

# Fast and ultrafast endocytosis

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## Highlights

- Reaction to stress, chemotaxis or synaptic vesicle exocytosis requires fast endocytosis.
- Clathrin-mediated endocytosis may be too slow to regulate fast physiological responses.
- Several fast clathrin-independent endocytic processes have been identified.
- Ultrafast endocytosis mediates rapid membrane removal at synapses.
- Fast-endophilin-mediated endocytosis (FEME) is triggered upon receptor activation.

## Abstract

Clathrin-mediated endocytosis (CME) is the main endocytic pathway supporting housekeeping functions in cells. However, CME may be too slow to internalize proteins from the cell surface during certain physiological processes such as reaction to stress hormones ('fight-or-flight' reaction), chemotaxis or compensatory endocytosis following exocytosis of synaptic vesicles or hormone-containing vesicles. These processes take place on a millisecond to second timescale and thus require very rapid cellular reaction to prevent overstimulation or exhaustion of the response. There are several fast endocytic processes identified so far: macropinocytosis, activity-dependent bulk endocytosis (ABDE), fast-endophilin-mediated endocytosis (FEME), kiss-and-run and ultrafast endocytosis. All are clathrin-independent and are not constitutively active but may use different molecular mechanisms to rapidly remove receptors and proteins from the cell surface. Here, we review our current understanding of fast and ultrafast endocytosis, their functions, and molecular mechanisms.

## Introduction

Endocytosis is essential for all eukaryotic cells to internalize macromolecules and proteins such as receptors, channels and transporters from plasma membrane. Endocytosis controls the levels of receptors at the cell surface and thereby regulates their signaling [1]. It also mediates synaptic vesicle recycling to support the rapid recovery of vesicle pools following synaptic transmission [2, 3]. Internalized receptors (also referred to as endocytic 'cargoes') are sorted in endosomes for recycling back to the plasma membrane to sustain signaling or for degradation in lysosomes to induce long-term desensitization of the cell [4, 5]. Many viruses and bacterial toxins exploit endocytosis to gain access into

eukaryotic cells and infect or poison cells [6, 7]. There are several pathways of endocytosis, defined by their distinct morphological features or by their requirement of key cytosolic components. Clathrin-mediated endocytosis (hereafter, CME) is the best characterized endocytic pathway and support the uptake of a wide range of cell surface proteins [1, 8]. In CME, cargo receptors are sorted by adaptor proteins that bridge them to clathrin triskelia. Clathrin then polymerizes into ‘soccer ball’ looking coats during the formation of endocytic pits [8]. All other endocytic pathways are referred to as clathrin-independent endocytosis (CIE) [1, 9]. Each CIE pathway is typically named after its morphology (coat-less invaginations emanating from the plasma membrane), cytosolic proteins markers or cargoes (such as viruses, IL2R $\beta$ , MHC class I, CD44 or Shiga toxin [10-15]). However, some CIE do not appear to have specific cargoes or markers and can only be identified by morphology (macropinocytosis).

Compared to CME, our understanding of CIE is lagged likely because i) these pathways are typically not constitutive and only activated upon specific stimuli, ii) molecular players may not be specific to the CIE pathways and iii) some CIE events might be too fast to be recorded by classical methods used to study endocytosis. This review focuses on recent advances revealing fast and ultrafast clathrin-independent endocytosis occurring upon activation of discrete receptors and at synapses.

## The need for fast endocytosis

Clathrin-mediated endocytosis is the dominant endocytic route to support housekeeping functions in cells (Figure 1a) [8, 16]. However, some physiological processes require very rapid and scalable cellular response and need to be swiftly controlled to prevent exhaustion of the response. These processes include reaction to stress hormones (‘fight-or-flight’ reaction), membrane flux during directed cell migration (chemotaxis) or compensatory endocytosis following exocytosis of synaptic vesicles or hormone-containing vesicles. Additionally, signaling arising from some receptors need to be tightly regulated to avoid overstimulation. For example, epidermal growth factor receptor (EGFR), is internalized almost exclusively via CME at low doses of EGF (<1-2 ng/mL), and the internalized receptors recycle back to the cell surface to sustain mitogen-activated protein kinases (MAPK) ERK signaling for cell proliferation [5]. At higher doses (>10-20 ng/mL), however, EGFR triggers high ERK signaling and anti-apoptosis responses, often observed during tumorigenesis, and thus the receptors must be removed rapidly from the surface and routed to lysosomes for long-term desensitization and attenuation of the response [5]. The fast response in these cases is mediated by clathrin-independent endocytosis.

