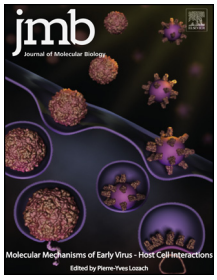

Microscopy in Infectious Disease Research—Imaging Across Scales

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Abstract

A comprehensive understanding of host–pathogen interactions requires quantitative assessment of molecular events across a wide range of spatiotemporal scales and organizational complexities. Due to recent technical developments, this is currently only achievable with microscopy. This article is providing a general perspective on the importance of microscopy in infectious disease research, with a focus on new imaging modalities that promise to have a major impact in biomedical research in the years to come. Every major technological breakthrough in light microscopy depends on, and is supported by, advancements in computing and information technologies. Bioimage acquisition and analysis based on machine learning will pave the way toward more robust, automated and objective implementation of new imaging modalities and in biomedical research in general. The combination of novel imaging technologies with machine learning and near-physiological model systems promises to accelerate discoveries and breakthroughs in our understanding of infectious diseases, from basic research all the way to clinical applications.

Why microscopy?

Microscopy and infectious disease research have been inseparable partners ever since in the 1670s Anthonie van Leeuwenhoek used his newly invented microscope to examine a sample of plaque he had scraped from his own teeth, and observed—for the first time—bacteria and other microorganisms that share the world with us [1] (Fig. 1a). It took 200 years until the work of Robert Koch and Louis Pasteur in the second half of the 19th century demonstrated that bacteria can cause the diseases. Microscopy was used to examine the samples of infected people and animals, and it quickly became an indispensable research tool to investigate infectious diseases. In contemporary photographs, both Koch and Pasteur are often shown "as peeking through a microscope," and their microscopes are displayed in museums around the world.

Due to their small size, viruses were not visible with the contemporary light microscopes, so they were not discovered until the late 1890s. However, it was only after the first image of a virus (mouse ectromelia virus) was acquired and published by Helmut Ruska and colleagues in 1938, using an electron microscope [2] (Fig. 1b), that the scientific establishment finally accepted the existence of viruses as particles and not as "toxins or elements of ferment nature," as in the view held by many at the time. This was substantiated in the subsequent two decades when, owing to improvements in electron microscopy (EM) technology and sample preparation, EM micrographs revealed the beautiful structure of T bacteriophages [3] (Fig. 1c). These microscopy-based discoveries in the mid-20th century firmly opened the door to the field of virology. Undoubtedly, microscopy was fundamental in infectious disease research, as it was necessary for the discovery of infectious agents by direct observation and later on also for their diagnosis. However, microscopy in those early days of infectious disease research did not yet reveal its full potential.

A big boost to microscopy came when computer technology and digital detectors (such as charged-

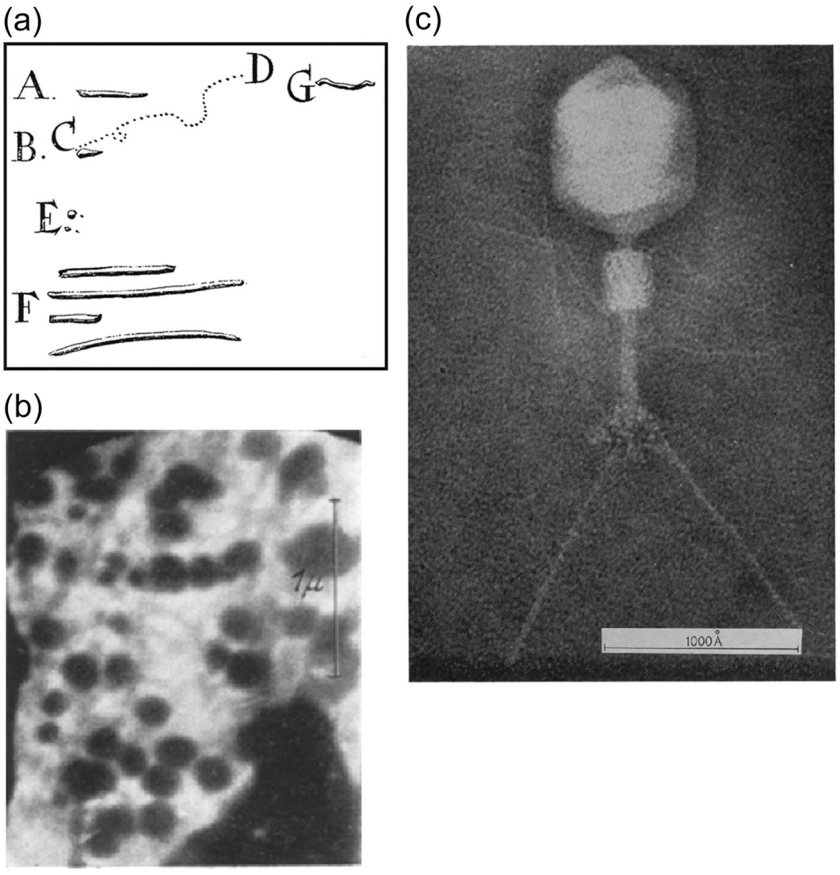

Fig. 1. Examples of first microscopy utilization in the discovery of infectious agents. (a) Early illustrations of dental plaque bacteria by Antonie van Leeuwenhoek in 1683 (adapted from Ref. [1]). (b) Electron micrograph of mouse ectromelia virus by Helmut Ruska and colleagues in 1938, the first virus whose size and shape were shown to the scientific world (adapted from Ref. [2]). (c) Electron micrograph showing intricate structural organization of the T bacteriophage (adapted from Ref. [3]).

