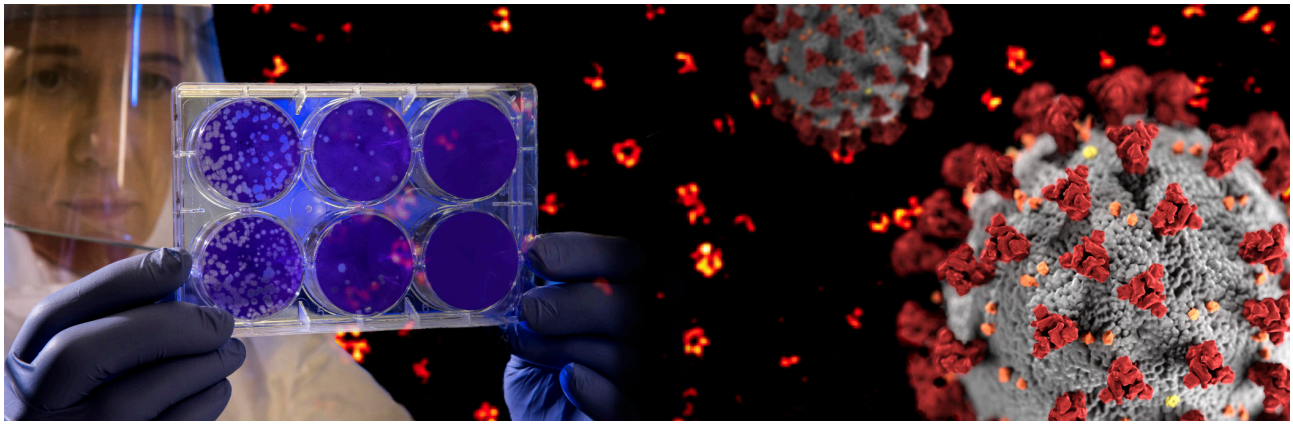
STED and virology



Super resolution microscopy is a widely used research method for studying biological features beyond the diffraction limit, such as nuclear pores, clathrin coated vesicles, the periodicity of the actin skeleton in neurons, and viruses. In this white paper we will focus on the advantages of STED microscopy and its contributions to the virology field. We will provide insight into the advances in virus research made possible by STED microscopy and give a hint to were the journey might go.

Virus particles vary in size across different virus species. One of the smallest viruses, the parvovirus, has a particle size of approx. 20 nm while the largest known virus particles of the Mimivirus family possess a capsid size of up to 500 nm, which already compares to the size of a bacterium (Fig. 1). Therefore, due to their size, virus particles and their substructures can be studied in detail using super-resolution microscopy methods, such as STimulated Emission Depletion (STED) microscopy or single-molecule localization methods, which offer resolution capabilities of about 20 nm. Numerous studies using super resolution imaging techniques have already expanded the scientific knowledge of the Human Immunodeficiency Virus (HIV-1) [1, 2], Influenza A virus [3, 4], Herpes Simplex Virus (HSV) [5], Respiratory Syncytial Virus (RSV) [6], Vaccinia Virus [7], Adeno-Associated Virus (AAV) [8], Nipah Virus (Niv) [9], Adenovirus [10] and many more.

- Super-resolution STED microscopy is a powerful technique to observe the finest structural details in cells and viruses
- It covers the full resolution spectrum required to study viruses even in a live environment and in interactions with the host cells
- MINFLUX will open completely new paths of virus imaging

STED microscopy for virus research

The gold standard technique for imaging virus particles was and still is electron microscopy (EM). The main advantage of EM is undoubtable the angstrom-range resolution it provides, which exceeds the resolution capabilities of all other available imaging techniques. The extremely good resolution, however, comes at the cost of highly fixed specimens with low contrast. Furthermore, labeling and detection of specific proteins inside large assemblies and cells is challenging with EM. On the other hand, light microscopy methods, and especially confocal fluorescence microscopy, generate high-contrast images, with specific labels for the proteins of interest. However, the resolution of these techniques is limited to about half the wavelength of light (~200 nm). The Nobel Prize winning methods of super-resolution microscopy, specifically STED microscopy and single molecule localization microscopy techniques, overcome this resolution barrier by exploiting the on-