During responses to high intensity stimuli, CME is likely insufficient to internalize proteins from the cell surface, requiring additional pathways to take over or complement CME (Figure 1b-c). The *de novo* formation of a clathrin-coated vesicle takes 30 to 120 s on average to form *de novo* [17, 18]. This time lag is not because of the concentration of free clathrin or adaptors available in the cytoplasm. Elevation of Pi(4,5)P<sub>2</sub> levels (lipid required for the recruitment of many CME proteins) induces the transient nucleation of up to 3 fold more clathrin-coated vesicles [19]. Instead, the kinetics of clathrin-mediated endocytosis is limited by the speed of cargo receptors selection (and exclusion of the ones that should not be internalized) and by the rate at which adaptors and clathrin triskelia are activated and recruited from the cytoplasm to the site of endocytosis. As a consequence, the majority of clathrin-coated pits abort before completion [8, 17-18]. In addition, the average density of clathrin-coated vesicles forming at the surface of fibroblasts or epithelial cells is in the order of magnitude of one new clathrin-coated pit per square micron every 2 minutes even during high stimuli [20]. Finally, the physical dimension of clathrin-coated vesicles limits the number of receptors retrieved via this pathway: typically, the number ranges between few units for large extracellular domain receptors or ligands (e.g. LDL receptor) to about 30 copies for smaller ones (M6PR or TfR) [21]. Thus, it would take over 30 minutes for CME to clear an abundant receptor (>1 million copies such as EGFR) from the cell surface. Therefore, a faster endocytic mechanism is required to remove receptors and proteins from the surface under certain physiological conditions.

In addition to retrieval of proteins from the surface, three factors at neuronal synapses warrant necessity for a faster endocytic mechanism. First, the number of synaptic vesicles is limited. For signal

transmission, vesicles containing neurotransmitters fuse with plasma membrane at synaptic terminals in response to neuronal activity [22, 23]. Typically, tens to hundreds of vesicles are used in a second [24, 25]. However, only a few hundred vesicles are available at synaptic terminals [26, 27]. Because transport of vesicle components from a cell body is too slow, synaptic vesicles must be regenerated at synaptic terminals through an endocytic mechanism [3]. Second, fusion of synaptic vesicles likely requires proper lateral tension in the plasma membrane [28]. Exocytosis of vesicles leads to the expansion of the cell surface, reducing the lateral tension of plasma membrane. Fast endocytosis is necessary to compensate for the surface expansion and restore the tension in the membrane. Third, the number of exocytic sites is limited. Synaptic vesicles fuse at the membrane domains enriched with calcium channels [29]. The number of these sites is predicated to be low at synaptic terminals [30]. Following exocytosis, vesicle proteins lodged at these sites may prevent incoming of new vesicles. Endocytosis can remove proteins from the surface, allowing rapid site clearance. Like in many other cell types, clathrin-mediated endocytosis is thought to be the predominant mechanism at synaptic terminals (Fig. 2a). However, the kinetics of CME is too slow, and thus a faster endocytic mechanism is likely required to accommodate these necessities.

### Molecular mechanisms of fast endocytosis

There are several fast endocytic processes identified so far: macropinocytosis, activity-dependent bulk endocytosis (ADBE), fast-endophilin-mediated endocytosis (FEME), kiss-and-run and ultrafast endocytosis at synapses (Figures 1 and 2). All are clathrin-independent and are not constitutively active and may use different molecular mechanisms to rapidly remove receptors from the cell surface.