coupled device cameras) had sufficiently matured to be used for microscopic imaging. By coupling the microscope to a digital detector and a computer [4, 5], the microscope was transformed from a tool that "only" magnifies an image to an exact, quantitative, analytical instrument. Another breakthrough was the introduction of fluorescence microscopy, dramatically increasing the contrast and allowing the observation of sub-resolution structures specifically stained with fluorescent labels (although they could still not be spatially resolved until recently).

Based on development of new imaging modalities, fluorescent probes and sensors, computer technology, image analysis algorithms and new biological model system, the field of biological microscopy is undergoing a revolution. Only now we are starting to appreciate and make use of the full potential of this microscopy-based approach. Modern microscopes have evolved into complex robotic instruments capable of automatically interrogating a wide range of biological processes at vastly different spatiotemporal scales and organizational complexities, from structural studies on macromolecular scale all the way to whole organ/body imaging in living animals (Fig. 2).

Microscopy is also an integral component of modern medicine, where it provides a diagnostic tool for many bacterial, fungal and parasitic infections (light microscopy) [6] as well as for viral infections (EM) [7]. In many developing regions, light microscopy is the most important procedure for the diagnosis of infections, facilitated by new low-cost, smartphone-based microscopes to be used in the field [8]. However, in this overview, I will concentrate on the role light microscopy-based imaging currently has in infectious disease research specifically focusing on novel imaging modalities, as well as highlight the challenges for the future.

The goal of infectious disease research is to obtain a comprehensive understanding of replication, spread and pathophysiology of infectious agents in order to develop new and better medical treatments. Classical biochemical, genetic and genomic approaches have been employed over the years to yield important insights in host–pathogen interactions. Most of these experimental approaches are population-based ("bulk"), end-point analyses where obtained information represents an average across the population and where important parameters can be missed as they become "averaged out" in the bulk measurement. It is important to realize that the different states of biological systems, such as, for example, the differences between the health and

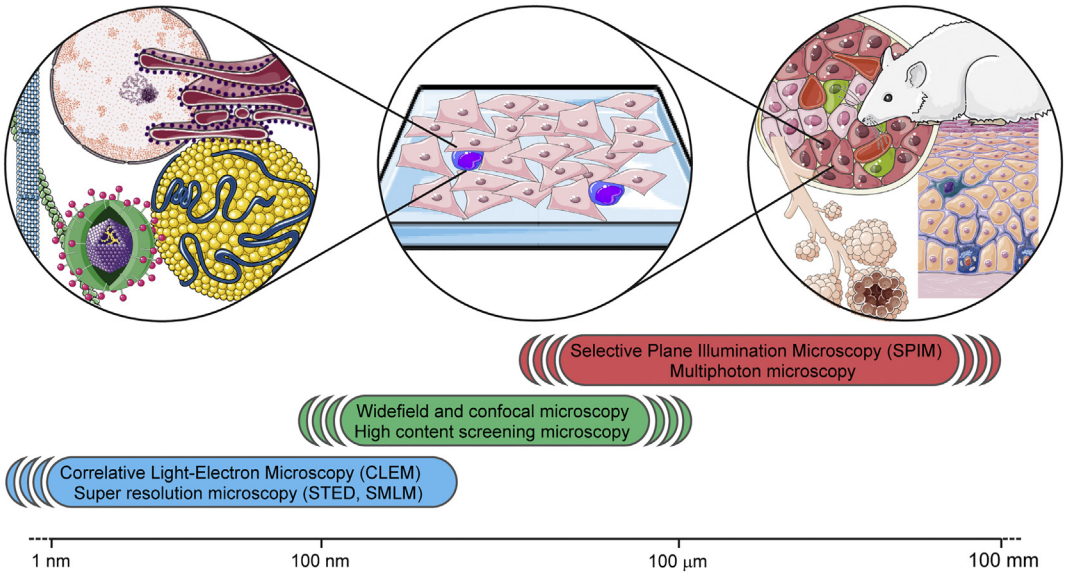

Fig. 2. Imaging across scales and organizational complexities. Development of new imaging modalities have enabled visualization and quantification of infectious diseases processes with high spatiotemporal resolution and at scales spanning many orders of magnitude in size—from nm to mm. Much of the infectious disease research was done on the cellular monolayers using conventional microscopy technologies. Now, using CLEM and super-resolution microscopy, it is possible to "zoom in" further and get the relevant information on molecular and structural level. But it is also possible to "zoom out" (using SPIM) and examine the same processes in more physiological setting, in the context of organoid, organ or living animal. (Individual images courtesy of medical illustrations database—https://smart.servier.com/)

disease, are ultimately governed by individual, stochastic and often rare molecular events. To truly understand different physiological states, we need to employ a reductionist experimental approach that is able to capture and quantitatively examine these individual molecular events. Due to recent innovations, light microscopy is particularly suited to sample complex spatio-temporal dynamics of living systems at sufficient resolution, and thus able to provide realistic representations of the biological systems. It is a big technical challenge to observe and quantify these stochastic molecular events. An even bigger challenge is to understand how they give rise to the different typical patho-physiological states that we observe in "bulk" measurements. In this respect, microscopy-based analysis yields quantitative information that can be integrated into predictive multiscale mathematical models of infection that will hopefully be used to reconcile the findings obtained by the reductionist approach with the ones that are based on population measurements.

