REVIEW ARTICLE

Focusing on clathrin-mediated endocytosis

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Investigations into the mechanisms which regulate entry of integral membrane proteins, and associated ligands, into the cell through vesicular carriers (endocytosis) have greatly benefited from the application of live-cell imaging. Several excellent recent reviews have detailed specific aspects of endocytosis, such as entry of particular cargo, or the different routes of internalization. The aim of the present review is to highlight how advances in live-cell fluorescence microscopy have affected the study of clathrin-mediated endocytosis. The last decade has seen a tremendous increase in the development and dissemination of methods for imaging endocytosis in live cells, and this has been followed by a dramatic shift in the way this critical cellular pathway is studied and understood. The present review begins with a description of the technical advances which have permitted new types of experiment to be performed, as well as potential pitfalls of these new technologies. Subsequently, advances in the understanding of three key endocytic proteins will be addressed: clathrin, dynamin and AP-2 (adaptor protein 2). Although great strides have clearly been made in these areas in recent years, as is often the case, each answer has bred numerous questions. Furthermore, several examples are highlighted where, because of seemingly minor differences in experimental systems, what appear at first to be very similar studies have, at times, yielded vastly differing results and conclusions. Thus this is an exceedingly exciting time to study endocytosis, and this area serves as a clear demonstration of the power of applying live-cell imaging to answer fundamental biological questions.

Key words: adaptor protein 2 (AP-2), clathrin, dynamin, endocytosis, fluorescent protein, live-cell imaging.

INTRODUCTION

'Bristle-coated vesicles' were first observed using electron microscopy in 1964 by Roth and Porter [1]. However, it would be over a decade before Barbara Pearse devised a method for isolating coated vesicles, and employed it in the purification of clathrin [2,3]. Along the way, there have been numerous groundbreaking contributions in this area. These include the exquisite electron microscopy of John Heuser, the structural studies of Tomas Kirchhausen, as well as the identification and analysis of cargo adaptors by people such as Margaret Robinson and Linton Traub, including the heterotetrameric AP (adaptor protein)-2 complex, in addition to a series of ‘alternative adaptors’ [4–10]. Work from researchers such as Sandra Schmid and Mark McNiven has provided great insight into the understanding of dynamin, a large regulatory GTPase involved in vesicle fission [11–14]. Furthermore, endocytosis at the synapse has been a focus of people such as Pietro De Camilli and Timothy Ryan [15–18]. Finally, contributions from researchers including Harvey McMahon, Peter McPherson, Alexander Sorkin, Francis Brodsky, Lois Greene and Alexandre Benmerah, as well as many others, have added to the understanding of clathrin-mediated endocytosis [7,19–31].

The general progression of clathrin-mediated endocytosis has been explicitly detailed in numerous reviews, and will only be summarized here (Figure 1) [32,33]. Integral membrane proteins (e.g. activated receptors) bind to cytosolic adaptors which form a link to the clathrin lattice. Accessory proteins, such as the GTPase dynamin, have the ability to affect the process of endocytosis, e.g. through promoting clathrin polymerization or inducing membrane curvature. Furthermore, both adaptors and accessory proteins can have the capacity to bind PtdIns(4,5)P2, a phospholipid concentrated at sites of clathrin-mediated endocytosis [23,24,34,35]. Important roles for actin have also been demonstrated in the process of clathrin-mediated endocytosis (see the Other proteins section below). Finally, the nascent coated vesicle is severed from the plasma membrane through a fission reaction thought to be mediated by dynamin [18].