*Macropinocytosis and ABE.* Strong stimulation of many signaling pathways induces indiscriminate engulfment of large portion of membrane containing activated receptors as well as nonspecific uptake of fluid and solutes (Figure 1c) [1, 31]. While macropinosomes form much more slowly than other endocytic carriers, they are huge by comparison (up to 10  $\mu\text{m}$ ) and thus internalize a much larger membrane area. This results in a ten fold increase in receptor uptake rate, protecting cells from overstimulation. A wide range of stimuli can induce macropinocytosis, including high doses of growth factors, integrin substrates, phosphatidylserine (PS)-containing apoptotic cell remnants, several viruses, and bacteria [31]. However, macropinocytosis is not selective - many other receptors present on the patch of membrane are also engulfed as 'collaterals'. The sorting of receptors takes place after internalization, either directly from macropinosomes or following fusion with early endosomes [31]. Inactive receptors are then sorted and recycled back to the plasma membrane by the retromer complex whereas activated receptors are sorted by the ESCRT machinery into multivesicular bodies (MVBs) and destined for degradation in lysosomes, resulting in long-term desensitization of cells [4].

Macropinocytosis is defined by the morphology of the process: large ruffles or blebs of plasma membrane collapsing back onto the cell surface, forming large, irregularly shaped, vacuoles (Figure 1c) [31]. Under this loose definition, several CIE pathways may be categorized into macropinocytosis despite the fundamental differences in molecular requirements (for example only some forms of macropinocytosis are dynamin dependent [1]). However, all macropinosome formation requires extensive and dynamic actin polymerization. Strong and sustained stimulation of growth factor receptors and integrins induce the robust activation of Ras, FAK and Src [31]. These activated molecules in turn induce the activity of Rac1 and Cdc42, which are at the center of N-WASP and WAVE-induced actin branching and polymerization required for membrane ruffling [31]. Several kinases and phosphatases such as PI3K, PAK1, PKC, PTEN, SHIP2 and INPP4 also play important roles by modulating  $\text{Pi}(3,4,5)\text{P}_3$  and  $\text{Pi}(3,4)\text{P}_2$  levels or by inhibiting or activating downstream proteins essential for the pathways to regulate macropinocytosis formation [31]. Some tumor cells have mutation in Ras that makes it constitutively active, bypassing the requirement of the activation through growth factor receptors. The resulting constitutive macropinocytosis in these cells mediates a high level of extracellular protein uptake, thereby supporting amino acid supply to sustain their elevated central carbon metabolism [32]. Macropinocytosis might also function to recycle periodically 'resident' channels, transporters and receptors that do not have endocytic motifs.

At synapses, high intensity stimulations trigger activity-dependent bulk endocytosis (ABDE), which is reminiscent of macropinocytosis (Fig. 2c) [33]. The fundamental difference between these two processes lies in the activation mechanism: ABDE is triggered by high loads of membrane addition through exocytosis of synaptic vesicles and elevated concentration of calcium in synaptic terminals. A rise in calcium activates the phosphatase calcineurin, which dephosphorylates and thus activates many endocytic proteins including Syndapin-I and dynamin [34]. However, as it is the case for macropinocytosis in non-neuronal cells, bulk endocytosis may also occur independently of dynamin [35], suggesting the existence multiple pathways producing large macropinosomes.