Super-resolution light microscopy

For a long time, the observation of molecular and cellular determinants relevant for host–pathogen interactions was hampered by the inability of a conventional microscope to spatially resolve any two objects that are closer together than the Abbe diffraction limit. In simple terms, if two objects are closer than approx. 200 nm, they will not be seen as two individual objects in a conventional microscope, but as one, and no further details can be seen below this limit. This fundamental limit of light microscopy cannot be overcome by building better optics. However, recently developed super-resolution imaging techniques have found ways to circumvent this limit, by only allowing a subset of molecules to emit fluorescent light at any one time. This can be done by using patterned light to spatially modulate the fluorophore emission [e.g., in stimulated emission depletion microscopy (STED) [9, 10]] or by temporally modulating the emission via fluorophore "blinking" [in single-molecule localization microscopy (SMLM) [11, 12]]. Since spatial resolution in STED microscopy scales inversely with the power of the depletion laser and in SMLM it is determined by the number of detected photons per blinking event [13], there is no more fundamental spatial resolution limit of fluorescence light microscopy. Indeed, a method able to localize single molecules with 1-nm precision and resolving emitters only 6 nm apart has been reported [14]. However, due to technical challenges connected mainly with fluorophore photostability and labeling density, the resolution in biological imaging typically achieved with super-resolution methods is in the range of 20–40 nm.

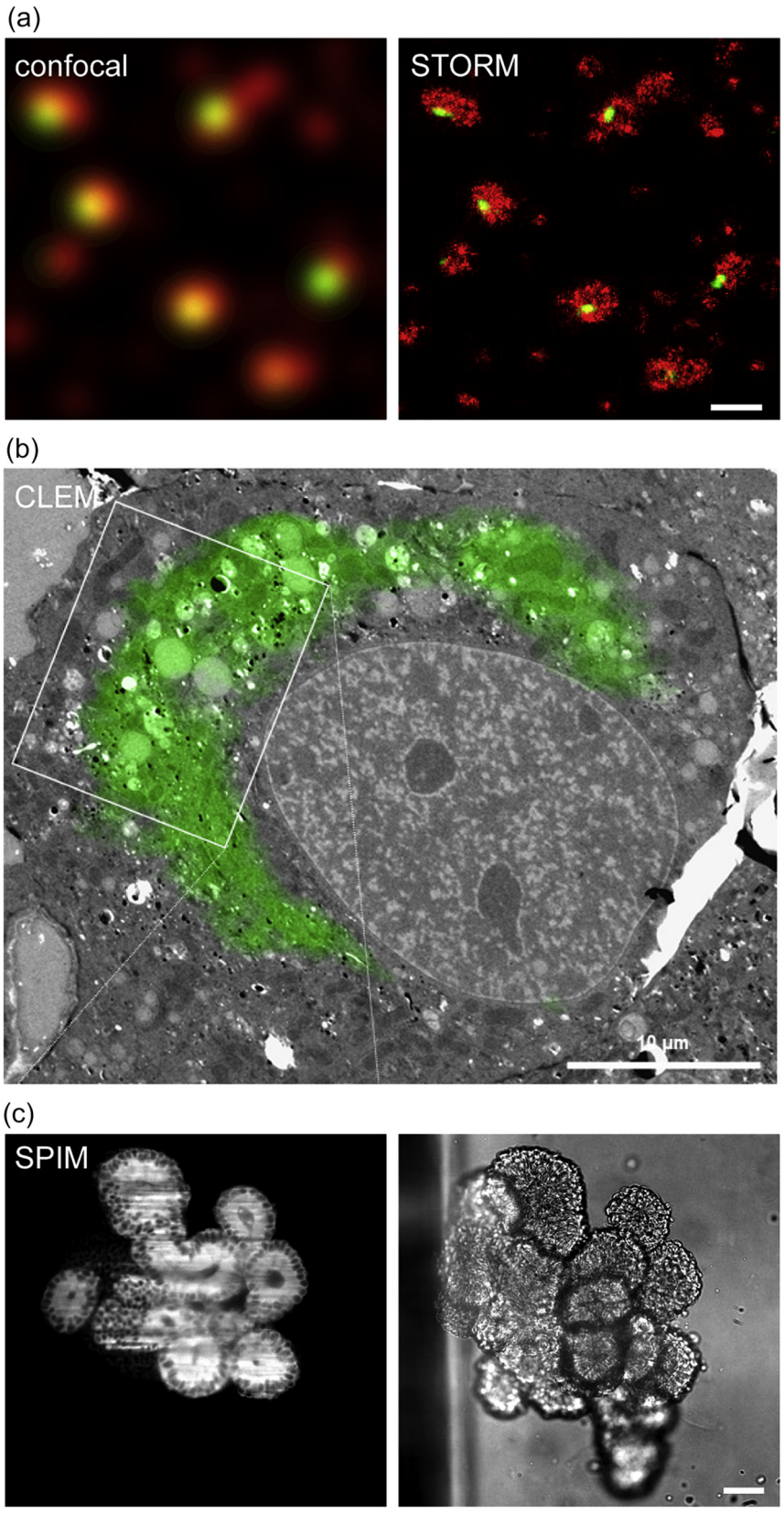

Fig. 3 (*legend on next page*)