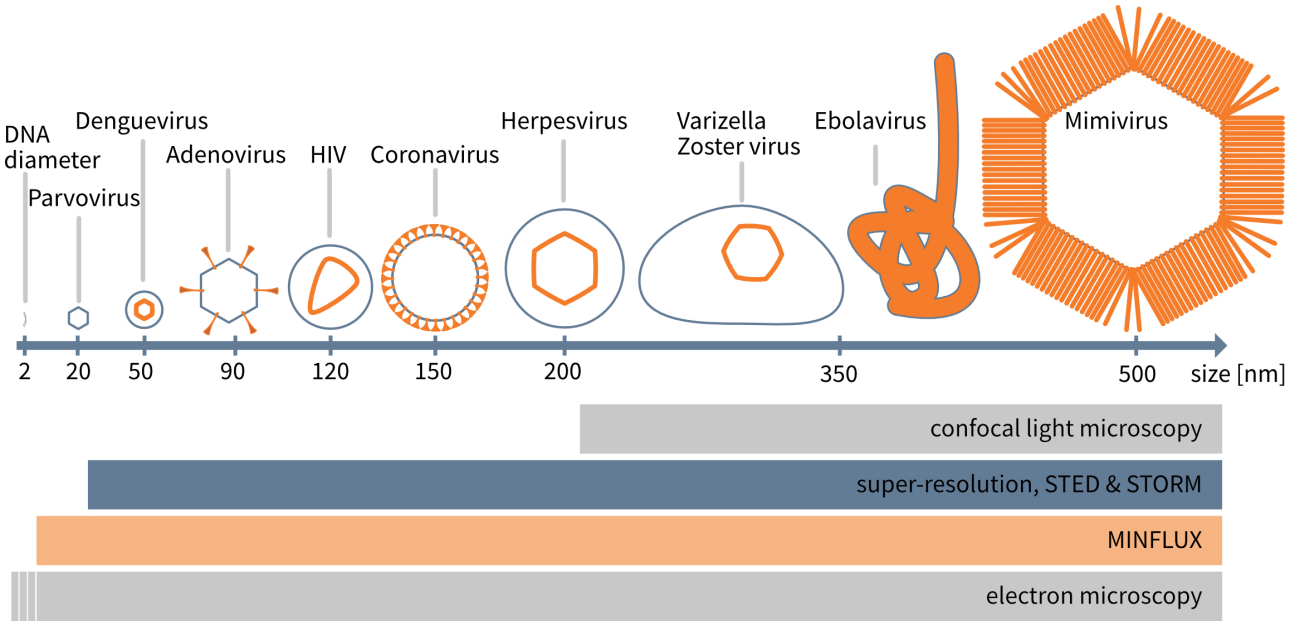


Fig. 1: Comparison of different virus particle sizes with the resolution of different imaging techniques.

off switching of fluorescent dyes. These techniques improve confocal resolution by roughly a factor of 10, achieving resolution capabilities closer to those offered by EM (see Fig. 1). Furthermore, the nanoscale resolution of STED microscopy combined with the less destructive sample preparation and labelling methods of fluorescence make it possible to study the structural information of native (unfixed) virus particles in the 100 nm-range [2]. An example of how STED microscopy has contributed to HIV-1 research is detailed in the box on page 3.

Protein-specific labeling coupled with the high-contrast imaging of light microscopy enables colocalization studies of different proteins to shed light on, for example, virus-host interactions. In conventional microscopy, however, our understanding of the protein position is blurred by diffraction. Super resolution microscopy techniques overcome this blurring effect and thus lend a much higher confidence to the results of colocalization experiments. STED microscopy is especially well-suited for these studies since resolution can be improved not only laterally, but axially as well, giving rise to isotropic resolution of up to 60 nm without any post-processing. Additionally, if two spectrally different dyes are used which can both be depleted by the same STED laser doughnut (e.g. STAR ORANGE and STAR RED, or Alexa594 and ATTO647N), there is no chromatic shift between the two channels. Because the central zero of the STED doughnut defines the point from which photons are emitted, if two channels share the same depletion doughnut, colocalization is intrinsically given. Various studies have used STED microscopy to visualize the HSV genome inside infected cells [11] or to study the virus-host interaction of the Influenza A virus [3, 4].