Until James Keen’s group was able to image fluorescent clathrin in live cells in 1999 [36], nearly all previous investigations of clathrin-mediated endocytosis had been performed in fixed cells (e.g. using electron microscopy or immunocytochemistry), through quantification of radiolabelled cargo uptake, or with isolated coated vesicles [37]. Subsequently, there have been numerous investigations employing live-cell imaging. These studies have come out of nearly all of the above-mentioned endocytosis laboratories, as well as from investigators for whom live-cell fluorescence imaging has always been the tool of choice (e.g. Christien Merrifield) [38,39]. The focus of the present review is to specifically detail the entirely new perspective on questions of clathrin-mediated endocytosis that has been permitted by developments in high-resolution live-cell fluorescence imaging. Finally, although several recent studies have elegantly employed similar techniques to study endocytosis in Saccharomyces cerevisiae, for the sake of clarity and brevity, this review focuses upon investigations in mammalian systems [40–43].

LIVE-CELL FLUORESCENCE IMAGING

In many types of live-cell studies, the use of chemical fluorophores can be hindered by the barrier imposed by the plasma membrane. However, in the case of endocytosis, the process being studied is

Abbreviations used: AP, adaptor protein; FRAP, fluorescence recovery after photobleaching; FRET, fluorescence resonance energy transfer; GAK, cyclin G-associated kinase; GFP, green fluorescent protein; siRNA, small interfering RNA; TIRFM, total internal reflection fluorescence microscopy.

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that of cargo entry. Consequently, fluorescent endocytic ligands have provided a valuable resource in the study of clathrin-mediated endocytosis [44,45]. Furthermore, as in many other areas of biology, the use of endogenously fluorescent proteins [e.g. GFP (green fluorescent protein)], has revolutionized the field [46,47]. Thus usage of fluorescently tagged proteins of interest (e.g. ligands, receptors, adaptors, and accessory and coat proteins) can be extremely powerful, assuming that the appropriate imaging technology is employed.

The initial report utilizing GFP-tagged clathrin light chain for live-cell imaging employed epifluorescence microscopy [36]. Even with a high NA (numerical aperture) objective lens, which provides efficient light collection, as well as excellent lateral and axial resolution, this configuration has the drawback that out-of-focus light can contaminate the observed region of interest (Figure 2). Thus, when imaging fluorescent clathrin by epifluorescence microscopy, puncta can only generally be resolved towards the cell periphery. This is due to the tremendous contribution of intracellular signal outside of the plane of focus resulting from clathrin at the trans-Golgi network, as well as certain populations of endosomes [48,49]. Furthermore, if intracellular structures can be imaged, how can one be sure that plasma-membrane-associated clathrin is being tracked?

Although post-acquisition methodologies such as deconvolution can be applied to these types of dataset to assign emitted light to the appropriate focal planes, drawbacks to these approaches exist [50]. Generally speaking, the most useful form of deconvolution involves reassignment of out-of-focus emission based upon point-spread functions either determined empirically or calculated. However, this requires the acquisition of images at multiple focal places, with precise z-stepping. This can greatly increase the time between acquisition of successive images at the focal plane of interest, which can make deconvolution time-prohibitive in studies of events occurring in live cells. Finally, the reassignment of out-of-focus light in ‘nearest neighbour’ deconvolution algorithms precludes many forms of quantitative data analysis, as pixel value read-out no longer corresponds directly to emission from the sample [50].

Confocal microscopy has also been applied in certain studies of clathrin-mediated endocytosis [30,31,51]. This technique has the benefit of optical sectioning: pinhole optics prevent the collection of out-of-focus light originating outside of the ~1-μm-thick plane of interest, generally speaking. Thus it is easy to see how this could avoid unintended imaging of intracellular structures. However, confocal scanning of a whole-cell field with high resolution can easily take 1 s or more. Thus, as with ‘three-dimensional’ deconvolution, rapid events such as internalization of nascent clathrin-coated vesicles cannot be readily resolved. Because of these issues, application of confocal microscopy to studies of individual events of clathrin-mediated endocytosis has been limited. One place where confocal imaging has had a major impact is the analysis of clathrin subunit exchange (see the Imaging clathrin section below) [30,31]. However, even in that area, other imaging methodologies have been shown more recently to be potentially more appropriate [52].