The development of super-resolution technologies provided a way forward across the field of biomedical sciences but had an especially significant impact on the infectious disease research. Not only is it now possible to examine the host–pathogen interactions on a nano-scale, such as human immunodeficiency virus (HIV)-induced microdomain organization on the plasma membrane of host cells during virus budding [15, 16] or intracellular membrane reorganization during hepatitis C virus (HCV) infection [17], it is also possible to examine molecular determinants on the pathogens themselves. This was demonstrated by showing Env receptor clustering during maturation of HIV virions [18] (Fig. 3a), by localization of the ESCORT machinery within HIV particle during budding from producer cells [19] and by structural organization analysis of herpex simplex virus [20]. Furthermore, super-resolution microscopy was used to determine 3D macromolecular structures of large cellular complexes such as the nuclear pore complex [21] and viruses [22, 23] by making use of particle averaging techniques originally developed for cryo-EM [24]. To facilitate the 3D super-resolution microscopy-based structural analysis of viruses, an open-source software package that combines super-resolution imaging and single-particle analysis to generate unbiased molecular models of intact viruses or viral substructures has been reported [25]. Although still far from the resolution limit of EM, light microscopy has been transformed into a new tool for structural biology and now can be used to tackle questions that were previously reserved exclusively for EM and X-ray crystallography studies with the benefit of higher throughput, specific labeling and possibility to obtain native structures "*in situ*".

However, as the resolution of the light microscopy is approaching that of the EM, some of the considerations well established in the EM field are becoming relevant for light microscopy-based approaches. All super-resolution imaging modalities require high density of labeling. Nyquist criterion dictates that the fluorophores should not be separated by a distance larger than half of the desired resolution. One consideration is that the labeling density necessary for extracting structural information by super-resolution imaging might induce unwanted, non-physiological macromolecular organization due to sterical interference between exogenously introduced dyes and tagged fluorescent proteins or due to non-physiological effects connected to high fluorescent protein overexpression. The second consideration is that chemical fixation typically employed in light microscopy is resulting in structural artifacts [26, 27] and is incompatible with high-resolution structural analysis. An interesting solution to this is to immobilize the biological material by vitrification process—a technique that allows maintaining the water content of cells by freezing them to a temperature around −190 °C within milliseconds. The fast freezing process prevents the water from crystalizing into hexagonal ice (occupies a volume 10% larger than liquid water) and forms a glass-like layer of amorphous vitreous ice. Vitreous ice occupies the same volume as water and therefore preserves cellular structures in near-native conditions without inducing structural changes [28]. This sample immobilization approach is typically employed in cryo-EM but not yet established for the light microscopy approaches. Super-resolution light microscopy under cryogenic conditions is a very promising new imaging modality with various technical challenges to overcome. Besides near-native structure preservation, light microscopy of vitrified samples will benefit from increased fluorophore photostability under cryogenic conditions [29]. It has been demonstrated that the single-molecule localization-based super-resolution approach is feasible under cryogenic conditions and able to yield resolutions below the diffraction limit of the light [30]. Furthermore, when coupled to cryo-electron tomography, the approach was able to identify different conformations of macromolecular complexes involved in a bacterial secretory system [31].

Another fundamental advantage of super-resolution light microscopy over EM is the ability to examine dynamic processes in living specimens. Despite pilot experiments reporting successful super-resolution

Fig. 3. Examples of applications of new imaging modalities in infectious disease research. (a) HIV budding sites (Gag, green; Env, red) on the surface membrane of HeLa cells shown in conventional microscopy (left) and super-resolution microscopy (STORM) (right). Only using super-resolution microscopy is it possible to realize the structure of the viral budding sites where Env molecules accumulate around the Gag cluster rather than directly co-localizing with it. The scale bar represents 200nm (Images courtesy of Walter Muranyi and Hans-Georg Kräusslich, Department of Infectious Diseases, Virology, University of Heidelberg, Germany). (b) Fluorescence image of GFP-tagged HCV subgenomic replicon overlaid with EM image in a correlative approach. Areas of high fluorescence intensity correlate with the sites of complex HCV-induced membranous compartments essential for HCV pathogenesis. (Image courtesy of Inés Romero-Brey and Ralf Bartenschlager, Department of Infectious Diseases, Molecular Virology, University of Heidelberg, Germany). (c) Organoid, derived from human small intestine cells, expressing innate immunity sensor IRF3-GFP shown in bright field (right) and imaged using SPIM (left). Upon sensing of viral infection, the IRF3-GFP sensor will translocate from the cytosol into the nucleus. With SPIM, it is possible to analyze this process within the context of complex close-to-physiological model system. The scale bar represents 50 μm. (Images courtesy of Megan Stanifer and Steeve Boulant, Department of Infectious Diseases, Virology, University of Heidelberg, Germany).

