Bioimaging contributes significantly to our understanding of plant virus infections. In the present review, we describe technical advances that enable imaging of the infection process at previously unobtainable levels. We highlight how such new advances in subcellular imaging are contributing to a detailed dissection of all stages of the viral infection process. Specifically, we focus on: (i) the increasingly detailed localizations of viral proteins enabled by a diversifying palette of cellular markers; (ii) approaches using fluorescence microscopy for the functional analysis of proteins in vivo; (iii) the imaging of viral RNAs; (iv) methods that bridge the gap between optical and electron microscopy; and (v) methods that are blurring the distinction between imaging and structural biology. We describe the advantages and disadvantages of such techniques and place them in the broader perspective of their utility in analysing plant virus infection.

Key words: correlative microscopy, fluorescent protein, in vivo interaction, membrane topology, RNA imaging, super-resolution.

INTRODUCTION

In order to infect a host cell and to spread systemically, viral pathogens have to usurp, manipulate and overwhelm numerous biological processes within cells. They often achieve this with a minimal set of multifunctional virus-encoded proteins, as well as through the recruitment of host factors [1–5]. Most of these ‘hijacked’ processes are compartmentalized within the host cell, and thus the virus and its gene products become localized to specific subcellular compartments during the appropriate stages of infection, often in a highly dynamic manner. The ability to visualize these events and their ensuing effect on the host cell have greatly enhanced our understanding of the infection process.

Since the 1950s, EM (electron microscopy), later coupled to immunogold detection of specific proteins and RNA, has been the mainstay of virological approaches, but is restricted to static snapshots of the infection process. Furthermore, the limited accessibility of antigenic epitopes in samples embedded and sectioned for EM makes it difficult to detect less-abundant proteins or to quantitatively assign visible structures to specific biomolecules [6]. The discovery of FPs (fluorescent proteins) reintroduced LM (light microscopy) as a major tool for cell biology in the early 1990s, and today fluorescence microscopy is one of the cornerstones of plant virology. Initially, virus-expressed FPs were simply used to monitor the spread of infection [7,8], but plant virologists quickly started to employ them as fusions to virus-encoded proteins. Nowadays, fluorescence microscopy is used increasingly to permit not just localization, but also functional analysis of proteins in vivo, and new approaches have allowed optical-based microscopy to break the diffraction barrier, long held as a fixed limit to resolution [9–11]. At the same time, EM has also made important advances, in particular through improved sample preservation using high-pressure freezing/freeze substitution [12] and the extension into 3D (three-dimensional) imaging using ET (electron tomography) [13,14]. Consequently, correlative approaches that combine LM and EM are currently being developed in many laboratories [15,16]. Cryo-EM tomography and AFM (atomic force microscopy) are starting to blur the boundary between imaging and structural biology, and are opening the door to a truly molecular understanding of the viral infection process. In the present review, we summarize some of these recent developments with a particular focus on the challenges and advantages that are specific to imaging plant virus infection.

ADVANCEMENT OF PROTEIN LOCALIZATION STUDIES

Since the first use of GFP (green FP) as a reporter of virus infection in plant cells [7], the palette of autofluorescent proteins available for localization studies has increased exponentially through the discovery of new natural source proteins and the engineering of new varieties. Generally, new FPs are selected to increase the number of spectral variants that can be separated in co-localization experiments, and to overcome problems with photostability, brightness, oligomerization and fusion tolerance [17,18]. For plant virus studies, however, brightness and photostability are often less important considerations than spectral properties because...
transient expression from agrobacteria, particle bombardment or viral genomes is the most common experimental approach. As a result, researchers usually deal with problems related to overexpression rather than detectability or dimness, and FPs for plant virus studies can simply be chosen based on their spectral separation. Because virus-expressed FP constructs can accumulate at extremely high levels, sequential image acquisition is preferable for co-localizations, at least as a control. Several sets of expression vectors are now available that enable fast generation of multiple FP-fusions from a single clone of the gene of interest based on Invitrogen’s proprietary Gateway in vitro recombination system [19–31], and additional vectors using more recently developed FP variants are continuously being developed [30,31].

As knowledge of cell biology increases, so does the number of available fluorescent markers for subcellular compartments and transport pathways [32,33], and plant virologists are taking advantage of this to determine the location of viral proteins in detail, and to follow their trafficking pathways throughout the cell. For instance, the 6K protein of potyviruses, which functions to establish viral replication sites, was known previously to target the ER (endoplasmic reticulum) [34]. Wei and Wang [35] have shown that the specific location of the 6K protein is the ER exit site, where 6K accumulation depends on both the COP (coatomer protein) I and COPII vesicle trafficking machineries, and that the 6K-containing membrane compartments travel onwards through the secretory pathway to chloroplast membranes where they accumulate to form the main replication sites [36] (Figures 1a and 1b). In another study, Taliansky and co-workers used a variety of markers of subnuclear bodies to follow the path of the ORF3 (open reading frame 3) protein of the umbravirus groundnut rosette virus, as it enters the nucleus [37,38]. Here it interacts with Cajal bodies, fuses them with the nucleolus and recruits the nucleolar localization sequence elements [42,43]. Viral genome sizes are often limited by virion packaging mechanisms, constraining FP insertions, and in RNA viruses, high recombination rates can quickly eliminate insertions. With the additional potential for FP-fusions to disrupt the (multi)functionality of viral proteins, it is not surprising that approaches in which the FP-fusion functionally replaces the wild-type gene in the viral genome (e.g. [44,45]) are the exception rather than the rule. Instead, various compromises have been used such as insertion under the control of alternative viral promoters (e.g. [7,34,46]), self-cleaving FP-fusions using a linker derived from the 2A protein of foot and mouth disease virus to release sufficient native viral protein for functional complementation [47] or expression from viral satellite genomes (e.g. [48]). Such viral expression strategies, together with agroinfiltration and particle bombardment, all comprise transient expression assays, which are convenient and produce fast results. They are also somewhat appropriate, as viral proteins in most cases are only ‘transiently’ expressed during a natural infection, and often to very high levels. Additionally, transient expression can be advantageous compared with transgenic plants stably expressing viral proteins, as the latter are often resistant to infection.

Where localization studies differ most significantly between viral and plant proteins is the nature of the virus as a self-contained ‘closed’ system. Redundancy between functionally similar genes or lack of mutant phenotypes is not usually a problem in virus systems. On the other hand, expression of individual viral proteins can never be representative for the context of the actual infection and, by whatever means, localizations should be studied in infected tissue [49]. Most likely, the viral protein of interest will be affected by interactions with other viral or infection-specific factors. If non-viral expression is the only option, 35S-driven viral replicons can be a useful tool to spatially and temporally control co-introduction of the FP reporter with the virus. This can also facilitate studies of movement-deficient or replication-attenuated viral mutants. However, some viral proteins may only function (and localize correctly) when expressed in cis. But the ability to compare localizations between uninfected and infected tissue provides an additional tool to dissect these interactions and functions, similar to genetic approaches using knockout mutations (Figure 2).

Although high transient expression levels are not problematic themselves, localizations should consider the timing and expression levels during infection. For example, plant virus MPs (movement proteins) are often expressed early and at low levels, whereas CPs (capsid proteins) are typically expressed later and at very high levels. Therefore prolonged overexpression of an MP–FP may need to be interpreted with more caution than a CP–FP fusion. After particle bombardment, FP fluorescence can often be observed after a few hours, and in agroinfiltration and viral expression systems, imaging may be possible as early as 1 day after inoculation. In view of the fact that many viruses replicate and move intercellularly within less than 1 day [50–53], but may continue to accumulate progeny virions until the host cell’s resources are exhausted, the localization of a viral protein should be studied as early as possible as well as at later stages to observe changes in localization and function during the infection cycle. Within a viral lesion, the differences in localizations between cells at the leading edge (= early) and the centre (= late) of an infection site offer a valuable reference in this regard. Owing to the maturation time of FPs and requirements for replication or subgenomic RNA production, virus-expressed FP-fusions are generally unsuitable to study the earliest events during the infection process. Virus-independent transient expression can be a way to circumvent this problem, as the protein of interest can be present prior to infection, for instance by allowing a virus infection to enter an area already expressing the FP-fusion of interest. Again, the potential requirement for expression in cis needs to be kept in mind.