It should be noted that several microscope companies are currently producing ‘rapid-scan’ or ‘swept-field’ confocal microscopes, which can increase acquisition rates by orders of magnitude. One such configuration involves the use of a line-scanning (rather than point-scanning) laser which is coupled to a linear pixel-array-type camera. Images are then constructed rapidly from the acquired series of lines. However, although these types of microscope have been available for some time, this technology has not been widely employed in cell biological studies, and has yet to be applied to the analysis of clathrin-mediated endocytosis [53]. One place where this type of imaging could be very useful would be in the tracking of individual coated vesicles from formation, to fission and subsequent internalization and entry into the endocytic system.

Spinning (or Nipkow) -disk microscopy uses an array of pinholes arranged on a disk that rapidly rotates [54]. This imaging
that are collected have a very high signal-to-noise ratio due to refractive indices, angle of incidence and wavelength [57].

TIRFM, this is usually less than \( \theta_c \) at which the intensity of the evanescent field decreases to \( 1/e \). (c) defined by Snell’s law \( \sin \theta_c = n_1/n_2 \) at an angle greater than the ‘critical angle’ \( \theta_c \) defined by Snell’s law \( \sin \theta_c = n_1/n_2 \), total internal reflection occurs (Figure 3). This results in production of the ‘evanescent field’, a standing wave that decays exponentially with distance from the interface between the two media. The penetration depth \( d \) is defined as the distance at which the intensity of the evanescent field decreases to \( 1/e \). In TIRFM, this is usually less than \( \sim 100 \) nm, and depends upon the refractive indices, angle of incidence and wavelength [57].

As fluorophores deeper into the cell are not excited, the images that are collected have a very high signal-to-noise ratio due to the elimination of out-of-focus light. Thus fluorophores entering the cell will be observed to disappear. In this way, events of endocytosis can be observed directly (Figure 4). However, several criteria have had to be satisfied in order to verify that clathrin-mediated endocytosis was indeed imaged. (i) In order to ensure that photobleaching is not interpreted as vesicle internalization, neighbouring spots should be analysed alongside putative events of endocytosis (e.g. Figure 4, spot B). (ii) Disappearance must be observed to be progressive over successive frames, and not be the result of lateral spot motility out of the field of view [36,44,58]. (iii) To ensure that the dynamics of other clathrin-positive compartments (e.g. endosomes) are not being observed, other markers for clathrin-mediated endocytosis (e.g. dynamin) should be coincident with sites of clathrin internalization [38,59]. (iv) Cargo for clathrin-mediated endocytosis (e.g. ligands and receptors) have to be present as well [39,45]. Thus, given the sensitivity, relatively high frame rates (e.g. 5–10 frames/s), ability to image multiple fluorophores simultaneously and quantitative read-out, TIRFM has emerged as the methodology of choice for imaging events of clathrin-mediated endocytosis in live cells [38,59].

Furthermore, some studies have combined TIRFM with alternating images acquired via epifluorescence [13,26,38,39,60,61]. This powerful approach has the added benefit of permitting imaging of events at the cell surface and deeper into the cytosol. However, it should be noted that, as the epifluorescence and TIRFM images have not been acquired simultaneously, care must be taken in subsequent analysis. One area where this technique has been particularly useful is the confirmation that clathrin disappearing from the evanescent field, while still present in the epifluorescence images, has not been lost due to photobleaching or coat depolymerization before endocytosis [38,39]. However, once lost from the evanescent field, all information collected will be subject to the limitations of epifluorescence microscopy described above.