live cell imaging in infectious disease research [32, 33], this application remains challenging. The necessary high sampling frequency in space and time required to obtain a super-resolution image invokes huge demands on the photostability of fluorescent reporters. There is a clear need for better, brighter, more photostable, fluorophores especially in the far red part of the visible light spectrum. Better fluorophores have been continuously developed over the past years and this will continue in the future [34]. However, another, more challenging, aspect to consider is the effect of increased light irradiation during live cell super-resolution imaging on normal cellular physiology. Higher sampling density induces more cellular photodamage and perturbs the biological processes under examination [35]. Therefore, even if we would have the hypothetical ideal fluorescent probe, it would not necessarily directly translate into successful live cell super-resolution imaging. One way to prevent photo-induced cellular damage is by using various scavengers of reactive oxygen species (ROS) such as Trolox [36] and similar compounds. Another way would be to design better model systems for live cell imaging. The processes behind light-induced cell death are not known. It is assumed that ROS generated by fluorophore photobleaching and triplet-state interactions are a major source of photodamage, but it is not clear if that is the only process. All the sources of ROS species and the exact mechanisms are not clear yet. There is evidence that some cell types are more photodamage-resistant than others and therefore more suitable for live cell imaging. For example, HeLa cells can be challenged with order-of-magnitude higher light doses than U2OS or COS-7 cells without apparent photodamage [35]. It is perhaps the time to investigate in detail what is the molecular and physiological basis for this resistance. Once we know the genetic basis for the resistance then, with the help of CRISPR–Cas9 technology [37], relevant biological model systems could be genetically modulated to become more photodamage-resistant. Lastly, the total light exposure could be reduced by implementation of innovative illumination schemes. A new illumination concept for STED microscopy has recently been developed that reduces the sample exposure to the STED beam by actively modulating the STED power on per pixel basis depending on the spatial distribution of the fluorophores in the sample so that it only uses as much on/off-switching light as needed to image at the desired resolution [38]. With this illumination scheme, it was possible to reduce the sample exposure to light by up to 2 orders of magnitude depending on the sample [38]. Another novel, super-resolution live cell imaging concept has been demonstrated that uses consecutive sequence of reversible, on-stage cryo-arrests followed by super-resolution image acquisition within living, but arrested, cells [39]. Many issues connected to live cell super-resolution approaches will be mitigated by optimizing the microscopy hardware; however, physical limits will be reached that cannot easily be overcome. Novel computational procedures that improve the quality of acquired microscopy images and enable biological observations beyond the physical limitations of microscopes are becoming increasingly important. A purely analytical approach has been described that is able to reconstruct super-resolved images from a sequence of 100 images acquired in a standard wide field or confocal microscope by taking into account temporal fluorophore intensity fluctuations and achieving resolutions in the range of 70 nm [40]. Using deep learning networks, it was possible to restore microscopy images even when using 60 times less photons during acquisition [41]. This remarkable achievement will allow for acquisition of low signal-to-noise super-resolved images using minimal light exposure but without the compromise on the final output and could become standard in biological image restoration in the years to come. Hardware developments together with the design of new photostable fluorescent probes, image restoration by machine learning and photodamage-resistant model systems will pave the way to more robust super-resolution live cell imaging.

Correlative light-electron microscopy

Despite the advances in super-resolution technologies described above, EM still remains the method of choice when ultrastructural analysis (below 10 nm) of isolated viral particles or virus-induced intracellular structures needs to be conducted. EM analysis is usually performed on cellular volumes that are only 50–300 nm thick and a few microns wide. Finding a molecule or a molecular event of interest in such minute volume is a major challenge and a limitation of an EM approach. This is especially relevant for infectious disease research where one often needs to look for very rare or very transient molecular events. Examples are latent HIV infections, which only affect one in a million CD4+ T cells in vivo [42], unmodified HCV, which will only replicate in one out of hundred thousand hepatocyte-derived cells (Huh7) [43], or HIV-reporter HeLa cells, which internalize hundreds of HIV particles, but only a few virions undergo reverse transcription and eventually enter the nucleus [44]. Finding the proverbial needle in a haystack is a major bottleneck in addressing questions in infectious disease research. Another limitation of the EM is the difficulty to distinguish a molecule of interest from the surrounding cellular context which is also visualized. Specifically labeling molecules in EM by immunogold methods is possible but only available for a small subset of molecules where we are lucky enough to have a working antibody–antigen combination and is usually only possible on the section surface. Correlative light-electron microscopy (CLEM) has been