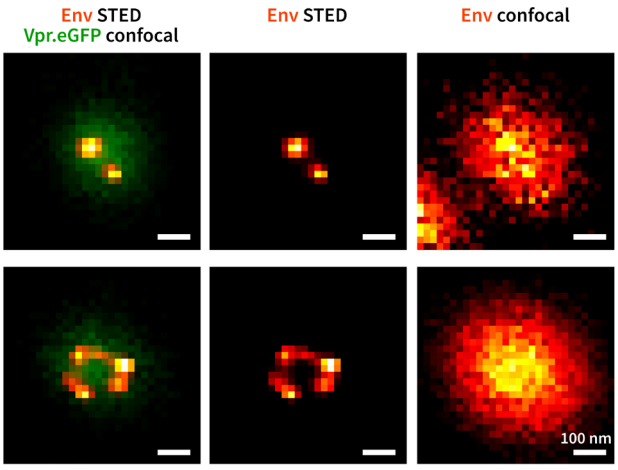


Fig. 2: Env protein distribution (red/yellow) on single HIV-1 particles (green). Vpr.eGFP was used as a marker for HIV-1 particles. Env clusters on the virus particle surface were labelled by Fab fragments coupled to STAR635P. Only with super-resolution microscopy can single Env clusters be revealed (compare middle column (STED) and right column (confocal)). Images by Jakub Chojnacki (used with permission).

The possibility of live-cell imaging and the tracking of dynamic events over time is a great advantage of fluorescence microscopy, and STED microscopy uniquely combines this advantage with nanoscale resolution. Live-cell STED microscopy has proven valuable in many studies [12-14]. A major concern in live-cell STED microscopy is bleaching and phototoxicity. While bleaching can be overcome by using photostable dyes or exchangeable fluorophores [18], minimizing phototoxicity is less straightforward. Nevertheless, with STED microscopy, it is often overlooked how resolution and laser power are interdependent. Due to the square-root dependence of resolution on STED power, high STED laser power is only needed to squeeze out the last few nanometers of resolution. In STED microscopy, the achieved resolution is tunable via the STED laser power and this can be increased only as much as required to answer a given scientific question. Furthermore, novel technologies such as pulsed STED combined with adaptive illumination methods (e.g. MINFIELD, DyMIN and RESCue) [15-17], allow for prolonged imaging at high resolution with low light levels and thus less phototoxic effects. An example on HIV-1 imaging is shown in Fig. 3. Here, it is also important to note that since a virus particle contains only a small number of proteins and hence labels, gentle adaptive illumination methods help minimize photobleaching, thus allowing one to image with higher resolution, or for a longer time in a live experiment.

Especially sparse events are difficult to image in EM and can usually only be found by correlative imaging of light microscopy and EM. Identification of sparse events, such as cell infection or virus particles binding to enter a cell, or nucleus, are the perfect target for STED microscopy. Since every STED microscope is inherently a confocal microscope (with the STED laser turned off), it is possible to scan the sample in confocal mode with a low magnification objective lens to easily identify rare events. After finding the event, a region of interest can be chosen to target the event, and the STED laser can be turned on to acquire a super-resolution image. The imaging of capsid positive objects after HIV infection of cells close to the nuclear pore [19] is one example of how STED microscopy can be used to study rare events.

Scientific research involving virus imaging requires a large range of imaging scales, from a single virus particle on the coverslip [2], to whole infected cells [19] or even intact living mice [21] to, for example, observe

A glimpse into HIV-1 studied by STED microscopy

Human Immunodeficiency Virus 1 (HIV-1) maturation occurs concomitantly with cleavage from the plasma membrane of its host cell. During maturation, the protease is active, and cleaves the polyprotein Gag into its components, leading to a rearrangement of the virus proteins. Mature HIV particles enter cells more efficiently. However, there is no apparent change of the surface protein (envelope protein, Env) structure or composition. Compared to other virus particles, only a few envelop proteins are incorporated on the HIV surface (7-14 Env proteins per virus particle). The question remains of how the internal morphological change influences the Env protein on the particle surface, and what the surface effect is that increases entry efficiency into new host cells.

In 2012, the research team of Hans-Georg Kräusslich answered these questions using STED microscopy, when the method was still in its infancy. In a collaboration with Stefan Hell's group, they showed that Env proteins on single virus particles form clusters after virus maturation, and that this clustering is induced by maturation through an interaction between the Env tail and the internal virus proteins. Since virus particles (diameter ~120-150 nm) are much smaller than the diffraction limit of light, only direct observation by super-resolution microscopy was able to resolve this clustering (see Fig. 2) [1].

In a follow-up study at the University of Oxford, researchers were able to determine the mobility of the Env trimers on a single virus particle. This study showed that the HIV-1 lipid envelope is a low mobility environment due to its high lipid order. The standard technique to image mobility is Fluorescence Correlation Spectroscopy (FCS). However, since the virus particles are smaller than a conventional focused spot, intensity fluctuations due to particle movement can only be observed by a combination of STED and FCS, to reduce the focal volume [23].