Finally, one area that is developing particularly rapidly is that of automated data analysis [39,55]. This provides the ability to identify and quantify many more events than could be evaluated manually. Thus greater statistical power can be applied to specific questions still being addressed at the level of individual events. However, it should be noted that automated data analysis carries potential caveats which must be taken very seriously. Although specific bias can be removed with regards to inclusion or exclusion...
of events on a case-by-case basis, this has now been replaced by systematic decisions which can affect all data analysed. A very interesting example of the complexity of automated data analysis came with the publication of two papers analysing similar questions, published in the same journal, less than 1 year apart [39,55]. Although it cannot be known to what extent the vast differences in conclusions stemmed from the use of different cell lines, microscopy modalities or automated data analysis paradigms, this does provide evidence that all aspects of methodology, including analysis regimes, can potentially affect experimental outcomes.

**IMAGING CLATHRIN**

Numerous observations were made in the initial report by Gaidarov et al. in 1999 [36]. These included the determination that GFP-tagged clathrin light chain incorporates into clathrin triskelia, localizes properly and does not perturb endocytosis. Furthermore, it was determined that individual clathrin spots could be seen to increase in intensity (forming coated pits), decrease to background (internalization) and move laterally in the plane of the plasma membrane (vesicle motility). Additionally, the striking observation of a population of clathrin spots which did not seem to disappear, but seemed to be sites from which nascent clathrin-coated vesicles could bud and internalize, was quite surprising. This result has been validated by numerous subsequent studies employing TIRFM (Figure 5) [44,45,61]. However, the determination that specific sites on the plasma membrane serve as hotspots for repetitive de novo coated-vesicle formation has been more elusive.

Two general paradigms exist for the formation of nascent clathrin-coated vesicles (Figure 6). The ‘iterative budding’ model suggests that flat clathrin lattices are present on the cytosolic leaflet of the plasma membrane from which coated vesicles form repeatedly. This model draws support from both classical electron microscopy studies, as well as more recent TIRFM data (Figure 5) [33,39,44,45,61–64]. Interestingly, one report from the group of Sandra Schmid clearly demonstrated that these types of separation events are spatially and temporally correlated with transient flashes of actin [61]. Thus localized increases in actin concentration seem to occur when and where the nascent vesicle separates from the static spot [61]. One tantalizing hypothesis arising from these types of observation is that cargo could be sorted from flat clathrin lattices into adjacent forming vesicles. However, direct evidence regarding this aspect of the model is lacking. It is possible that these issues will be more clearly elucidated by the application of new imaging techniques (see the Future directions section below).

Evidence for the apparent production of clathrin-coated vesicles from bare membrane initially, and subsequently, devoid of clathrin (‘de novo formation’) has also been reported [38,55,59]. Ultimately, both mechanisms for coated-vesicle production most likely occur. However, whether some cargo enters preferentially through one pathway or the other has not been adequately investigated. Finally, although it might seem logical that receptors which undergo stimulated endocytosis following ligand binding internalize through clathrin-coated vesicles formed de novo, this has not been observed. Both G-protein-coupled receptors and the epidermal growth factor receptor seem to cluster at pre-formed clathrin spots following activation ([51,65] and J. Z. Rappoport and S. M. Simon, unpublished work). Thus this would suggest that, at least in these systems, receptor activation does not induce the formation of new clathrin-coated pits.

Another area of active investigation involves studies of FRAP (fluorescence recovery after photobleaching). Led by the group of Lois Greene, a series of studies have yielded very interesting results potentially relevant to the formation and uncoating of clathrin-coated vesicles [27,30,31,66]. FRAP of clathrin puncta at the plasma membrane (with a t1/2 for recovery of ~16 s) demonstrated that the clathrin coat is not a fixed entity [30]. Instead, polymerized subunits are constantly exchanging with those that are freely diffusible in the cytosol. Furthermore, clathrin subunit exchange proceeded in the face of perturbations which can inhibit vesicle internalization (e.g. dominant-negative dynamin or cholesterol depletion), but not those shown previously to elicit global structural changes in the clathrin coat (e.g. hypertonic sucrose treatment or potassium depletion) [30].