*FEME*. Recently, a novel clathrin-independent endocytic pathway has been discovered. This pathway is mediated by the endocytic protein endophilin and induce the formation of endocytic vesicles quickly (1-10 sec) upon certain stimulations (Figure 1b). Thus, it was named fast endophilin-mediated endocytosis (FEME) [36]. Unlike CME, FEME is not constitutive and is triggered upon activation of specific receptors by their cognate ligands. Receptors identified so far are G-protein coupled receptors (GPCRs -  $\beta$ 1 and  $\alpha$ 2a adrenergic, dopaminergic D3 and D4 and muscarinic acetylcholine receptor 4), interleukin-2 receptor (IL2R) and receptor tyrosine kinases (EGFR, HGFR, VEGFR, PDGFR, NGFR and IGFR) [36]. IL2R is long known to enter cells independently of clathrin [10]. In addition to these receptors, endophilin mediates rapid internalization of Shiga and Cholera toxins [37]. In the case of toxin uptake, however, endophilin likely simply accumulates on the membrane curvature, initially formed by the interaction of these toxins to lipids (Gb3 for Shiga and GM1 for Cholera) and clustering of the toxin on the extracellular face of the membrane [37]. Therefore, activated receptors and these toxins can both be internalized by endophilin, but potential mechanisms for the recruitment are not identical. To account for these differences, FEME is defined by the morphology of its endocytic carriers (tubulo-vesicular and coat-less), its transient activity, and the speed to generate endocytic carriers following receptor activation (Figure 1b).

FEME is mediated by specific molecular machineries and initiates at particular membrane domains within a cell. Endophilin binds to activated receptors as well as to key regulatory proteins such as dynamin and synaptojanin through its SH3 domain. It induces and stabilizes membrane curvature through its BAR domain and amphipatic helices, both modules being critical in deforming the membrane but also in assisting dynamin to induce scission from the cell surface [37, 38]. Unlike clathrin that uncoats soon after vesicle budding, endophilin travels with internalized FEME carriers inside cells (Figure 1b). The initiation sites of FEME within cells are not homogenously distributed on the plasma membrane. For example, they are abundant at lamellipodia of migrating cells where  $\text{Pi}(3,4)\text{P}_2$  is enriched. This particular lipid is produced from phosphorylation of plasma membrane enriched  $\text{Pi}(4,5)\text{P}_2$  by PI3K and the subsequent dephosphorylation of  $\text{Pi}(3,4,5)\text{P}_3$  by SHIP1/2 phosphatases. Lamellipodin then accumulates at the foci, recruiting endophilin (Figure 1b). Additionally, active actin polymerization controlled by Rac1 and Cdc42 is critical upstream for endophilin enrichment at lamellipodia and priming of cells for FEME [36]. The pre-enrichment of endophilin prior to stimulation allows rapid response to the stimuli, thereby accelerating the kinetics of the receptor uptake. In absence of stimulation, the foci disassemble within a few seconds. Thus, FEME mediates the rapid internalization of a subset of plasma membrane receptors following their activation. The role for such targeted receptor uptake and a more complete understanding of the molecular mechanism of FEME should be revealed in the near future.

*Ultrafast endocytosis*. Clathrin-mediated endocytosis was thought to be the predominant mechanism for recycling of synaptic vesicles. However, recent experiments using an innovative electron microscopy technique have suggested an alternative rapid pathway for vesicle recovery in *C. elegans* motor neurons [22] and mouse hippocampal neurons [23]. The flash-and-freeze approach induces synaptic transmission by stimulating neurons using optogenetics and captures the subsequent membrane dynamics by freezing neurons at defined time points [39]. After a single stimulus, vesicle membrane is recovered at the sites lateral to the fusion sites within 100 ms. This endocytic pathway does not require clathrin [22, 23, 40]. The amount of membrane internalized equals the amount of membrane exocytosed, suggesting that this

mechanism is compensatory and is likely the major pathway [22, 23]. In fact, the number of clathrin-coated pits does not increase in the synaptic terminals. Instead, clathrin acts on the endosomal membrane after fusion of the internalized membrane with an endosome [40, 41]. These data have suggested a novel mechanism for synaptic vesicle recycling (Fig. 2d).