The high lipid order observed led to a collaboration between labs in Spain, France, Germany and Australia to study the lipid environment of budding HIV particles in cells by STED-FCS. The study showed that around an HIV-1 budding site, the virus seems to generate its own lipid environment, which is likely due to the recruitment of specific lipids through the main structural polyprotein Gag [24].

In summary, major contributions to basic research on HIV-1 were achieved using STED microscopy. Also in the future, STED and super-resolution microscopy in general will be instrumental in the research on small structures such as viruses.

the immune response in lymph nodes. Thus, the ideal super-resolution microscope needs to be flexible to perform all given imaging tasks. STED microscopes reliably meet this need and can be adapted to a variety of imaging experiments. Objective lenses and mounting media can be chosen as needed for the experiment. Adaptive optics allows STED and confocal imaging deep inside the sample by using a deformable mirror to correct for aberrations induced by the sample. With these tools at hand, the STED microscope is able to image across scales and highly adapts to the experimentalist's needs.

Conclusion and Outlook

In this article, we provide a brief overview of super resolution microscopy, focusing on STED microscopy and its contributions to virus imaging and virus research. Developments in STED microscopy instrumentation are rapid and, while the technique has already seen widespread use in scientific research, we are confident that it's potential can only increase in the future. One should not forget that the first ever images on a home-built STED microscope were acquired only less than two decades ago. Since then, tremendous amounts of engineering and innovation have made STED microscopy what it is today: a reliable, easy-to-use super resolution imaging technique that can be operated as intuitively as a confocal microscope, by everyone.

The potential of super resolution microscopy extends far beyond STED. The novel technique MINFLUX [22] offers isotropic resolutions up to 2 nm – the size of a single fluorescent molecule. Already STED microscopy was able to change the way we look at virus particles, so the impact MINFLUX will have on scientific research cannot be underestimated. This new level of resolution is expected to have a tremendous impact on virology research. MINFLUX tracking can be performed at extremely high speeds with true molecular precision, making it possible to observe rearrangements inside a single protein over time, or to follow single virus particles over a long time with unprecedented precision and speed.

Got questions?

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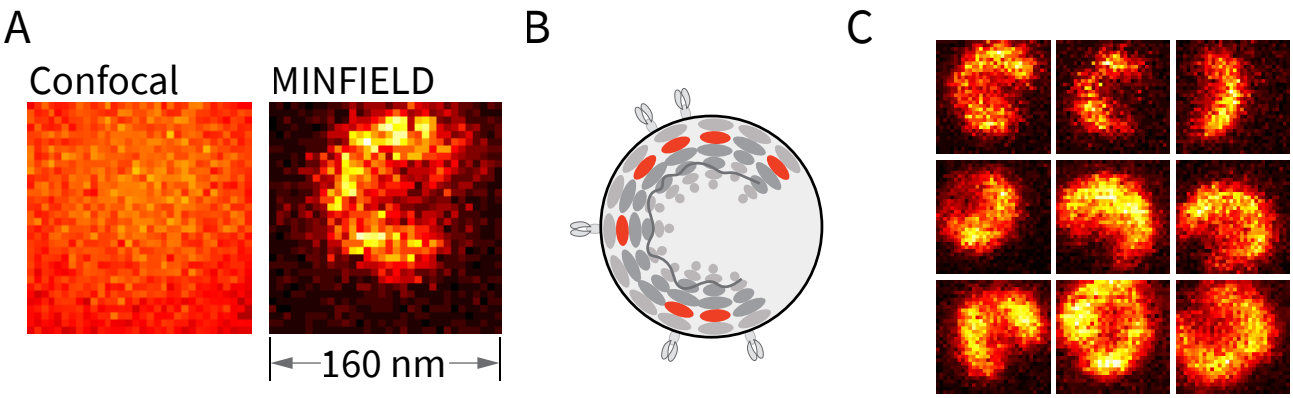

Fig. 3: MINFIELD STED presented on human immunodeficiency virus type 1 (HIV-1 labelled with SNAP-tag between matrix and capsid in the Gag protein and stained with Siliconrhodamine). (A) Confocal (left) and MINFIELD STED (right) images with a field size of 160 nm. (B) Scheme of the immature HIV-1 particle with labels indicated in red. (C) Imaging examples of single HIV-1 particles.

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