximum transmission of light lower than 300 nm. The microscope is equipped with quartz objectives Neofluar 10x/NA 0.30, Ultrafluar 32x/NA 0.40 and Ultrafluar 100x/NA 1,25 (glycerol immersion).

Images are acquired by a UV-sensitive video camera (C8000-30, Hamamatsu Photonics). It employs an ultra-high sensitivity back-thinned CCD sensor made by Hamamatsu, which offers extremely high quantum efficiency (QE) in a wide spectral range including UV.

Standard CCD cameras have practically no sensitivity in the UV spectral range. In front illuminated CCD sensors, pixels have their light sensitive surface covered by a semi-transparent Poly-Si electrode for the charge transfer of electrons converted photons. As this Poly-Si film absorbs in the UV spectral
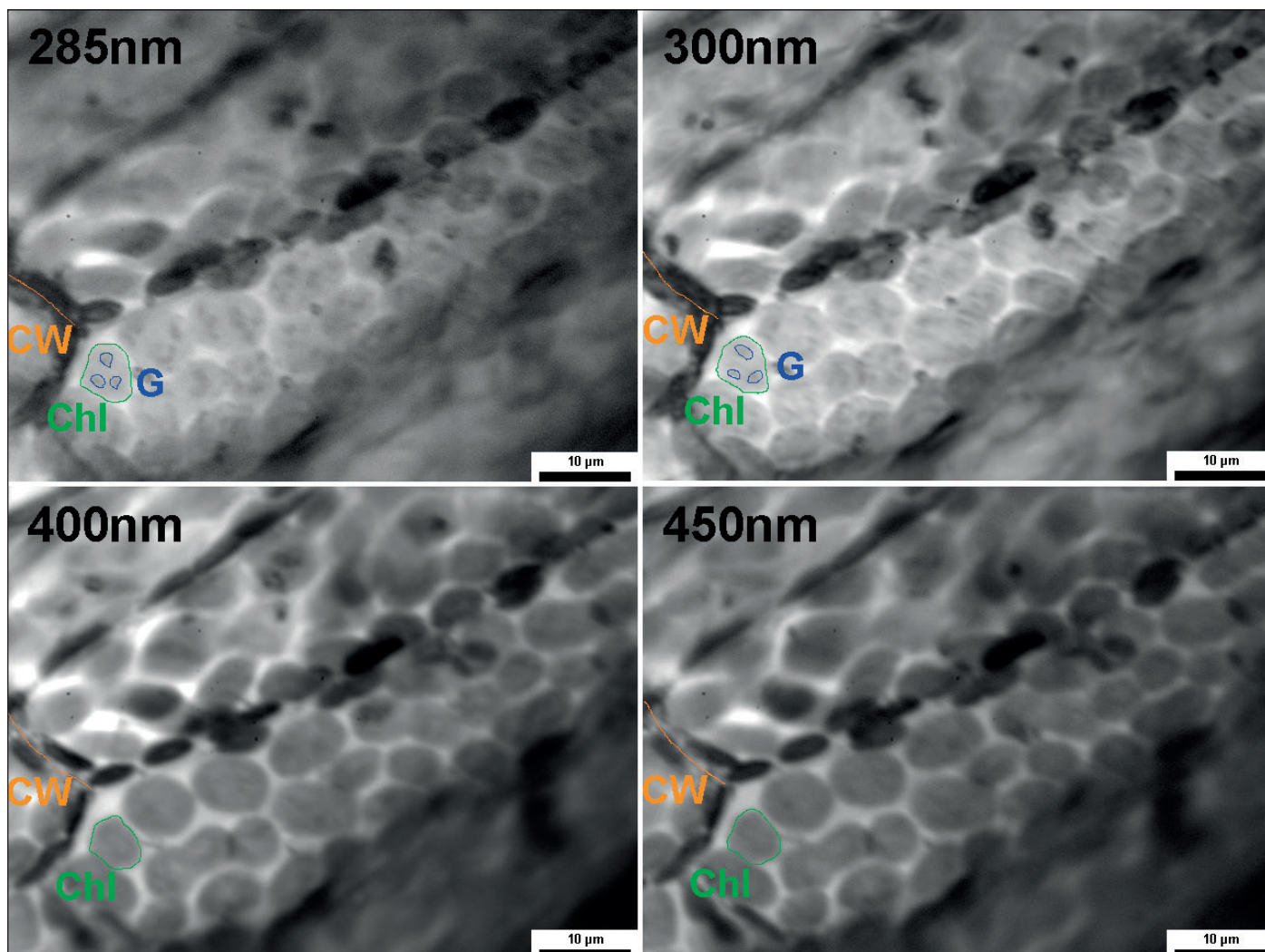
Figure 2: The 12-bit RAW images show gametophore cells of the moss *Physcomitrella patens*. Imaging the cells at wavelengths of 450 nm resolves the chloroplasts inside the cells. However, no details are visible. Decreasing the wavelength of illumination to below 300nm resolves the fine inner structure of the chloroplasts (grana) as dark spots Chl: chloroplast, G: grana, CW: cell wall