In transient experiments using non-native expression, relative ratios of potential interaction partners also have to be considered. For example, the overlapping ORFs of the second and third ‘triple gene block’ MPs of different viruses are expressed from a single subgenomic messenger RNA, with the downstream TGB3 (triple gene block 3) ORF translated by leaky ribosome scanning after

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Imaging plant virus infections

Figure 1  Various applications of live-cell fluorescence microscopy suitable for plant virus imaging

I. Detailed localizations within the context of the host cell. (a) Polyviral 6K replication protein induces ER-derived vesicles that co-localize with the ER-to-Golgi exit site marker Sar1 (i, ii). A dominant-negative Sar1 mutation abolishes 6K vesicle formation (iv, v). At later stages, the 6K-induced vesicles transfer to the chloroplast envelope (iii), the putative viral replication site. Transport depends on the actin cytoskeleton, shown by reduced plastid labelling after overexpression of a dominant-negative fragment of the motor protein myosin IX-K (vi) (reproduced from [35,36] with permission from American Society for Microbiology). II. Study of protein dynamics. (b) FRAP of PD-localized TMV MP shows that MP is delivered actively to PD; the metabolic inhibitor azide (lower panels) blocks fluorescence recovery (modified from [83] with permission from John Wiley and Sons). (c) Use of the green-to-red photoswitching protein Kaede to localize translation sites. At time 0 min, a patch of Kaede-labelled potassium channel Kv1.1 in a neuronal dendrite (outlined) is converted into red. Within 60 min, newly synthesized green Kv1.1-Kaede has accumulated, which is again converted into red at time 60 min. Only green fluorescence is shown (modified from [89]. Reprinted with permission from AAAS). III. Detection of protein–protein interactions. (d) FLIM–FRET of tomato spotted wilt virus replication proteins. N protein co-localizes with both GP and Gn, but only interacts directly with GP, as shown by reduced CFP fluorescence lifetime (co-localization and FLIM–FRET images show different cells) (modified from [189] © 2009, with permission from Elsevier). IV. Topology analysis of integral membrane proteins. (e) BiFC-based membrane
initiation at the upstream TGB2 ORF. This results in an excess of TGB2 protein over TGB3 [54]. Recently, Lim et al. [48,55] have shown that the TGB2/TGB3 ratio profoundly influences the TGB3-dependent localization of the TGB2 protein of barley stripe mosaic virus.

Finally, the choice of host system needs to be considered. Compared with animal virologists, researchers working on plant viruses are in the advantageous position of having both intact plants and isolated cells for infection, but both come with certain limitations. In some cases, for instance with fusion-intolerant viral proteins (often replicases) or specific RNAs (discussed below), immunofluorescence is a better option than FP-fusions. However, to make intracellular epitopes accessible to antibody labelling, fixation and membrane permeabilization are usually insufficient in plant tissues due to the presence of the cell wall, requiring additional embedding and sectioning. The use of protoplasts can overcome this limitation and offer the additional benefit of permitting synchronized infection of an entire culture, i.e. defined infection timing [56]. Protoplasts are also more accessible for invasive delivery methods such as membrane permeabilization or microinjection that may open new avenues in the investigation of early infection events (see below). However, even when prepared from fresh tissue rather than cell culture, they represent profoundly altered non-native host cells. Additionally, many membranous organelles, which are important in viral processes such as replication and movement, are strongly perturbed by fixation and embedding. For imaging, especially in vivo, intact tissue should be the preferred choice, with protoplast systems reserved for more specialized experimental problems.

**LABELLING FUSION-INTOLERANT VIRAL PROTEINS WITH SMALL FLUORESCENT TAGS**

Besides expression level and context, the most important factor influencing the accuracy of fluorescence-based localizations is the effect of the fluorescent tag on the protein of interest. FPs may mask functional domains, interfere with interactions with other macromolecules, or have a stabilizing or destabilizing effect. Simply increasing protein size by adding a ~27 kDa GFP moiety...
may influence localization, for instance a protein’s ability to move through PD (plasmodesmata) [57] or nuclear pores [58]. Some FP-fusions are unstable, and proteolytic processing may release a significant pool of the free FP resulting in nucleo-cytoplasmic labelling [7,39,59]. When this is suspected, Western blotting against GFP should be carried out to test for degradation of the fusion protein. If mistargeting is suspected, valuable information may still be gleaned from the data. For instance, a secondary or transient localization may only become apparent when the main targeting site of a protein of interest is disrupted. This is especially relevant in the case of multifunctional viral proteins. In most cases, the orientation of the FP-fusion protein will have the greatest effect on localization. In a recent study of the TGB MPs of grapevine rupestris stem pitting-associated foveavirus, Rebelo et al. [60] systematically investigated the effects of FP-fusions to each TGB protein on both N- and C-termini. Such a thorough approach may not always be necessary, but with the available repertory of Gateway fusion vectors it can be done relatively quickly. If only one fusion orientation is used, publications should clearly state which, and for what reason it was selected. On some occasions, the best approach may be to introduce the fluorescent tag internally at a boundary between different protein domains [61]. Few reports using such strategies have been published in plant virology to date (e.g. [62]), probably because there is surprisingly little structural information available for most plant viral proteins. An approach for rapid production of internal FP-fusions based on the Gateway technology has been described [61].

Some of these difficulties might be overcome if smaller, less disruptive fluorescent tags were available, which would additionally help to limit the detrimental effects of introducing the tag into viral genomes. One novel FP that meets some of these requirements is iLOV [63], derived from the light-, oxygen- or voltage-sensing LOV2 domain of the Arabidopsis thaliana photoreceptor phototropin 2. With a size of ~300 bp and 17 kDa, iLOV is approx. 1.5–2-fold smaller than GFP and has been demonstrated to outperform GFP in some virus-specific contexts [63]. For example, TMV (tobacco mosaic virus) expressing unfused or MP-fused iLOV moved faster cell-to-cell and systemically than the corresponding GFP constructs, indicating advantages both in terms of genetic load and functionality of the MP-fusion.

If a protein-specific antibody is available, immunofluorescence microscopy can make tagging altogether unnecessary, but it is limited to fixed samples. A potential compromise permitting live-cell imaging could be to use a small non-fluorescent tag that is rendered fluorescent only by addition of a specific interacting compound (probe). A variety of such tag–probe labelling systems have been developed (reviewed in [64–66]), but unfortunately the majority are cell-impermeant and therefore unsuitable for plant studies. Of those which permit intracellular labelling, the proprietary SNAPTM and CLIPTM tag systems based on O'-alkylguanin-DNA alkyltransferase (New England Biolabs; [67,68]), HaloTagTM (Promega; [69]), based on haloalkane dehalogenase, and LigandLinkTM, based on Escherichia coli dihydrofolate reductase (ActiveMotif; [70]), all utilize the reaction between small enzymes and substrate analogues to link a fluorophore covalently to a protein of interest, but provide only a modest size advantage over GFP (27 kDa) with tag molecular masses between 18 and 33 kDa. Only HaloTagTM has been tested in plants, with good tissue permeability [71]. A tag based on the human immunophilin FKBP12 (FK506-binding protein 12) [72,73] adds only 12 kDa, but has not been tested in plants yet. The smallest available tags with cell-permeant fluorescent probes are a 38-amino-acid peptide that binds Texas Red with picomolar affinity [74], which also has not yet been tested in plants, and the tetracysteine tag/biarsenical ligand system [75,76]. The optimized, tetracysteine-containing peptide tag is of similar size as an epitope tag (12 amino acids [77]). The biarsenical probes FlAsH (fluorescein arsenical helix binder) and ReAsH (resorufin arsenical helix binder; [78]) are membrane-permeant non-fluorescent fluorescein derivatives that become fluorescent only when covalently bound to the tetracysteine tag. This ‘switch on’ is an advantage over the Texas Red-binding tag, and particularly useful as it eliminates the washing out of unbound dye, which is time-consuming and a problem when the tissue needs to be studied rapidly. Despite its promise, the tetracysteine tag/FlAsH system has seen little use in plants so far [79]. The biarsenical probes can cause cytotoxic effects [76] and some background staining may result from non-specific reactions with other cysteine-containing proteins [64,80]. From our own experience, the greatest problem in plants may be insufficient permeability through (and non-specific labelling of) the cell wall (P. Boevink, N.M. Christensen and K.J. Oparka, unpublished work). Tag–probe labelling systems could be an ideal approach to localize fusion-sensitive viral proteins, but so far none is entirely suited to plant systems.