Unlike other endocytic mechanisms, molecular mechanisms of ultrafast endocytosis are not well understood due to the very recent discovery. In the initial studies, a few key factors have been discovered. First, calcium influx itself is not sufficient to induce ultrafast endocytosis. In exocytosis-deficient synaptic terminals (*unc-13* or Munc13 mutants), ultrafast endocytosis does not initiate following calcium influx, suggesting that addition of membrane is the pre-requisite [22, 23]. Although calcium influx may enhance the rate of endocytosis [42], it does not likely trigger ultrafast endocytosis. Second, filamentous actin, F-actin, is required for initializing the membrane curvature [23]. However, it is still not known whether actin plays an active role through polymerization of actin around the endocytic site or a passive role through the maintenance of the surface tension in bending membrane. Third, dynamin function is required to pinch off the ultrafast endocytic vesicles [22, 23]. Fourth, ultrafast endocytosis takes place at the distinct membrane domains lateral to the fusion sites (typically within 200 nm). When multiple rounds of exocytosis are triggered, ultrafast endocytosis seems to remove the excess membrane from the same site [23], indicating a potential specialized lipid or protein organization at synapses. These key factors would likely help us make predictions and reveal the molecular mechanisms in more exquisite details in the near future.

Why is ultrafast endocytosis necessary at synaptic terminals? The physiological role of ultrafast endocytosis is also not well understood. The most likely explanation is that the excess membrane must be removed rapidly from the plasma membrane to restore fusion sites and keep membrane area and tension constant during a high load of membrane turnover. Consistent with this idea, synapses experience faster depression when experiments are performed under conditions that disrupt ultrafast endocytosis [43]. Furthermore, recent studies indicate that rapid site clearance is more rate-limiting than the regeneration of synaptic vesicles during sustained activity [30]. Ultrafast endocytosis likely allows rapid restoration of the surface area and thereby tension. However, it is not known whether ultrafast endocytosis mediates recovery of vesicle proteins, which is the most necessary function of endocytosis at synapses. Ultrafast endocytosis initiates within 30 ms in *C. elegans* and 50 ms in mouse hippocampal neurons. To remove proteins that are on the recently exocytosed vesicles via this endocytic pathway, those proteins must diffuse about 50-200 nm within the time periods. The diffusion of proteins on the cell surface is likely fast enough [44], but given that ultrafast endocytosis takes place at a specialized location at synapses, not all proteins will be recovered via this pathway. The major synaptic proteins, however, reside in the plasma membrane and are, perhaps, pre-sorted for rapid retrieval. Recent optical experiments seem to suggest the existence of such arrangements at synaptic terminals [45, 46]. Therefore, in addition to membrane internalization, ultrafast endocytosis likely plays its essential function - protein recovery.

What distinguishes ultrafast endocytosis from kiss-and-run and ADBE? Kiss-and-run is predicted to take place within an active zone and internalizes the same vesicles that have undergone exocytosis (Figure 2b). Ultrafast endocytosis occurs in a membrane domain just outside the active zone (Fig. 2d). The size of endocytic vesicles is larger than that of synaptic vesicles, suggesting that exocytosed and endocytosed vesicles are different. Ultrafast endocytosis may share the same molecular mechanisms as ADBE but is different from ADBE for three reasons. First, ultrafast endocytosis completes within 100 ms while ADBE requires seconds to minutes. Second, the amount of membrane internalized via ultrafast endocytosis is much more uniform (~60-80 nm in diameter). Third, internalized membrane fuse with an endosome for protein sorting following ultrafast endocytosis. In ADBE, the internalized membrane, or cisternae, serves as the sorting station. Thus, ultrafast endocytosis is likely different from ADBE. In the future, it will be interesting to find out how synapses toggle through different modes of endocytosis.