range, short wavelength photons are not able to reach the pixels. A solution to this problem is to use the chip back to front. Its backside is thinned to 10-15 μm. Those back-thinned CCD chips are then mounted with their electronics covered front facing inward. Light travels through the thinned silicon layer into the light sensitive part of the pixels. In this way, higher quantum efficiencies of >90 % and a much larger spectral range is achieved.

Although back-thinned cameras have larger pixels and therefore usually lower spatial resolution, the resolution of this system is more than doubled by using UV light for illumination. In addition, the contrast is improved due to the absorption of the UV light by proteins, DNA and other biological structures (figure 2). However, exposure times should be kept short to prevent cell damage. Furthermore, cell compartments with excessive content of UV-absorbing substances, e.g. in the cell wall or vacuoles are challenging and sometimes impossible to resolve.

3. Example Measurements

Here, we present some examples of different cell types, human and plant respectively, to demonstrate the possibilities of the described setup. In moss (*Physcomitrella patens*, Funariaceae) gametophore cells, we show the different absorption levels of proteins in UV and blue light wavelengths (figure 2). Four 12-bit images acquired with the UV microscope are shown at different wavelengths (450, 400, 300 and 285 nm). Wavelengths of 450 nm, which may also be achieved by modern standard light microscopes, do not resolve details of the chloroplasts. In contrast, at wavelengths shorter than 300 nm fine details of the grana structure can be observed. The image sequence also demonstrates the different absorption properties of the chloroplasts at different wavelengths. Whereas at 285 nm and 300 nm the chloroplasts appear light grey, they are much darker due to absorption at wavelengths above 400 nm.

The same moss sample is used to show the increased resolution in UV light, further improved by specific LUT settings (figure 3). This option is particularly interesting for analysis of structural details at the limit of resolution. We focus on the organization of a small, transparent membrane structure that is pinned to the inner side of plant cell walls. The so called Hechtian reticulum and Hechtian strands [1] become visible after plasmolysis, a process to detach the living protoplast from the cell wall using hyperosmotic solutions [4-11] (see arrow in figure 3). Again, the setting described allows for detailed investigation of intracellular structures and provides new insights in the vital and physiological functions of living organisms. Especially since the organization and attachment sites of the Hechtian reticulum are still under debate [8, 10] it is therefore favorable to gain highest possible resolution of the membrane structures. In addition, a good software can facilitate image analysis by offering tools such as additional contrast gain by setting the lookup table (LUT) or to invert an image (figure 3d). The software used (HoKaWo, Hamamatsu Photonics Germany) also allows for background subtraction to eliminate disturbing structures such as the close cell wall.
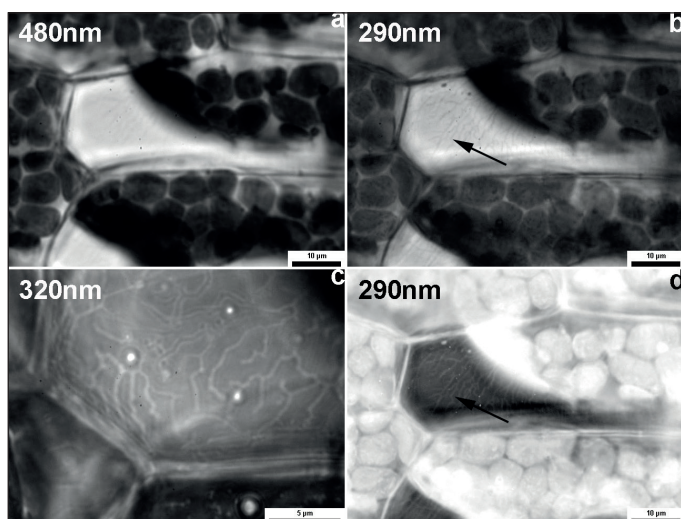

Figure 3: After plasmolysis, the protoplasts detach from the cell wall and the chloroplasts are pushed together. The resolution increased from 480 nm (a) to 320 nm (c) and 290 nm (b and d), respectively. Again, the lower wavelength allows the identification of detailed structures within the cell (Hechtian reticulum, arrow). The image at the bottom right is an inverted image which highlights the fine structures even more. On the lower left another inverted image is shown which was acquired with a 100x objective lens.