**TRACKING PROTEIN DYNAMICS AND LOCALIZING TRANSLATION SITES**

Many proteins are not static in the cell and some may take different pathways to the same subcellular localization. Therefore significant functional information can be gained from analysing protein dynamics. With standard FP-based fluorescence microscopy, this is possible only to a limited extent, e.g. by following protein localization over a time course. A range of techniques has been developed that permit more detailed insights into protein transport pathways, mobility and also the site of their synthesis. Tracking approaches are particularly valuable in the study of multifunctional viral proteins during an infection cycle, while determining the site of viral protein translation may help to link viral replication and protein function.

**FRAP (fluorescence recovery after photobleaching) and FLIP (fluorescence loss in photobleaching)**

One way to gain dynamic information from FP-fusion localization studies is to use photobleaching approaches [81,82]. High-excitation light intensities ‘overload’ the energy absorption of fluorescent molecules and destroy them permanently, leading to photobleaching. With the spatially restricted light intensities that can be achieved with lasers, it is possible to locally bleach a specific fluorophore within a cell in a selected ROI (region of interest). Because all cellular proteins, including FP-fusions, participate in dynamic interactions with their environment, the non-fluorescent bleached molecules from the ROI will subsequently mix with their still-fluorescent counterparts elsewhere in the cell. The kinetics of this process can be measured and used to draw conclusions about the mobility and transport pathways of FP-fusion proteins. In FRAP experiments, the return of fluorescence to the bleached area is monitored. If the FP-fusion protein is freely diffusive within the cell, fluorescence will return rapidly and with a simple kinetic based on cellular diffusion constants. If the protein has limited mobility, e.g. by interactions with other cellular macromolecules, fluorescence recovery will be slowed and will follow a more complicated kinetic. For instance, Goodin et al. [49] were able to distinguish two intranuclear pools of the N (nucleocapsid) protein of *Sонchus* yellow net rhaddovirus. One pool of N in the nucleoplasm showed fast FRAP kinetics and was probably present in a soluble form. The other pool
was membrane-associated and showed very slow FRAP kinetics, indicating that the protein is immobilized. N interacts with another viral protein, P, but FRAP experiments on N/P-co-expressing uninfected cells showed similar kinetics to soluble N, indicating that viral factors other than P are responsible for immobilizing N on membranes. If the bleached pool of an FP-fusion is compartmentally isolated, no fluorescence recovery may occur at all. Goodin et al. [49] used this FRAP approach to show that the membranous intranuclear replication sites of Sonchus yellow net virus are continuous with the nuclear envelope. Both the replication sites and the inner nuclear envelope were labelled by a GFP-fusion of the human lamin B receptor, and photobleached replication sites quickly recovered their fluorescence, indicating redistribution from the connected cellular endomembranes.

FRAP can also give information on the trafficking pathway a FP takes to reach the bleached area. Wright et al. [83] combined FRAP of the PD-localized TMV 30K MP with cellular inhibitors to analyse the intracellular route of the protein to PD. Microtubule-depolymerizing drugs had no effect on 30K PD targeting to PD, whereas actin cytoskeleton inhibitors significantly inhibited MP targeting (Figure 1c). Also, high concentrations of brefeldin A sufficient to distort the cortical tubular ER network and treatment with the general metabolic inhibitor sodium azide both significantly reduced fluorescence recovery at PD, indicating that the 30K protein is actively transported along the cortical ER/actin network.

In contrast with FRAP, the depletion of fluorescence from the surrounding non-bleached cell areas is monitored in FLIP experiments. Similar to FRAP, this allows conclusions to be drawn about protein-trafficking pathways as well as connectivity between compartments.

**Fluorescent highlighter proteins and tag–probe pulse–chase labelling**

FRAP and FLIP are not suitable for continuous tracking of the same protein pool or for distinguishing newly synthesized from pre-existing protein. The latter is of particular interest to the study of virus infections because the location of viral protein synthesis is of considerable functional relevance. The native location of viral proteins in the course of infection may result from both their intrinsic properties and their site of synthesis. For instance, plant virus MPs must associate with the genome they are transporting, but so far none has been characterized as sequence-specific nucleic acid binders [4]. In some cases, specific interactions between the MP and other viral proteins, such as CPs, may aid specificity (e.g. [84]), but the spatial proximity of the MPs to progeny genomes, i.e. ‘co-compartmentation’, may also play a role which could be achieved by synthesis and retention of the MP in the vicinity of the replication site. Clear identification of a specific subpopulation of proteins in a cell requires differential labelling. This can be achieved using either FPs whose spectral properties can be changed in vivo, so-called photoconvertible or ‘highlighter’ FPs (pc-FPs; reviewed in [18,64,85,86]). Alternatively, tag–probe systems can be employed that permit pulse–chase labelling with differently coloured probes. Both approaches permit the selective highlighting of the most recently synthesized protein pool and can thus identify translation sites. The ability to change the colour of the fluorescently labelled protein locally in a subcellular ROI and track its movement through the cell is, however, limited to pc-FPs.

One group of pc-FPs, including PA (photoactivatable)-GFP and PA-mCherry, is switched irreversibly from a near-non-fluorescent dark state to green or red fluorescence respectively by strong UV illumination [18,64,85,86]. Activatable pc-FPs permit selective tracking of subcellular molecule populations, but do not allow pulse–chase labelling. Additionally, they are difficult to detect prior to photoactivation, which therefore has to be done ‘blindly’ or using other cellular markers for orientation. A different group of pc-FPs are ‘switched’ on and off reversibly, and include the variants of Drosha, Padron, KFP1 (Kindling), rsFastlime and rsCherry, IrisFP and DsRed Timer (see reviews [18,64,85,86] for comparisons of spectral properties). These proteins permit repeated on/off switching (in some cases many cycles), and can be used for repetitive FRAP or tracking experiments within the same cell. They are also important for new subdiffraction-resolution microscopy approaches (discussed below).

The most relevant group of pc-FPs for pulse–chase labelling are true photoconverters, i.e. irreversibly colour-changing proteins. These include PS-CFP2 (where CFP is cyan FP), which switches from cyan to green fluorescence, and numerous green-to-red switching pc-FPs: Kaede, KikGR (Kikume), EosFP variants and Dendra2; also KFP1 (kindling FP1) can be green-into-red converted irreversibly (for details of spectral properties, see [18,64,85,86]). Additionally, it was recently discovered that many red FPs can be photoswitched, including Katushka, mKate and mRed1 (from red to green) and mOrange1 and 2 (from red to farred), making them suitable as highlighters [87]. A conversion into cyan fluorescence has also been described for the YFPs (yellow fluorescent proteins) EYFP (enhanced YFP), Venus and Citrine [87,88].

Colour-changing pc-FPs are ideally suited for both tracking and pulse–chase labelling experiments. The label is easily visible before photoconversion, and the colour change leaves both switched and unswitched protein pools visible simultaneously. Movement of selected particles, organelles etc. can thus be followed, whereas newly synthesized protein can be visualized by first (irreversibly) photoswitching the entire pc-FP population in a cell and then observing reappearance of the unswitched colour due to de novo translation. This approach has been used to identify localized translation sites of a potassium channel in neuronal dendritic appendages [89] (Figure 1d). Translation inhibitors such as cycloheximide and puromycin can be used for controls. Also, both FRAP and PA-GFP were used to show that the NS4B and NS5A proteins of hepatitis C virus remain associated with putative viral replication sites when expressed from the virus, but are motile in uninfected cells [90,91]. One potential problem with the use of pc-FPs in plants may be that the experimental organism has to be kept in the light. Since natural daylight contains a UV component, this can lead to undesired photoswitching (some pc-FPs are also switched by visible wavelengths). A potential solution is to keep the plants in the dark for some time before microscopy.

Pre-existing and newly synthesized protein pools can also be pulse–chased using tag–probe labelling systems. The existing protein pool is first labelled with one fluorophore, then subsequent labelling with a different fluorophore selectively highlights only the most recently synthesized protein, identifying the translation sites. The tetracystene tag/biarsenical ligand system has been used to localize β-actin translation sites in vivo with FIASH/ReAsH pulse–chase labelling [92]. Among the other tags which can be used intracellularly [64–66], the HaloTag™ [69], SNAP/CLIP-tag™ [67,68] and LigandLink™ [70] systems also offer different probe colours for pulse–chase labelling. However, a disadvantage over FIASH/ReAsH is that these probes are permanently fluorescent. Non-fluorescent probes are available for blocking the pre-existing protein pool instead of changing the label colour, but the tissue still has to be washed to remove non-specific fluorescence. Lang et al. [71] have reported washing...
times of between 4 and 18h for HaloTag™, the only one of these systems tested in plants. Unless the tissue is fixed, these times are too long for detecting translation sites. Lin et al. [93] recently introduced another alternative technique, TimeSTAMP, for selective labelling of translation sites. The protein of interest is tagged with an epitope tag via a linker that encodes both the NS3 protease from hepatitis C virus and its NS4A/B cleavage site. Autoproteolysis constantly removes the tag until a specific membrane-permeant protease inhibitor is added. Any protein synthesized after addition of the inhibitor will remain labelled and can be localized by immunofluorescence microscopy. The permeability of the inhibitor through plant tissue remains to be tested.