More recently, observations from both the Greene group, as well as that of Tomas Kirchhausen, have shown that the uncoating factor GAK (cyclin G-associated kinase) and the neuronal homologue auxillin are recruited to clathrin spots immediately...
before internalization [26,67]. This was demonstrated further to occur subsequently to dynamin recruitment, suggesting that uncoating might begin immediately after vesicle fission. However, this understanding seems to be inconsistent with previous suggestions that clathrin exchange is catalysed by these same factors, in concert with Hsc70 (heat-shock cognate 70), and might function to convert flat lattices into curved coated pits and vesicles [68]. If auxillin/GAK mediates clathrin subunit exchange, but recruitment of these factors occurs only after that of dynamin, it might not fit that subunit exchange reflects the induction of curvature before vesicle fission.

Finally, FRAP of clathrin has also been observed in two studies employing TIRFM [52,69]. In the first, it was very clearly demonstrated that subunit exchange occurs more rapidly than suggested by previous studies [52]. As confocal imaging will bleach ∼1 μm of cytosol, whereas TIRFM only bleaches approx. 1/10 of this, it is not hard to imagine that the pool of free clathrin in the earlier studies was effectively depleted during the photobleaching step. Furthermore, this study demonstrated FRAP for the clathrin heavy chain, and very interestingly, that it occurred with different kinetics from that of clathrin light chain A, but not light chain B. Finally, the second study employing FRAP through TIRFM specifically focused upon the ‘static’ population of clathrin spots [69]. The results of these experiments provided further evidence that, as subunit exchange is observed, static spots do not reflect non-functional clathrin aggregates. Overall, these live-cell imaging studies have provided a more complex and potentially heterogeneous view of clathrin dynamics than envisioned previously, as well as many more exciting questions to answer in future experiments.

**DYNAMIN**

In the nearly 20 years since being identified as a mechanochemical enzyme suggested to mediate microtubule sliding, dynamin has continually shown a tendency to confound reductionist models [70]. Implicated in processes ranging from regulation of nitric oxide synthesis to degradation of extracellular matrix, dynamin is clearly a complex molecule with numerous cellular roles [71,72]. Confusing things further, dynamin exists in three isoforms, each with numerous splice variants [11,73–75].

Dynamin-1 and dynamin-2 have been shown to play roles in clathrin-mediated endocytosis [76–78]. Although dynamin-1 is expressed only in cells from neuronal lineage, dynamin-2 is found ubiquitously [11,77]. Before the application of live-cell imaging to the study of endocytosis, the groups of both Sandra Schmid and Mark McNiven employed tagged dynamin constructs to permit specific analysis of individual isoforms and splice variants [11,76]. More recently, several studies have utilized GFP-tagged dynamins in studies of clathrin-mediated endocytosis by live-cell imaging [13,26,38,55,59,67].

The first such study came out of Wolf Almers’ laboratory, and clathrin, dynamin-1 and actin were imaged in various combinations [38]. This provided, for the first time, a functional molecular time course analysing the relative recruitment of endocytic proteins in real time. This paradigm of comparing spatiotemporal kinetics has been applied in numerous subsequent studies. One observation from the 2002 study of Merrifield et al. [38] was that dynamin-1 fluorescence intensity seemed to increase rapidly just before clathrin internalization. Although such data have been interpreted as the self-assembly of dynamin at the neck of the budding vesicle immediately before fission, it has yet to be determined whether this reflects dynamin recruitment from the cytosol, or the redistribution of dynamin from the vesicle to the neck. Importantly, there is electron microscopy evidence suggesting that dynamin can indeed be found at all stages of coated-vesicle production, from flat lattice onwards [12,79].