In a third set of experiments, we want to prove the advantages of UV imaging in moving samples. For the documentation of dynamic processes in real-time and time lapse recording, the Hamamatsu Photonics camera C8000-30 is ideal due to the high frame rates. Short exposure times of 30.8 ms allow frame rates of 31.4 fps at full resolution. Using binning, the frame rate is further increased to 101.8 fps maximum at a resolution of 180 x 120 pxls. At the same time, the signal to noise ratio is improved by a factor of 4.

To illustrate this, we have recorded living cells of a human blood sample. A time series of a granulocyte, taken at 340 nm, is given in figure 4. The time stack shows the rolling amoeboid motion of white blood cells. Again, the finest structures inside the granulocytes (bright and dark spots) can be resolved which allows for precise motion analysis. To better visualize cell movement, an image series was acquired and post-processed to generate a stack of subtraction images, whereby each pixel value in frame x was subtracted from the corresponding pixel value in frame x+1. A maximum intensity projection was then performed on the subtraction images, shown in the bottom right image. Additionally, the shape at the beginning (yellow) and the end (blue) is highlighted, depicting the complete movement of the cell during the 5.6 s acquisition time. It is interesting to note that the cell moved mainly in the bottom part where the cell is dented. This part also shows the highest intensity.
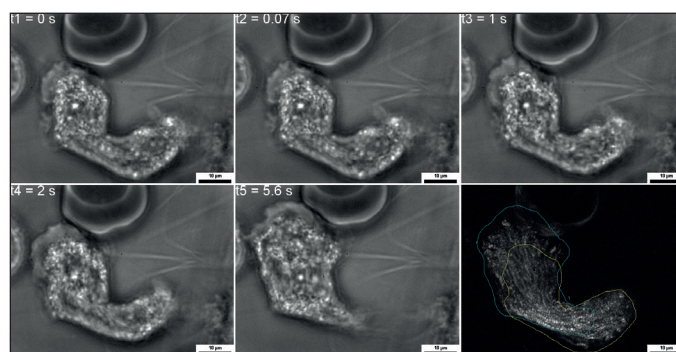

Figure 4: Amoeboid motion of granulocytes (white blood cells) at a wavelength of 340 nm. Time points of 0, 0.07, 1, 2 and 5.6 s are given. The image at the bottom right shows an overlay of intensity changes of the movie to highlight the movement of single cell compartments. The yellow line represents the cell outline at the beginning of the movie (0 s) and the blue the end (5.6 s). The fast readout of the camera allows the detection of fast processes (70 ms) at full field of view.

4. Conclusion

In this article, the advantages of a high performance UV sensitive camera (C8000-30, Hamamatsu Photonics) mounted to a bright-field UV-microscope are highlighted.

The Hechtian reticulum which connects the protoplasts to the cell wall of moss gametophore cells was imaged with a resolution surpassing that of a standard light microscope. It was demonstrated that illumination at 290 nm increased the resolution by a factor of 1.6 compared to images recorded at 450 nm. Some cell structures can still be seen but at 290 nm illumination, smallest structures of cellular compartments are resolved and natural protein absorption causes contrast levels to increase. Furthermore, Hechtian strands and the Hechtian reticulum which connect the protoplasts to the cell wall in plant cells are resolved in high contrast without any labelling. The dedicated and user-friendly software (HoKaWo, Hamamatsu Photonics) makes it easy to manipulate the 12-bit RAW images, e.g. by setting the limits of the lookup table or inverting the image. This is a major tool to depict structural details and describe the cell properties.

To prove the ability of fast imaging with our UV microscope setup, the movement of granulocytes (white blood cells) is recorded. The high speed of the UV camera allows acquisition at video rates and full field of view. Compared to other super-resolution techniques, this is a key advantage as these suffer from complicated setups and computational image reconstruction limiting their ability of fast imaging of dynamic biological processes.

To summarize, the UV imaging system is a powerful tool to image fine structures which are normally hidden due to the Abbe diffraction limit. Although this is achieved using biologically destructive UV light, no computational reconstruction or complicated sample preparation is necessary. This feature renders the system particularly interesting for imaging very fast and dynamic biological processes at a very large field of view and frame rate.

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