DETECTION OF PROTEIN–PROTEIN INTERACTIONS

Subcellular localization of a protein needs to be put into a spatial and functional context to permit conclusions about its role during infection. Co-localizations with other proteins including organelle markers can provide such a context. However, co-localizations do not provide proof of macromolecular interactions. The resolution limit of confocal microscopes in the focal plane is approx. 200–250 nm and even lower in the z-axis. A spherical volume with a diameter of 200 nm may contain up to 140 000 GFP molecules that could be co-localized without any physical interaction [94]. Only in special situations can co-localization be a sufficient indicator of protein interactions, for instance when one FP-fusion causes a clear redistribution of another, or when this is coupled to a functional phenotype. Therefore fluorescence microscopy applications have been developed to more specifically identify protein–protein interactions in vivo.

BiFC (bimolecular fluorescence complementation)

BiFC, reviewed in [39,94–96], is the reconstitution of a FP from two non-fluorescent fragments. BiFC can be used to detect protein–protein interactions because FP reconstitution depends on the FP halves being in close proximity long enough for refolding, a process thought to require up to several minutes. To detect interactions, the split-FP halves are translationally fused to two proteins of interest, whose interaction then facilitates BiFC, thereby ‘switching on’ fluorescence. In contrast with other protein fragment complementation approaches such as the yeast-two-hybrid system, BiFC not only allows direct microscopic visualization of the interaction but also provides additional information on the localization of the interaction within the cell. As for conventional FP-fusions, several sets of plant BiFC vectors compatible with restriction cloning [97–101] or Gateway technology [30,31,98,102] have been developed to facilitate rapid and easy experiments.

FPs are commonly split at either of two suitable splitting positions, between amino acids 154 and 155 or 172 and 173 respectively (actual positions may vary slightly depending on the FP variant) [95]. In both cases, the N-terminal fragment (FPN) is larger and contains the side chains forming the fluorophore. The smaller FPC (C-terminal fragment of split FP) is probably required to close the β-barrel tertiary structure that protects the fluorophore and allows its maturation. There do not seem to be any obvious benefits or disadvantages between the 154/155 and 172/173 splitting positions and they are being used rather indiscriminately at this point. However, FPN172 fragments can be complemented by FPC154 counterparts, whereas FPN154 fragments cannot be complemented by FPN173 halves [103], indicating that the refolded FP structure tolerates a small peptide ‘overlap’, but not a gap. This is of practical relevance as cross-complementation between different split-FP variants leads to the formation of FPs with intermediate spectral properties and thus allows simultaneous detection of different protein–protein interactions in the same cell [103]. Multicolour BiFC can, for instance, be used to test two putative or competing interaction partners (fused to different FPN fragments) of the same bait protein (fused to an FPC). Cross-complementation competencies and resulting spectral properties of the hybrid FPs are reviewed in [95,103]. Notably, GFP-derived FPC does not complement any of the other FP variants tested. Multicolour-BiFC has been used in plants, and suitable vector sets are available for both restriction-based [100,101] and Gateway [31,102] cloning, so this developing technique awaits exploitation by plant virologists.

Because of its relatively straightforward use, BiFC has quickly become the most popular technique to detect protein–protein interactions in vivo, and has been used in numerous plant virus studies. It is therefore worth being aware of the method’s complications and pitfalls, which are also reviewed in [94] and [95]. A general drawback of BiFC is that the FP reconstitution is practically irreversible, making the technique unsuitable for monitoring dynamic interactions. This irreversibility potentially increases BiFC sensitivity, although it can also result in the accumulation of non-specific background fluorescence. For several experimental systems, no fluorescence was reconstituted when unfused FPN and FPC fragments were co-overexpressed. However, for some FPs, background fluorescence has been observed (e.g. [62,94]). Li et al. [104] have recently found that shortening the N-terminal fragment of YFP split at position 154 to YFP-N152 eliminates non-specific background, albeit at the cost of reduced signal intensity. In any case, unfused split-FP fragments are not a sufficient control in BiFC experiments, as the fusion to proteins of interest may influence their folding and stability so that non-specific fluorescence occurs for fused FP fragments even if no background was observed with unfused FPN+FPC [94,105]. Therefore fusions to known non-interacting proteins are a more adequate control. Additionally, the splitting position and the orientation of the split-FP-fusions may influence both undesired background signal and the detection of genuine interactions. The sterical constraints imposed on the FP reconstitution by fused interacting partners appears to be complex (see [94]). Sometimes one orientation will yield fluorescence, whereas another will not [62,97], and sometimes fusion orientations have no effect. Including information on splitting positions and fusion orientations in publications may help to improve the comparability between different experiments. Finally, both non-specific FP complementation and relative brightness of the BiFC signal will be increased by molecular crowding, i.e. if the FP-fusions are confined to a small subcellular compartment. Studies of such compartments require particularly rigorous controls, and the use of strong promoters is best avoided in BiFC experiments. However, even non-specific background fluorescence in BiFC can be used to obtain valuable information about the topology of integral membrane proteins (see below).

FRET (fluorescence resonance energy transfer)

FRET is the direct, nonradiative transmission of excitation energy from one fluorophore (FRET donor) to another (FRET acceptor), resulting in fluorescence of the acceptor after excitation of the donor. For this, the fluorophores have to come into very close proximity (≤10 nm), as FRET efficiency decreases with the sixth power of distance. This makes it ideally suited to study molecular interactions (reviewed in [39,94,106]). For this purpose, similar to BiFC, the FRET partners are linked to the macromolecules whose interaction is to be studied. In contrast with BiFC, which is based
on the properties of a specific protein tertiary structure, FRET can occur between any kind of fluorophore, and is therefore much more versatile. For instance, it can be used to study interactions between proteins and other molecules such as nucleic acids [107]. FRET can also occur intramolecularly between different fluorophores attached to the same macromolecule, allowing microscopic study of conformation changes (e.g. [108]). Perhaps most importantly for in vivo applications, the interaction between the FRET fluorophores themselves remains transient so that no non-specific background signal can accumulate. However, non-specific FRET can result from random collisions of fluorophores, and this probability increases with fluorophore concentrations and decreasing experimental volume. FRET measurements therefore always require adequate controls.

In the most straightforward method for FRET detection, the donor is excited with an appropriate wavelength, and fluorescence emission is then detected in the spectral window of the acceptor (‘sensitized emission’). For this approach, well-separated excitation and emission spectra respectively of donor and acceptor are desirable to avoid bleedthrough (donor fluorescence in the acceptor channel or direct excitation of the acceptor by donor-‘specific’ wavelengths). On the other hand, efficient FRET requires sufficient overlap between the donor emission and acceptor excitation spectra. Controls that are good practice for FRET experiments of this type include measurement of FRET efficiency for the non-interacting (unfused) FRET pair (background level due to random collision), fluorescence intensity measurements of the individually expressed FP-fusions with both donor- and acceptor-specific filters (to quantify bleedthrough) and ideally also a positive control, usually a direct fusion between donor and acceptor FPs. An alternative approach, which can also be used as an additional control in sensitized emission experiments, is acceptor photobleaching. Here, the quenching (reduction) of donor fluorescence by interaction with the FRET acceptor is measured. Selective photobleaching of the acceptor restores the full fluorescence intensity of the FRET donor (unquenching), allowing quantification of FRET efficiency. For this, the spectral properties of the FRET pair need to permit selective bleaching of only the acceptor. All of these methods depend on the measurement of light intensities, i.e. photon counts, and are therefore dependent on fluorophore concentrations. However, reproducible, consistent levels of the same construct are difficult to achieve in independent in vivo experiments, leading to a certain level of inaccuracy in all intensity-based FRET measurements.