Also, consistent with a potential role for dynamin before vesicle fission are two reports which demonstrated a more gradual increase of clathrin and dynamin-2 together before endocytosis [55,59]. However, there are also reports which have shown a rapid increase in dynamin-2 relative to clathrin just before internalization, more like the dynamin-1 paradigm [26,67]. As each study was performed in a different cell line, it might be that the behaviour of dynamin-2 depends upon the cell type in which it is expressed. Interestingly, however, the kinetics of dynamin-1 have been relatively consistent across several studies [13,26,38,59]. One important caveat is that, to date, no published study has investigated the behaviour of dynamin-1 at sites of clathrin-mediated endocytosis in cells in which it is endogenously expressed. This is of particular importance as there are dynamin-1-binding partners which share the neuronal specific distribution [28,80]. However, it should be noted that we have recently observed that, in dynamin-1-positive PC12 (phaeochromocytoma) cells, the kinetics of recruitment are identical with those reported in other cells [80a].

Similarly to the question of whether dynamin functions in clathrin-coated vesicle production before fission is the issue of when and how dynamin signal drops. The study by Merrifield et al. [38] claimed that a proportion of dynamin seemed in fact to gradually internalize along with clathrin, while the majority disappeared more rapidly. The later might reflect diffusion following the dissolution of self-assembled dynamin subsequent to GTP hydrolysis. Our recent studies in PC12 cells have demonstrated that both dynamin-1 and dynamin-2 demonstrate a biphasic drop in fluorescence: ∼30% of the signal drops away at a much higher rate than the remaining ∼70% which internalizes along with the nascent clathrin-coated vesicle [80a].

Thus it does seem as though dynamin might have a role beyond the vesicle neck. However, clearly more experiments are required to determine exactly what, if anything, dynamin does apart from mediating fission. It will be very interesting to see whether studies employing new technologies and data analysis strategies (see the Future directions section below) will be capable of determining the nature of any dynamin function before fission, as well as the origin of the rapidly brightening population of dynamin. Finally, whether dynamin truly plays a functional role in the nascent vesicle is not currently clear.

**AP-2**

The first clathrin adaptor identified, the AP-2 complex, consists of α, β2, μ2 and σ2 subunits, and is able to link clathrin to cargo [81]. Furthermore, AP-2 serves as a ‘hub’ for protein–protein interactions, essentially clustering numerous proteins at locations where clathrin-coated vesicles form [82]. AP-2 can also bind to PtdIns(4,5)P2, which is required for the proper localization of clathrin, as well as progression of endocytosis [34,35].

As AP-2 can be found within a clathrin-coated vesicle preparation, it seemed reasonable to predict that it would be internalized as an inherent part of the nascent vesicle [9,81]. However, it is possible that production of these types of preparations also results in vesicularization of the plasma membrane. Interestingly, two quantitative proteomic studies have suggested that clathrin-coated vesicles derived from non-neuronal sources contain, as a whole, much more AP-1 than AP-2 [83,84]. Thus this would suggest, among other possibilities, that either most of these vesicles derived from compartments
other than the plasma membrane, or that vesicles produced via clathrin-mediated endocytosis are depleted of AP-2. Interestingly, using an in vitro assay, removal of ~90% of AP-2 did not significantly decrease clathrin-coated vesicle formation [85]. Finally, biochemical evidence has been obtained suggesting that AP-2 can be released independently, leaving membrane-associated clathrin coats behind [86]. Although the heterotetrameric nature of AP-2 provides numerous sites where fluorescent proteins could be appended, not all GFP-fusions have appeared to be reliable markers. GFP-tagged β2-adaptin was observed to mislocalize to endosomal AP-1 complexes when overexpressed [44]. Similarly, the distribution of σ2-adaptin–GFP appeared to include partial localization to the nucleus [55]. Although mislocalization does not preclude targeting to the proper sites as well, and does not necessarily prevent acquisition of useful data, it should be avoided if possible. To this end, GFP-tagged α-adaptin constructs have been demonstrated to properly localize to clathrin-coated pits [31,87]. However, even in that case, longer expression times (e.g. 48 h) can be required to minimize gross cytosolic signals [45].