developed to overcome these limitations. In CLEM approach, the sample is first examined by fluorescent light microscopy, exploiting the light microscopy advantages such as the possibility to specifically label molecules of interest with fluorescent probes, track dynamic and transient events as well as examine large fields of view. Subsequently, the same sample and the same object of interest are examined by EM to provide the detailed structural information and cellular context at ultrastructural resolution. This way CLEM combines the strengths of fluorescent light microscopy with those of the EM and is particularly suitable for "needle in a haystack" type of experiments as well as to complement the analysis of dynamic, transient molecular events with ultrastructural analysis. For these reasons, the CLEM approach proved to be extremely powerful in addressing the open questions in the field of infectious disease research. Although rudimentary implementations of correlating light and electron imaging modalities to address host–pathogen interactions has been around since 1985 [45], the power of the CLEM approach for infectious diseases research was really demonstrated in proof of principle studies where single, fluorescently labeled, HIV-1 particles were observed on the cellular surface during the internalization process using the light microscopy followed by high-precision localization of the same particles and ultrastructural analysis by 3D EM [46]. In recent years, a similar approach has been employed to analyze molecular composition, ultrastructural architecture and spatiotemporal development of virus-induced intracellular alterations such are "viral replication factories" induced by HCV [47, 48] (Fig. 3b), dengue virus [49] and semliki forest virus [50], nuclear aggregates induced during herpesvirus varicella-zoster virus assembly [51] or virological synapse during retrovirus murine leukemia virus spread [52]. CLEM can also be combined with super-resolution imaging to derive high precision correlation between light and electron imaging modalities as it was done in the study of the molecular machinery and underlying ultrastructural morphology involved in HIV-1 budding [19]. Light microscopy can be incorporated in the EM workflow at almost any stage; however, two main CLEM approaches can be distinguished—when light microscopy is performed before embedding (pre-embedding CLEM) and after embedding (post-embedding CLEM). The main advantage of pre-embedding CLEM is that the light microscopy and EM can be performed using conventional light microscopy and EM protocols resulting in generation of high-quality light microscopy/EM data. Main disadvantage is that the structures of interest may change between light microscopy and EM steps resulting in low-accuracy correlation. Post-embedding CLEM provides excellent correlation but a compromise must be found in the sample preparation procedure between optimally preserving sample structure, and optimally preserving the fluorescent signal essentially yielding sub-optimal light microscopy and EM data quality. Regardless of which CLEM approach is employed, the use of embedding resins and heavy metal stains prevents achieving nanometer to sub-nanometer structural resolution. Furthermore, chemical fixation of samples within the CLEM workflow could introduce structural artifacts [26, 27]. This led to a development of a special implementation of CLEM called cryo-CLEM which performs the entire correlative workflow under cryogenic conditions. The sample is first immobilized by vitrification [28], followed by consecutive light and electron microscopic analysis, while keeping the sample in a vitrified state during the entire procedure. In cryo-CLEM, contrast is generated directly by the electron density differences within the molecules, while objects of study and their history or specific state are first identified by their fluorescence profile. By using vitrification and avoiding resin embedding as well as any heavy metal staining procedures, cryo-CLEM permits the morphological and structural characterization of defined biological objects near their native state and at the highest possible resolution. Cryo-EM combined with single-particle averaging/subtomogram averaging is capable of determining macromolecular structures at resolutions typically around 3–4 Å, although resolutions below 2 Å (resolution where individual carbonyl bonds and aromatic rings are resolved) have been reported [53]. The same approach has been used to solve the structure of immature HIV-1 capsids [54] and mature Dengue virus [55] at 3.9- and 3.5-Å resolutions, respectively. Furthermore, cryo-EM has been used to determine the structure of large macromolecular complexes such as 26S proteasome [56] and nuclear pore complex [57] at below 30-Å resolution directly in vitrified cells ("*in situ*") by single-particle averaging/subtomogram averaging of approximately 1000 particles. It is expected that the combinations of cryo-CLEM and single-particle averaging/subtomogram averaging approaches will be able to yield 3D structures of relevant macromolecular complexes for infectious disease research "*in situ*" and at single-digit nanometer resolution, which will have a major impact on the molecular understanding of host–pathogen interactions.

Selective plane illumination microscopy

Molecular biology studies of host–pathogen interactions are often performed in monolayer or suspension cells of established transformed cell lines. It is becoming increasingly clear that such systems are fundamentally different and often only partially recapitulate the relevant physiology of the complex biological processes as they occur in infected patients or animals. Relying solely on such model systems creates a risk of fabricating an oversimplified and potentially skewed view on these fundamentally important patho-physiological processes. Therefore, there has been a strong tendency in