The most accurate method for FRET detection is FLIM (fluorescence lifetime imaging) of the FRET donor [109,110]. Energy transfer to the FRET acceptor shortens the average time that a donor molecule remains in the excited state, which for FPs is usually in the order of nanoseconds. Reduction of donor fluorescence lifetime is independent of fluorophore concentrations and depends only on the FRET pair characteristics and fluorophore distance. FLIM requires sophisticated special equipment: for ‘time-domain’ FLIM, where donor fluorescence decay is measured directly, a pulsed laser capable of producing bursts of excitation light sufficiently shorter than the decay times is required, as well as similarly fast time-resolved photon detection. ‘Frequency domain’ FLIM measurements use an excitation laser that produces continuous light with a sinusoidally modulating intensity. Fluorescence lifetime is deduced from the phase-shift between the intensity curves of excitation and emission fluorescence, again requiring specialized detection equipment. Because of these technical demands and prohibitive costs, FLIM–FRET, although considered the ‘gold standard’ for microscopic detection of protein–protein interactions, is not yet widely available.

FRET experiments are also dependent on the choice of FRET pair. Many FP pairs have been used for FRET, and a complete listing is beyond the scope of the present review (see [17,18,39,94,106,111] instead). The most commonly used FRET pair, CFP/YFP, has a number of problems. YFP has a small Stokes shift (distance between excitation and emission maxima), making it harder to avoid CFP bleedthrough. Additionally, EYFP can change into a CFP-like blue form in response to intense UV light during acceptor photobleaching, leading to overestimation of the FRET contribution to CFP unquenching [88]. This effect has been disputed [112], but was recently also reported for the YFP variants Venus and Citrine [87]. CFP is comparatively dim and its fluorescence decay curve follows a double exponential, complicating FLIM quantification [113]. Brighter CFPs with single-exponential fluorescence decay are available [94,114]. Recently, TagGFP/TagRFP (where RFP is red FP) has been promoted as an optimized FRET pair [115]. Importantly, in contrast with several other FPs, TagRFP shows no photoconversion under strong UV illumination, avoiding potential artefacts during acceptor photobleaching [87]. Also of note is a non-fluorescent YFP variant, REACh [116], which permits FLIM- and intensity-based FRET measurements with a GFP donor, free of any spectral bleedthrough.

Perhaps due to the technical sophistication required for FRET experiments, BiFC has so far proven the more popular method to study protein interactions in vivo among plant virologists. Recently, both approaches have been combined to study ternary protein interactions in planta [117]. However, because of its reliance on transient molecular interactions and applicability to any type of fluorophore, FRET has tremendous potential for the functional study of plant virus replication, including protein–protein interactions within the replication site (e.g. [118]), protein–RNA interactions ([107,119]) and potentially even RNA secondary structure changes (discussed below). Ideally, both BiFC and FRET protein–protein interaction experiments should be verified by genetic approaches such as yeast two-hybrid or split-ubiquitin complementation assays.

**ANALYSIS OF PROTEIN MEMBRANE TOPOLOGY**

Many plant viruses encode integral membrane proteins, including various MPs [4]. So far, very few molecular structure data are available for these proteins. Of particular interest is information on the topology of viral transmembrane proteins, to gain insights into the position of functionally important groups with regard to the cellular surroundings and thus potential interaction partners. Integral membrane proteins may have one or several hydrophobic transmembrane helices and, depending on their mode of membrane insertion, the N-terminus can be directed towards the cytoplasm or towards the lumen of the ER (corresponding to the apolast for plasma membrane proteins), also determining the orientation of other transmembrane domains and connecting loop regions. Membrane topology can be analysed by immunofluorescence microscopy. A tag is fused to either end of the transmembrane protein (or internally) and its accessibility to antibody labelling at the cell surface (non-permeabilized) and intracellularly (membrane-permeabilized) is compared (e.g. [120]). However, this is unsuitable for intracellular endomembrane-resident proteins. Additionally, it requires fixation and permeabilization. Analogous biochemical approaches, e.g. using protease accessibility, require disruption of the cell. None of these approaches permits analyses in vivo.

Two new live-cell imaging methods have been developed for analysis of membrane topology. Zamyatnin Jr et al. [62] used the
non-specific fluorescence that occurs in BiFC assays: if one half of a split FP is fused to a transmembrane protein, the complementing split FP fragment then has to be localized to the same compartment for restoration of fluorescence. They demonstrated that a FPN fragment fused to either of the N- or C-termini of the potato mop-top virus TGB2 protein, which has two transmembrane domains, reconstituted fluorescence only in combination with cytosolic FPC. On the other hand, an internal fusion of FPC within the loop region connecting the transmembrane helices led to BiFC only in combination with FPN targeted to the ER lumen. Accordingly, both N- and C-termini are located on the cytosolic face of the ER and the loop domain in the lumen (Figure 1f). This approach could also be modified for plasma membrane transmembrane proteins: only their cytoplasmic domains will enable BiFC with intracellular split FP fragments. The authors did find, however, that unfused cytoplasmic FPN was able to trap the smaller ER-targeted FPC in the cytoplasm, resulting in cytoplasmic fluorescence. Therefore they used only the larger ER-targeted N-terminal YFP fragment to detect ER-luminal BiFC [62].

An alternative method uses the redox-sensitivity of a GFP variant together with redox differences in the cytoplasmic and ER-luminal compartments, and is therefore limited to endomembrane-resident transmembrane proteins (ReTAR, redox-based topology analysis; [121]). In this approach, the transmembrane protein of interest is fused to the redox-sensitive GFP variant roGFP2 [122], which is strongly excited at 405 nm in its oxidized form, but only very weakly excited in the reduced state. The ratio of fluorescence after 405 nm and 488 nm excitation can thus be used to determine the position of roGFP2 in the cytoplasm (more reducing, low 405/488 nm excitation ratio) or the ER lumen (more oxidizing, high 405/488 nm excitation ratio). When fused to a membrane protein of interest, the 405/488 nm excitation ratio reveals the orientation of the fused protein domain in the ER or cytosol (Figure 1g). These new tools, together with the analysis of protein–protein interactions in vivo will greatly facilitate our understanding of the membrane-associated protein complexes involved in plant virus infections.

RNA IMAGING

Since the advent of molecular cloning in the 1970s, the functional investigation of viral genomes, including sequence determination, analysis of expression strategies and identification of regulatory sequence elements [42,43], has been at the forefront of modern virology. The link between such data and the current wave of FP studies is the location of the viral genome in relation to its encoded proteins and host cell structures. Where, and in what cellular context, do the genetically characterized infection events actually take place? In situ hybridization at the light and electron microscopic level can provide some of this information, but is experimentally demanding and reveals only snapshots of dynamic processes. RNA localizations by in situ hybridization have often been carried out on protoplast systems due to better permeability and because some temporal resolution can be achieved by stopping synchronized infected cultures at defined timepoints (e.g. [123,124]). However, all RNA viruses replicate on cellular membranes which they reorganize extensively into a VRC (viral replication centre) [3,5] and the sensitivity of cellular membranes to standard aldehyde fixation protocols commonly employed in in situ hybridization is another reason for making in vivo RNA tracking a preferred choice.

Tools for sequence-specific live-cell RNA detection have been available since 1998 (reviewed in [125,126]). The first such system, based on the coat protein of bacteriophage MS2 [127], and a more recent version using a 22-amino-acid peptide from the N protein of bacteriophage λ (λN22) [128], employ fusion of a sequence-specific RNA-binding protein to GFP. The MS2 CP and λN22 peptides recognize RNA hairpins with which the RNA of interest needs to be tagged. In order to separate unbound and RNA-bound reporter, the GFP-fusion is usually targeted to the nucleus. Cytoplasmic fluorescence then indicates redistribution in response to RNA binding. The MS2 system has been used in numerous studies, and MS2 and λN22, together have enabled simultaneous tracking of two RNAs in the same cell [129]. Zhang and Simon [130] tracked turnip crinkle virus infection using the MS2 system, but did not localize the viral RNA at the subcellular level, although the technique is principally suited to this. Sambade et al. [131] showed that a nuclear expressed, MS2-tagged RNA encoding the TMV MP formed motile granules in tobacco leaf epidermal cells which co-localized with MP at PD. A limitation of the MS2 and λN22 systems for virus imaging is the introduction of additional sequences with extensive secondary structure into the viral RNA, which may affect infectivity, recombination rates or RNA localization [43,126,129]. Additionally, multiple tandem copies of the hairpin tag have to be introduced to obtain sufficient sensitivity (commonly 6–24, but up to 96 repeats have been used; [125]). Zhang and Simon [130] used only a single MS2 hairpin to tag turnip crinkle virus, which was sufficient for relocation of MS2 CP–GFP from the nucleus to the cytoplasm, but this may permit subcellular RNA localization for very highly accumulating viruses.