In recent years, numerous studies have been performed imaging clathrin and AP-2 in live cells [33,35,44,45,55,60,69,87]. There are currently a number of differing perspectives on whether AP-2 serves a functional role as a cargo adaptor within the nascent vesicle or whether it is mainly involved in cargo sorting at the cell surface. Our work employing TIRFM has repeatedly demonstrated that AP-2 does not seem to internalize along with clathrin, although the two do demonstrate significant colocalization in static images [33,45,69,87]. Two hypotheses were put forth in our initial report on this subject: (i) AP-2 is excluded from the forming vesicle before endocytosis; and (ii) AP-2 enters the coated vesicle, but localizes to the cytosolic pole, a sufficient distance away from the origin of the evanescent field as to preclude excitation [87]. However, as was noted, the penetration depth in this study nearly matched the diameter of a clathrin-coated vesicle.

More recently, it has been shown in two studies that AP-2 exits the evanescent field significantly before clathrin [60,69]. Furthermore, when imaged by both TIRFM and epifluorescence microscopy, AP-2 could be seen to exit the evanescent field before loss of the wide-field signal [60]. Thus it was concluded that the second hypothesis was in fact occurring. Importantly, a previous study from the same group which employed spinning-disk confocal microscopy suggested that AP-2 and clathrin disappeared simultaneously [55]. This determination seems to have arisen from the relatively deep acquisition volume of spinning-disk microscopy relative to TIRFM. Thus it would seem that combining the shallow penetration of TIRFM with conventional epifluorescence microscopy has provided a more accurate view of these events.

If the localization of AP-2 during clathrin-mediated endocytosis is becoming clearer, this cannot be said of the underlying mechanisms. Numerous questions still remain which might be addressed through the application of new technologies (see the Future directions section below). What proportions of AP-2 exit the forming vesicle, are left behind at the plasma membrane or polarize to the cytosolic side of the coated vesicle? Is this the same for all events? What about vesicles formed de novo compared with those produced via iterative budding? Finally, what about comparing neuronal and non-neuronal cells, or vesicles containing cargo which is internalized following ligand binding, relative to constitutive entry?

Finally, a recent report from the laboratory of Margaret Robinson has cast doubt on all studies employing GFP-tagged α-adaptins [88]. In this study, the authors were unable to rescue the phenotype of endogenous α-adaptin knockdown with a version that had been mutated to become ‘siRNA (small interfering RNA)-resistant’, and was linked at the C-terminus to GFP (α-GFP). These results led the authors of this study, and of several subsequent review articles, to conclude that all studies of α-adaptin dynamics might be severely flawed [88–90]. However, it must be noted that, although some studies of AP-2 FRAP and AP-2 and clathrin dynamics have employed α-GFP, all of our studies imaging clathrin and AP-2 have involved the use of α-adaptin linked at the N-terminus to GFP (GFP-α) [31,33,44,45,69,87]. This is a very important point, as there are examples of tags to one terminus of a protein perturbing function, whereas tags to the opposing end do not [91]. Finally, in order to test this directly, we have recently attempted rescue experiments with GFP-α, and can report that it does indeed complement the phenotype resulting from siRNA silencing of endogenous α-adaptin (J.Z. Rappoport and S.M. Simon, unpublished work).

**OTHER PROTEINS**

In addition to other adapters and accessory proteins, the actin cytoskeleton has also been an active area of research. The paper by Merrifield et al. [38] included data from imaging clathrin, dynamin and actin in various combinations and found that a local increase in actin signal was detected at the site of endocytosis, beginning between the rise in dynamin and decrease in clathrin [38]. Subsequent work from the Merrifield group showed that this rise in actin fluorescence was also marked by similar increases in the actin associated proteins N-WASP (neuronal Wiskott–Aldrich syndrome protein), Arp2/3 (actin-related protein 2/3) complex and cortactin [39,92]. Furthermore, results from Sandra Schmid’s laboratory extended these observations implicating actin at multiple steps during clathrin-coated vesicle formation [61].