biomedical research in recent years to move toward more physiologically relevant model systems of increasing complexity (such as complex 3D cell and organotypic cultures, organs, tissues, entire animals or samples from biopsies of patients) [58]. For a long time, confocal and multi-photon microscopy was the only method that allowed for microscopy-based research on complex 3D model systems. Confocal and multi-photon microscopy technology continues to evolve with major improvements introduced at a steady pace by commercial providers and academia alike. Confocal microscopy has entered the super-resolution regime with introductions of new detectors and image restoration routines (Airyscan by Zeiss, HyVolution by Leica and OSR by Olympus). Developments in adaptive optics ensure better signals and resolutions at increased depth [59–61]. Confocal and multi-photon microscopy is still the method of choice when examining non-transparent model systems that extend up to 1–2 mm along the optical axis or for microscopy performed in living rodents (intravital microscopy) [62]; however, these methods are fundamentally limited in speed and employment of high-power lasers is incompatible with high spatial/temporal sampling or long-term observation of living samples due to phototoxicty considerations. Visualization and quantification of pathogen behavior in the complex 3D systems requires microscopy methods that allow for high temporal and spatial resolution with minimum phototoxicity. In recent years, selective plane illumination microscopy (SPIM, also known as lightsheet microscopy) has emerged as the technical advance that provides the essential basis for such studies. SPIM is a fluorescence microscopy technique where only a thin slice (usually a few hundred nanometers to a few micrometers) of the sample is illuminated perpendicular to the direction of observation [63]. As only the actually observed section is illuminated, this method reduces photodamage and stress induced by light on a living sample. In fact, this is the most gentle fluorescence microscopy technique to date. Because SPIM scans samples by a plane of light instead of a point and employs a high-sensitivity camera for detection, it can acquire images with high signal-to-noise ratios at speeds several orders of magnitude faster than those offered by point-scanning methods such as confocal and multi-photon microscopy. The SPIM approach has revolutionized developmental biology research by allowing for long-term, high-resolution observation of developing embryos [64]. Together with advancement of tissue clearing methods [65, 66], it led to the generation of detailed, single-cell resolved, highly multiplexed models of complex organs relevant for infectious disease research such as the lymph node, brain, liver and so on [66]. Despite the mentioned benefits of SPIM technology and the fact that it has been available for more than a decade, the potential of SPIM technology has not been fully exploited in infectious disease research. SPIM has been employed in proof-of-principle studies to analyses HIV-1 assembly sites dynamics in host cells [33] or parasite *Toxoplasma gondii* behavior in 3D epithelial model systems [67]. It has also been used to study zika virus (ZIKV) tropism in cerebral organoids [68] and *Trypanosoma brucei* life cycle in tsetse fly [69]. However, there are several potential reasons for low application of the SPIM in infectious disease research. The SPIM approaches are difficult to implement in general due to high demands on IT and computational infrastructure that are necessary to support the storage, transfer, visualization and analysis of data sets ranging from 100s of GB to multiple TB in size that are produced in a typical experiment. As high-end IT infrastructure is getting less expensive and both commercial (Arivis, Imaris, etc.) and open source software for SPIM data visualization and analysis [70–72] are becoming more robust and readily available, it is expected that SPIM will have wider use in the field of infectious disease research and others. More specifically, model systems purposefully developed for infectious disease research that fill the gap between simple cellular monolayer cultures and living animals (such as spheroid and organoid 3D cultures) amenable for SPIM are largely missing (Fig. 3c). That is certainly going to change in the next years as development of near-physiological organoid model systems derived from human stem cells has become an area of significant interest [73, 74] (Fig. 3c). Furthermore, objectives, detectors and lightsheet thickness in SPIM systems are not fully optimized for sub-cellular resolution which have prevented addressing many open questions in infectious disease research. Recent implementations of new illumination strategies such as Bessel beams [75] and lattice lightsheet [61, 76] achieve lightsheets that are thinner than the depth of focus of the detection objective and uniform across a large field of view. With such illumination strategies, nearly isotropic 3D resolution (230 nm laterally and 350 nm axially) has been demonstrated, thus allowing for analysis of single-molecule dynamics across multiple cells simultaneously within a 3D stem cell spheroid or formation of a T-cell immunological synapse in a 3D matrix at diffraction-limited resolutions and at speeds of 1 volume/s [76]. Recently, by combining structured illumination microscopy and SPIM, a 100-nm lateral resolution was demonstrated [77]. Although lattice lightsheet-based SPIM system is commercially available, instrumentation utilizing new SPIM modalities is still very rare worldwide and not widely used by the scientific community. Specific advantages and disadvantages of these approaches will have to be evaluated by the scientific community in the years to come. Being able to analyze physiology of host–pathogen interactions in 3D model systems at spatiotemporal scales offered by SPIM approaches in combination with innovative illumination techniques

promises major advances in the field of infectious disease research.

Microscopy by machine learning

One major limitation of all mentioned imaging modalities is their low experimental throughput. Super-resolution microscopy (especially SMLM techniques), (cryo)CLEM and SPIM tend to require manual interventions during the entire experimental workflow, from laborious adjustments of image acquisition parameters, over subsequent quality control to—often non-intuitive—tweaking of the data processing parameters, for image acquisition, processing and analysis. This severely limits the data that can be obtained. A snapshot of one or two objects of interest clearly does not constitute a data set from which conclusions can be derived. In order to derive statistically significant information and turn the bioimage data into scientific discovery, one often needs to collect and analyze a large number of images. Furthermore, this needs to be done over many experimental conditions and preferentially in an unbiased manner. This issue is further aggravated by the trend of moving the biomedical research toward more physiologically relevant 3D model system (such as stem cell-derived organoids, spheroids, entire organs/organisms and other 3D model systems) where suddenly large volumes of biological material need to be swept in order to find objects of interest and derive relevant quantitative information. One approach to tackle this issue is to develop fully automated microscopy workflows which would automatically acquire and process the data. There has been a substantial progress in the last decade in automating microscopy acquisition and analysis using standard imaging modalities (such as confocal and widefield microscopy) [78, 79]. This approach, referred to as high-throughput high-content screening (HT-HCS), has been extensively used to study the effects of systematic downregulation (via siRNA technology) or upregulation (overexpression) of each gene in the genome on biological processes across biomedical disciplines including infectious disease research [80–82]. Extending a decade of HT-HCS know-how to new imaging modalities proved to be more challenging than anticipated. It is difficult to extend some of the established HT-HCS pipelines to new imaging modalities due to more complex acquisition and analysis procedures that still require substantial human input. However, the need for HT-HCS approach in new imaging modalities has been recognized and automated acquisition and analysis pipelines are being developed for super-resolution [83, 84], CLEM [85] and SPIM [86] approaches.