Because of these limitations, a novel approach based on the RNA-binding domain of the translational repressor human Pumilio1 [132] held promise for virus studies. The PUMHD (Pumilio homology domain) has a modular structure of eight imperfect repeats that each bind one nucleotide with just three amino acids per repeat involved in the protein–RNA interaction. This makes it possible to alter the sequence specificity of the PUMHD in a defined way with relatively little genetic engineering of the protein [133,134]. Instead of tagging the viral RNA, the detection system can be modified to recognize the unaltered RNA. Additionally, the PUMHD has a higher affinity (K₅₇ = 0.48 nM) to its target sequence than either MS2 CP (6.2 nM) or λN22 (22 nM; reviewed in [126]), and binding neither requires nor introduces RNA secondary structure. Also, the need for nuclear sequestration of the unbound reporter was avoided by instead using BiFC between two PUMHD variants engineered to bind to closely adjacent target sites on the same RNA and fused to the two halves of a split FP [132]. This system enabled detailed mapping of the distribution of unencapsidated RNA of TMV and PVX (potato virus X) in their respective VRCs (Figure 1h), as well as the co-introduction of an RNA reporter and a 35S-driven TMV replicon to study early vRNA (viral RNA) distribution beginning ~6 h after co-bombardment [105]. The perinuclear VRC of TMV contained granular RNA ‘hot spots’, whereas that of PVX showed circular RNA ‘whorls’ arranged around aggregates of the viral MP TGB1, indicating that these structures show considerably different arrangements in different viruses. The PUMHD variants were modified to recognize unaltered TMV genomic RNA, but subsequent tagging of the PVX genome with the same TMV-derived sequences demonstrated that this short (21 nt) non-structured tag had no disruptive effect on the virus [105]. The PUMHD-BiFC reporter has also been used to localize potyviral RNA to replication sites in membrane invaginations of the chloroplast envelope [36].

More recent structural studies of the PUMHD protein have revealed that its sequence specificity is not as high as initially believed [134]. A certain degree of binding promiscuity is probably balanced by the requirement of BiFC on binding of two PUMHD fusions to generate a signal, but the BiFC approach
is itself not free of background signal. Additionally, altering PUMHD specificity often results in a marked reduction in affinity [132,133] and thus sensitivity. PUMHD-BiFC has enabled the most precise in vivo localizations of viral RNA genomes so far, but further developments are probably required to provide plant virologists with a generally applicable live-cell RNA reporter.

Extremely high sensitivity and signal-to-noise ratios can be achieved by methods that require invasive delivery of the RNA reporter into cells. In direct RNA labelling, the viral genome itself is rendered fluorescent by incorporation of fluorophore-coupled nucleotides during in vitro transcription and then microinjected [135]. Although low-throughput, this approach overcomes the temporal limitations inherent in virus-expressed FP reporters, and permits insights into the very early stages of infection. Microinjected TMV RNA was found to attach to the ER/actin network immediately after entry in a cap-dependent manner [135], possibly in connection with the host translation machinery [136]. Microinjection of directly labelled viral RNA encapsidated in fluorescence-conjugated CP allows the visualization of virus unencapsidation in vivo, also revealing the cellular context in which this infection step happens [135,137]. It is conceivable that such approaches could be expanded further, utilizing FRET to study changing macromolecular interactions during early infection events [119]. For instance, FRET between an RNA-intercalated dye and a protein FP-fusion was recently used to detect protein–RNA interactions in vivo [107]. Similarly, directly labelled viral RNA could be microinjected into cells expressing an FP-fused replication factor to study early interaction events. Alternatively, molecular beacons could be used. Molecular beacons are short synthetic oligonucleotides linked to fluorophores that are used for sequence-specific RNA detection in vivo and in vitro (reviewed in [125,126,138]). Molecular beacon signal is limited to the target-bound form because in the unbound state secondary-structure formation of the beacon brings a fluorophore and a quencher group in close contact, preventing fluorescence emission [139]. The beacons could be microinjected to detect viral RNAs, but another approach would be to hybridize them to the viral genome in vitro and then microinject beacon-labelled vRNA. The binding of the molecular beacon is sensitive to secondary structure of the target RNA [125,126,138,140]. Therefore disappearance of the fluorescent signal could be used to monitor displacement of the molecular beacon by RNA secondary-structure rearrangements or protein binding in vivo. Protoplasts would probably be the easiest system for such experiments.

For some questions, no in vivo imaging techniques are currently available. For instance, double-stranded RNA replication intermediates are a particularly good indicator of replication sites. Currently they can only be visualized by immunofluorescence microscopy with dsRNA (double-stranded RNA)-specific antibodies (e.g. [123,124]), although the use of preferentially dsRNA-binding cell-permeant dyes may provide an in vivo alternative [137]. Newly synthesized RNA can be visualized by incorporation of bromo-UTP and subsequent detection with specific antibodies (e.g. [123,124]), an approach that is also suitable for DNA viruses, in which bromo-dUTP is used instead (e.g. [53]). With the available toolbox of RNA-imaging techniques, genetic and microscopical plant virus studies can now be linked at the subcellular level.

**TRACKING THE COMPLETE VIRAL LIFE CYCLE IN FOUR DIMENSIONS**

For some animal viruses, the complete viral lifecycle can now be followed microscopically (e.g. [52,141]). All viral gene products can be localized throughout infection either in living cells or cell cultures fixed at specific time points. Viral nucleic acids can be visualized and the interactions of viral components with host cell factors and among each other can be detected in vivo, individual viral genomes or particles can be tracked [142], and even transient and complex events such as virus entry [137,143], packaging [144] and exit/cell–cell transfer [145] can be observed. For plant systems, the same tools and methods are also available, with certain imaging limitations due to autofluorescence from the cell wall and chloroplasts [64]. It can be expected that the spatial and temporal relationships between plant viruses and their host cells will soon be understood in similar detail.

The earliest events in the viral infection cycle are the most difficult to visualize. Usually, only a few viral genomes and a very low level of their encoded proteins are present in the host cell. Additionally, virus-expressed fluorescent reporters only become detectable after a few hours, due to a lag in expression and fluorophore maturation. One way to study early events, such as unencapsidation of virions and the initiation of translation, is to label viral components in vitro and then introduce them invasively, which may not differ all that much from the ‘natural’ delivery of plant viruses via mechanic wounds or insect feeding tubes. If unincorporated fluorescent molecules are removed after labelling, in vitro labelling yields extremely high signal/noise ratios. Also, larger pools of virions can be introduced than would be the case in a natural infection. This may lead to artefacts in some cases, but also overcomes some of the limitations resulting from low virus levels. Studies such as those of Christensen et al. [135], which investigated the initial localization and unencapsidation of microinjected CP- and RNA-labelled TMV virions could be expanded to RNA–protein and protein–protein interaction dynamics by FRET, as discussed above. Alternatively, transfection of entire double-labelled virus particles into protoplasts might be possible using electroporation, poly(ethylene glycol) precipitation or membrane-permeabilizing agents.

Very early infections events can also be studied indirectly. Cotton et al. [146] infected the same cells with two different potyviral genomes expressing either GFP- or RFP-fusions to the 6K protein that establishes the replication complex on the ER. The replication complexes were either green or red, rarely both, leading the authors to conclude that each replication site is established from a single infectious genome and that the p6 protein remains associated with the same replication site. Not all viral systems permit double infections, but where this is possible, 35S promoter-driven infectious clones are particularly suitable as different viruses can be co-introduced into the same cell with nearly 100% co-transformation efficiency through particle bombardment [147].