Other clathrin adaptors [e.g. ARH (autosomal recessive hypercholesterolaemia) and Dab2] and accessory proteins (e.g. Snx9 and eps15) have also been shown to localize to sites of clathrin-mediated endocytosis in live cells [13,44,51]. These observations include the determination that Snx9 recruitment is spatially and temporally coincident with dynamin, and that Snx9 promotes dynamin function [13]. Furthermore, TIRFM imaging has been employed to demonstrate that the clathrin adaptor/accessory protein epsin enters the cell as part of the nascent vesicle [35,69].

Epsin has been shown to play a role in inducing membrane curvature, and also functions as an adaptor for ubiquitinated cargo [23,93]. Previous biochemical studies were unable to resolve whether epsin represents an integral component of the clathrin-coated vesicle, and it has been suggested that epsin might be displaced before internalization [20,94–96]. However, live-cell TIRFM was able to unambiguously demonstrate that epsin internalizes along with clathrin [35,69]. This demonstrates the importance of direct observation of the machinery implicated in particular biological processes.

**FUTURE DIRECTIONS**

Technical advances in the area of live-cell imaging are continuing to provide ever new options for conducting experiments. The application of automated data analysis to the study of clathrin-mediated endocytosis will certainly continue to permit questions to be addressed with an ever increasing degree of statistical power. The use of newly developed microscopy apparatus will hopefully provide data sets more amenable to techniques such as particle tracking. Increasing signal-to-noise ratios, and reducing exposure...
times, thus minimizing blur associated with motile structures, are both areas where advancements would be useful.

Improvements in imaging technology are already driving analysis to greater spatial, temporal and spectral degrees. So called 'super-resolution imaging' has recently been applied, albeit in fixed cells, to analyse the morphology of clathrin-coated pits at nearly one order of magnitude higher lateral resolution than can be offered by traditional light microscopy [97,98]. Other advances are being made in stride with the development or combination of advanced imaging instrumentation. TIRFM imaging of FRET (fluorescence resonance energy transfer) will certainly permit the analysis of protein–protein interactions relevant to clathrin-mediated endocytosis. This could be particularly applicable to studies of signal-transduction events following receptor activation.

Analysis of polarized emission from fluorophores illuminated via TIRFM can provide the ability to measure membrane curvature [99]. Thus this could enable observation of the progressive invagination of coated pits in living cells. Furthermore, it could be employed to unequivocally demonstrate that static clathrin spots reflect flat clathrin lattices from which curved vesicles can form and bud. When put together with recently developed methods to identify the moment of vesicle fixation, all steps in the endocytosis cycle could be independently identified in real time [39].

Although four-colour emission splitters have been marketed, alignment issues have hindered use in high-resolution studies such as imaging clathrin-mediated endocytosis. However, use of such technology could provide the simultaneous analysis of cargo, adaptor and accessory proteins. This could be employed to determine whether cargo is transferred from AP-2 to alternative adaptors as nascent coated vesicles form from flat clathrin lattices. Another potentially relevant advance would be the simultaneous acquisition of donor, acceptor and FRET emissions, if polarized imaging is employed [100]. Thus the stage is set for another wave of technologically driven advances in this area.

CONCLUSIONS AND PERSPECTIVES

Clathrin-mediated endocytosis is an important topic which represents a synthesis of biochemical, biophysical and cell biological issues. The application of live-cell fluorescence imaging has created somewhat of a revolution in this area, both permitting new types of experiments and the re-evaluation of old hypotheses. Numerous examples of seemingly inconsistent data have arisen, and these can, in some cases, be attributed to differences in imaging methodology, cell type and/or marker protein. However, the gains in our understanding of clathrin-mediated endocytosis in the last decade have been phenomenal and have been driven in large part through the power of microscopy.

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