A particularly powerful approach, applicable to any imaging modality, would be combining the automated image acquisition with machine learning to perform automated morphological profiling and image analysis [87]. Machine-learning technology gives computers ability to learn patterns in the data without being explicitly programmed and then predict those patterns in the new data. Spearheaded by large IT companies such as Google, Microsoft, IBM and others, machine-learning techniques are already present in many aspects of modern society from web searches and speech recognition in smartphones to self-driving vehicles [88]. They have proved to be very powerful at discovering the intricate structure of high-dimensional data such as bioimages [89] which typically contain multiple phenotypes whose discriminating morphologies are not easily described by a few parameters. If a car can already segment, classify and understand its complex surrounding environment and make autonomous decisions by harnessing the power of deep neural networks, we can ask ourselves, why microscopes would not be able to do the same within the field of biomedical research. Automated acquisition and analysis approaches using machine learning are expected to have a major impact on how microscopy is performed in biomedical research in the future. This would increase the objectivity, reproducibility and sensitivity of analysis of very heterogeneous pathophysiological processes, as well as be able to identify new and rare phenotypes that might escape human attention. However, the restrictions and limitation of machine learning approach as well as the extent of its applicability in biomedical research still need to be carefully evaluated by the scientific community before adopting it for a wider use.

High-throughput imaging where the entire specimen is indiscriminately recorded at maximal spatial and temporal resolution may not be a feasible approach due to both phototoxicity and computational restrictions (such as for example for the SPIM-based imaging approach). For this, but applicable to literally any microscopy-based approach, we need an automated image acquisition scheme, probably based on machine learning approaches (e.g., by using deep neural networks) to interrogate the biological specimen in its entirety quickly at low resolution and decide what needs to be acquired at higher resolution or from which areas it is worth to extract a time lapse information in case of live cell imaging. This way, those data that do not contain the interesting information are not part of the acquired data set significantly relieving the pressure on IT infrastructure for storage and image analysis. Such approaches would have a significant impact on infectious disease studies where already the search for individual transient and rare molecular events provides a significant bottleneck in experimental throughput. Such software has been under development for classical imaging modalities [90, 91], also by implementing aspects of machine learning [92], but this needs to be made easy to use, compatible with various commercial hardware implementations and extended to new imaging modalities.

Conclusion

In infectious disease research, discoveries by direct visual observation have always been the crucial step toward understanding. With major breakthroughs in the last decade, microscopy has advanced to the point where we can literally visualize molecular mechanisms in model systems of increased complexity and even *in vivo*. New imaging modalities such as super-resolution microscopy, CLEM and SPIM have opened the possibility to interrogate our model systems at vast ranges of spatio-temporal scales—from nanometers to millimeters and from microseconds to days (Fig. 2). These technologies are essential for achieving a comprehensive understanding of the infection process, as they are the only ones in our experimental arsenal that allow sufficient spatio-temporal resolution to capture and analyze individual events that govern pathophysiological states in living systems. Technologies such as super-resolution microscopy and CLEM have already been established in infectious disease research and are providing major progress in the field. However, technical developments are still needed in the years to come to enable good structural preservation of fixed specimens for super-resolution and analysis of infection processes in living systems. Further technical developments are necessary to achieve automated image acquisition with minimal human intervention which will dramatically increase the experimental throughput. SPIM approaches have not gained a strong foothold in the infectious disease field yet. However, this is expected to change as new technical developments enable infectious disease research on close-to-physiological model systems (such as human stem cell-derived organoids and other complex 3D model systems) and at subcellular resolution. SPIM, together with human stem cell-derived organoid model systems, allow for comprehensive dissection of the structure–function relationships between diverse cell types, pathogens and their surrounding microenvironments. This will have a major impact in infectious disease research as shifting basic research toward close-to-physiological biological systems will yield results with higher predictive value for estimating pathogenicity or pathogen control (immunological or drug-mediated) in the infected host and therefore higher translation potential.

However, the complexity of living systems and host–pathogen interactions in health and disease require more than visual inspection in order to derive comprehensive understanding of these processes. Modern microscopy-based research requires quantitative measurements and statistics of a larger data set to identify rare events and judge the variability and significance of the findings which can only be achieved by an approach that utilizes fully automated acquisition and analysis protocols. Automated large-volume sampling combined with computational systems analysis using machine learning approaches (support-vector machine or deep neural networks) will provide a way forward. Taken together, modern microscopy technology is currently uniquely positioned, like at the time of the field's pioneers, van Leeuwenhoek, Koch and Pasteur, to propel the infectious disease research to a new frontier.

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Abbreviations used:
EM, electron microscopy; STED, stimulated emission depletion microscopy; SMLM, single-molecule localization microscopy; HIV, human immunodeficiency virus; HCV, hepatitis C virus; ROS, reactive oxygen species; CLEM, correlative light-electron microscopy; SPIM, selective plane illumination microscopy; HT-HCS, high-throughput high content screening.

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