Large imaging gaps are also still to fill in two areas where plant and animal viruses show the greatest discrepancies. These are virus entry and exit, both between neighbouring plant cells and between the host plant and vector organisms. Entry into and exit from the plant are usually achieved mechanically, i.e. through perforation of the cell wall, whereas cell-to-cell transport within the host organism occurs through the plasmodesmal pores traversing the walls. By contrast, the majority of animal viruses are membrane-coated and enter and leave cells by membrane fusion and budding events. Virus movement through PD is probably an early infection event for many plant viruses [50,51] and happens before virus-expressed fluorescent reporters can be imaged. Microinjected fluorescently labelled TMV vRNA was not detected in neighbouring cells even in MP transgenic plants although the infection had spread, as shown by a virus-expressed FP [135]. Trafficked genomes were either recruited.
exclusively from the (unlabelled) pool of progeny genomes after replication, or the amounts of fluorescence that were transported across the cell–cell boundary were too small for detection. Other studies have found cell–cell transport of viral RNA when MPs were co-injected (e.g. [148]), possibly due to pre-formation of movement complexes in vitro. Imaging of cell–cell transport through PD is also hindered by difficulties in imaging the wall-embedded PD, which is prone to the risk of optical artefacts due to autofluorescence [64] and reflection [149]. Systemic transport in the phloem is even more difficult to image due to the embedding of the vasculature deep within the plant tissue. But perhaps most challenging is imaging the release and uptake events that occur from and into vector organisms, as direct visualization of the phloem is even more difficult to image due to the embedding of the plant endomembrane system and cytokinesis (e.g. [162,163]), to only selected structure outlines of interest. ET can thus enable the analysis of 3D parameters such as volume, surface area or connectivity of subcellular structures at unprecedented resolution. Volumetric analysis of isolated macromolecular complexes by cryo-ET even achieves sub-nanometer resolutions sufficient for macromolecular structure determination, which is particularly valuable when crystallization is problematic as in the case of membrane proteins [151].

Electron tomography

Similar to LM, EM has made significant advances in the last two decades [14], including expansion into 3D imaging through ET (reviewed in [13,14,16,150]). In ET, relatively thick specimens (100–500 nm) are tilted over typically ±65° in 1–2° increments along an axis perpendicular to the electron beam during acquisition of 50–100 images. This set of images allows computational 3D reconstruction with a z-resolution of 2–10 nm, removing the limitation set by section thickness (≈20–100 nm) of conventional EM. The digitized 3D model can then be reduced to only selected structure outlines of interest. ET can thus enable the analysis of 3D parameters such as volume, surface area or connectivity of subcellular structures at unprecedented resolution. Volumetric analysis of isolated macromolecular complexes by cryo-ET even achieves sub-nanometer resolutions sufficient for macromolecular structure determination, which is particularly valuable when crystallization is problematic as in the case of membrane proteins [151].

Electron tomographic studies have yielded remarkable insights into the structure of animal viral replication sites (e.g. flock house virus [152]; Figure 3a) and SARS (severe acute respiratory syndrome)-coronavirus [153]. The flock house virus study permitted calculations of replication site stoichiometry: virus-induced invaginations of the outer mitochondrial envelope membrane each contain approx. 100 molecules of the replicative transmembrane protein A, as well as an average of three RNA replication templates, and remain connected to the cytoplasm by a 10 nm membrane channel wide enough to permit export of progeny RNA [152]. Such information is highly valuable as it connects directly to recent biochemical insights into how the oligomeric organization of viral RNA-dependent RNA polymerase complexes assist in their overall function [154], which may ultimately lead to the discovery of new antiviral drug targets. ET has also been used to study animal virus entry [155,156], uncoating [157], encapsidation [158] and exit events [159], and cryo-ET has facilitated the study of complex, membranous virus particles [160]. Most recently, successive sample abrasion with a focused ion beam has been used as an alternative to a tilt series for gaining 3D information in EM. This approach revealed the connectivity of intracellular membranous compartments containing HIV particles with the plasma membrane [161]. For plant viruses, ET studies are still missing, although EM-tomography has been very successfully applied to the study of the plant endomembrane system and cytokinesis (e.g. [162,163]), and was also used to study rice dwarf virus in its insect vector [164,165]. It can be expected that plant viruses, also, will soon be imaged within their plant hosts by ET.

For highly resolving EM approaches, high-pressure freezing has become a standard technique that preserves sample structure much better than traditional aldehyde fixation protocols, especially in the case of membranous organelles [12]. Under high pressure, samples up to a few hundred nanometers thick can be frozen sufficiently fast to maintain cellular water in an amorphous (vitrified) state, preventing tissue damage by ice crystals. Frozen-hydrated samples can be sectioned (cryo-ultramicrotomy; reviewed in [13,14]) and imaged directly (cryo-EM), with resolutions up to 3–5 nm on cellular samples, enough to identify known macromolecular complexes by their shape [166,167]. However, unstained vitrified specimens provide only phase contrast to generate detail and are also extremely sensitive to the electron beam. This results in low signal/noise ratio and makes it very difficult to pre-select a ROI, so that tomograms sometimes have to be acquired “blindly” [16]. Therefore alternative or complementing approaches are required to increase contrast, and permit molecular identification of cellular components.

The cellular water in high-pressure frozen samples can be replaced with organic solvents and then embedding plastics without thawing the sample (freeze substitution). Freeze substitution permits the use of stains to increase contrast, and embedded samples are stabilized for further sectioning at room temperature as well as being less sensitive to the electron beam although mostly retaining their preservation [168]. Truly specific labelling of proteins in ET can be achieved by immunogold labelling and similar immunodetection of dsRNA [153] or of bromo-UTP-labelled newly synthesized RNA [152], is also possible. However, antibodies cannot be applied in organic solvents or on frozen samples. Therefore high-pressure frozen/freeze-substituted specimens commonly used for ET can only be treated after embedding and sectioning, effectively limiting immunogold labelling to the surface of the specimen. Conversely, pre-embedding labelling gives greater sensitivity and higher labelling levels [6], but requires chemical fixation. As a compromise, several approaches have been developed to combine ultrathin cryosectioning, which provides a better label/sample volume ratio, with immunolabelling. This requires thawing and therefore also chemical fixation at some stage. Fixation can be done prior to freezing (Tokuyasu method), after thawing or by thawing and rehydrating high-pressure frozen/freeze-substituted samples followed by re-freezing and cryosectioning (see [169] for comparison). All of these approaches lose some of the pristine sample preservation of direct cryo-EM/ET, and chemical fixation also generally inactivates a proportion of the accessible epitopes, preventing quantitative labelling [13,14]. As a result, macromolecule-specific labelling techniques for EM/ET currently fall short of complementing the available spatial imaging precision. An EM-equivalent to FPs, i.e. a genetic tag than can be directly detected throughout an EM sample would be highly desirable [14].

CLEM (correlative light and electron microscopy) and ‘super-resolution’

An alternative, or even preferable, approach would be to combine the advantages of LM and EM (specific, quantitative protein labelling and high resolution respectively) by correlative imaging of the same sample (CLEM; reviewed in [15,16]). The promise of CLEM has increased even further with the recent development of various LM approaches that break the diffraction barrier, long viewed as a limit to resolution in LM [9–11,170]. Because
light is subject to diffraction in all lens-based optical systems, a fluorescent spot will appear as a PSF (point-spread function), i.e. enlarged. Two fluorescent spots whose point spread functions overlap cannot be resolved in a conventional light microscope and according to the principle described by Ernst Abbe in 1873, this limit to resolution is approximately half the wavelength of the emitted light, $\sim 250 \text{ nm}$ in the focal plane ($x$-y) and $\sim 500 \text{ nm}$ along the optical axis ($z$) for fluorescence microscopy. A number of different approaches now achieve resolutions beyond the diffraction barrier and are collectively referred to

Figure 3  Bridging the imaging gaps

I. Extending EM to 3D by electron tomography. (a) FHV (flock house virus) induces invaginations of the outer mitochondrial membrane, where it replicates (i). Tomographic 3D reconstruction of these invaginations (ii) shows them to be individual compartments, each of which is linked to the cytoplasm by a $\sim 10 \text{ nm}$ channel (iii and iv). Using biochemical and structural information, the potential packing arrangement of the replicative viral protein A within the spherule can be modelled (v) (reproduced from [152] under the Creative Commons Attribution License). II. Correlative light 'super-resolution' microscopy and EM. (b) Diffraction-limited TIRF (total internal reflection fluorescence) microscopy (i), ‘super-resolution’ PALM and transmission EM (iii) of the same cryosection showing mitochondria. Overlay of PALM and EM images (iv). Reprinted from [173] with permission from AAAS. III. Combination of LM, EM and AFM (c) The ORF3 protein of groundnut rosette virus localizes in cytoplasmic inclusions (i) that contain fibrillar material (ii). In EM cross-sections, the fibrillar material can be seen to consist of ring-like protein complexes encapsidating the viral RNA (inset in ii). Fluorescence microscopy of ORF3-GFP (iii) and anti-fibrillarin immunofluorescence (iv) shows that ORF3 targets the nucleus and leads to redistribution of nucleolar fibrillarin into the cytoplasmic inclusions (v). In vitro, ORF3 and fibrillarin are both necessary to encapsidate viral RNA (vi–ix), but can form ring-like complexes in the absence of RNA (viii). These are similar to the structures observed in infected tissue (ii, inset) and AFM reveals them to consist of alternating ORF3 and fibrillarin subunits (x) (reprinted by permission from Macmillan Publishers Ltd: EMBO Journal [37] © 2007, reproduced from [38] © 2007 National Academy of Sciences, U.S.A., reproduced from [187] with permission from American Society for Microbiology, reprinted from [188] © 2008 with permission from Elsevier). c-RNP, cytoplasmic RNP inclusion; DC, densely coated RNA; F, protein filaments; N, nucleus; No, nucleolus; V, vacuole; XB, cytoplasmic inclusion. Scale bars: (a, panels i and ii), 100 nm; (a, panels iii and iv), 25 nm; (b), 1 $\mu\text{m}$; (c, panels i, iii, iv and v), 5 $\mu\text{m}$; (c, panel ii), 100 nm; (c, panel ii inset), 25 nm; (c, panels vi, vii, viii and ix), 100 nm; (c, insets in panels vi, vii, viii and ix), 50 nm; (c, panel x), 20 nm.

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as ‘super-resolution’ or ‘far-field’ microscopy or ‘nanoscopy’. These include STED (stimulated emission depletion) microscopy, which increases \(x\)-\(y\) resolution to tens of nanometers by focusing a second laser beam on the excitation spot which is ring-shaped and of an appropriate wavelength to quench excited fluorophores, effectively restricting excitation to the dark centre of the quenching laser [171]. SIM (structured illumination microscopy) accesses subdiffraction information by moving a diffraction grid over the sample and calculating additional spatial information from the different diffraction patterns resulting from different grid orientations [172]. Both STED and SIM are suitable for in vivo imaging. PALM (photoactivation localization microscopy; [173]), and STORM (stochastic optical reconstruction microscopy) is the highest-resolving super-resolution technique [174], achieving up to 5 nm lateral resolution. Imaging of individual fluorophores without interference from overlapping point spread functions allows precise calculation of the position of the actual fluorescent spot within its PSF. In PALM/STORM, small numbers of photoswitchable fluorophores are activated for each image, localized and then bleached or switched off. This procedure is repeated until the population of fluorescent molecules has been exhausted and the collective image can be assembled from the localizations of individual fluorophores. Reversibly photoswitching pc-FPs such as Dronpa are particularly suited for this approach [175]. The high resolution and the slow image acquisition make PALM suitable mostly for fixed specimens. In all these techniques, additional modifications are necessary to achieve super-resolution also along the light path in the axial dimension. Various approaches have been developed including the use of two opposing objectives (4Pi microscopy), \(z\)-dependent lateral distortion of the image or axial interference patterns, and \(z\)-resolutions of approx. 50 nm can be achieved (reviewed in [10,170]). In vivo 4Pi microscopy has been correlated with EM to track transport of cargo molecules through single Golgi stacks and PALM has been combined with cryo-EM [173] (Figure 3b). The power of such correlative approaches is evident from the achievable labelling density: Betzig et al. [173] counted >5500 fluorophores in a subset of mitochondria in a single PALM crossection, whereas only 10–20 labels/section could be expected in a comparative immunogold experiment [86].

To image the same sample in CLEM, either live-cell imaging has to be carried out before fixation or the same fixed specimen has to be used in both LM and EM. The first approach makes full use of the benefits of LM, and ideally would allow the cessation of cellular processes at a precise moment of interest. Cryosectioning is ideal for the preservation of both fluorophores and sample ultrastructure, but continuous maintenance of samples at vitreous temperatures (<\(140^\circ\)C) is technically extremely challenging [16]. Less demanding is spatial matching of chemically fixed samples in both microscopies for which the first commercial setups are on the horizon (reviewed in [15]). Unfortunately, in fixed samples, the use of the fluorescent markers becomes problematic. Chemical fixatives denature proteins, and even though GFP-derivatives are very resilient and often retain fluorescence after mild fixation, signal intensity is usually significantly diminished, making this procedure unsuitable for low-abundance FP-fusions. If the sample is embedded in polymers for sectioning, additional problems may arise because conventional FPs require molecular oxygen for fluorescence. Such problems can potentially be solved by use of iLOV, which does not require oxygen (but does require presence of FAD) [63,176], or fluorescent labelling during/after embedding, e.g. with tag–probe labelling systems or using fluorescent antibodies. The best currently available tool for this purpose, which also comes closest to a ‘GFP for EM’, is the photo-oxidative precipitation of DAB (diaminobenzidine) by fluorophore bleaching [15,177,178]. Oxygen radicals generated in the vicinity of fluorophores during photobleaching oxidize DAB, which forms a granular polymer that can be osmium-stained, rendering it EM-visible. This technique has been demonstrated for FPs [178,179] as well as tetracysteine-/ReAsH-labelled proteins [180] and various fluorescent dyes [15,177,178].

Also suitable for direct LM/EM correlation are Qdots (quantum-dot)-labelled antibodies. Qdots are fluorescent nanocrystals which neither bleach [64] nor require molecular oxygen. Their spectral properties are defined by their size and they are large enough and sufficiently electron-dense to be directly visible in EM [181]. Use of Qdots for live-cell imaging has been hampered by their large size and incompatibility with biological molecules. However, CLEM may be the area where their full utility can be realized [15]. It can be expected that the combined imaging power of super-resolution LM and EM will provide many new insights into previously inaccessible aspects of plant virus infections in the near future.

Combined EM and AFM

At the far end of the resolution scale, EM can also be combined with AFM, which can achieve true atomic resolution [182]. In AFM, a very fine needle tip is used to scan the surface of the sample, either by touching it directly (contact mode) or by remaining at a small distance utilizing electromagnetic interactions (non-contact mode). Needle movements are measured with high accuracy and allow 3D reconstruction of the sample surface. The most significant contribution of AFM to plant virology so far has been in the study of isolated virions and viral RNPs (ribonucleoprotein particles).

Unlike EM, AFM does not require staining, which can obscure small features and is therefore useful for discovering asymmetric structures of viral RNPs which can then be identified by immuno-EM [183]. Interestingly, asymmetric binding of viral proteins to only one end of virions or viral ribonucleoprotein complexes has been found for a number of very different viruses (e.g. [84,183–186]) and may represent a general principle by which virions functionally interact with host and/or vector organisms. AFM and immuno-EM were used to investigate the destabilizing effect on the PVX virion of binding by the TGB1 MP to its 5\(^\text{′}\) end [84,184]. These experiments established that binding of TGB1 is one way to render the encapsidated viral RNA translation-competent and also identified an in vitro-assembled complex of viral RNA partially encapsidated in CP, termed a ‘single-tailed particle’ as the potential transport form of this virus [84]. Lim et al. [48] recently isolated viral RNPs from plants infected with a mutant form of barley stripe mosaic virus lacking the coat protein. The absence of CP prevents formation of true virions and these RNPs, which contained the TGB1 MP and positive sense genomic and subgenomic ssRNAs, were assumed to be viral movement complexes. Low amounts of unidentified high-molecular-mass protein components were also found, but the minor MPs TGB2 and 3 were not detectable, despite heterologous interactions between the TGBs [48]. Combined AFM and immuno-EM imaging of the complexes might be a useful approach to elucidate the potential presence, identity and position in the RNP of minor protein components. Similar approaches could be adopted for other viruses that do not require their coat protein for local movement, e.g. TMV.

In the case of the recruitment of nucleolar fibrillarin by the groundnut rosette virus ORF3 protein [37,38], described above, combined EM and AFM showed that fibrillarin forms part of the ring-like complexes that protect the viral RNA during...
long-distance transport [38,187,188]. This was the first direct demonstration of a plant host protein participating in a viral movement complex (Figure 3c). The study of viral RNPs by combined AFM and EM has provided some of the deepest insights into the elusive nature of putative plant viral movement complexes and will become even more valuable when more structural data on MPs become available. Collectively, the combinatorial power of the individual and correlative imaging approaches now at the disposal of plant virologists hold the promise that we will be able to gain a truly molecular understanding of all stages of the viral infection cycle within the not-so-distant future.

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