## Why was fast endocytosis missed?

Whilst macropinocytosis has been known for decades (but perhaps not recognized as a fast endocytic pathway), FEME, ABDE, and ultrafast endocytosis have been only recently identified. Why were they not discovered sooner? The first obvious reason is their speed: ultrafast endocytosis is an order of magnitude faster than typical membrane dynamics captured by conventional recording systems. In the case of FEME, the budding events are slower compared to ultrafast endocytosis but only few (10 to 50) carriers are visible at any given time in activated cells. In addition, most FEME carriers emanate from the leading edges of cells, which rapidly fluctuate upon rounds of actin polymerization and depolymerization, making it difficult to observe single endocytic events using conventional microscopy. In addition, some assays do not measure endocytosis directly. For example, pH-sensitive fluorescent probes (e.g. pHlourin) measures vesicle lumen accessibility prior to scission or vesicle reacidification. Capacitance measurement, on the other hand, can monitor bulk membrane flux at plasma membrane, but the technique is blind to the locations or natures of endocytic events. Fast endocytosis after synaptic vesicle fusion have been observed by capacitance measurements [47, 48]. These endocytic events have been attributed as either kiss-and-run or bulk endocytosis. Recent data have indicated that rapid capacitance drop is mediated by actin, endophilin and dynamin (but not clathrin) and is sensitive to the experimental temperature [49]. These are three key features of ultrafast endocytosis, suggesting that the capacitance drop likely reflects ultrafast endocytosis. These results warrant reconsideration and reinterpretation of the results in literature.

Other likely sources of oversight are details of the experimental procedures: fast and ultrafast endocytosis depend upon certain signals (specific receptor activation, mode of AP stimulation). Indeed, clathrin-independent endocytosis was found to be negligible in resting (i.e. non-stimulated) cells where CME is the dominant mode of endocytosis [16]. Importantly, details of the protocols used to measure endocytosis are critical for the mode of endocytosis. Many experiments are performed at room temperature or following a 4 °C pre-incubation to synchronize endocytosis, which reduce membrane fluidity. Yet, both ultrafast endocytosis and FEME are only observed at physiological temperature (37 °C for mammalian cells) [23, 36, 49]. Consistently, ultrafast endocytosis occurs on synaptic membranes that contain a high proportion of polyunsaturated lipids, a key determinant in membrane fluidity and endocytosis facilitation [50]. Serum starvation for several hours prior to ligand stimulation has been historically used to artificially increase the levels of receptors at the cell surface for electron microscopy studies. This protocol has been routinely used in many experiments since then. However, an artificial accumulation of receptor at the cell surface will likely change their mode of internalization and might favor their uptake by pathways that are not normally used.

## Conclusions

Fast endocytosis serves specialized and transient tasks and does not seem to have housekeeping functions in cells. It is likely that the immediate role for ABDE and ultrafast endocytosis is not to restore synaptic vesicle pools but to clear fusion site for other vesicles to come in and fuse in rapid succession. In contrast, FEME appears to be more geared toward controlling the signaling of specific receptors.

Two main mechanisms appear to explain the speed of the pathways: 1) indiscriminate rapid removal of receptors from the plasma membrane followed by sorting at endosomes (macropinocytosis, ABDE and possibly ultrafast endocytosis) or 2) pre-enrichment of endocytic proteins prior to receptor stimulation for rapid removal of activated receptor following activation (FEME). Even though the mechanism for ultrafast endocytosis is not understood yet, it is possible that it might use a combination of the two mechanisms as many endocytic proteins including endophilin are known to be enriched at synapses prior to stimulation or to be delivered there by exocytosis [51, 52]. A main convergence in their mechanisms is the exquisite requirement of actin polymerization and physiological temperature to function [36, 40], likely because of the force and membrane fluidity required for rapid endocytic carrier generation. Future studies should reveal the molecular basis and physiological functions of fast and ultrafast endocytosis and expand our understanding of receptor internalization and recycling of synaptic vesicles.

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## Figure legends

**Figure 1.** Fast endocytic mechanisms in non-neuronal cells. **(a)** Clathrin-mediated endocytosis is active ('CME: ON') and the main endocytic pathway functioning in resting, non-stimulated cells. Endocytosis by CME in resting cells support many housekeeping functions. Fast endophilin-mediated endocytosis is non active ('FEME: OFF') but endophilin is pre-enriched on specific zones of the plasma membrane. **(b)** Activation of several receptors (including  $\beta$ 1-adrenergic receptor, EGFR and IL-2R) by their cognate ligands activate FEME and induce their rapid uptake ('FEME: ON'). CME is active in such cells. **(c)** Hyper-stimulation of cells with many receptors, including EGFR, trigger macropinocytosis and the rapid uptake of large portions of the plasma membrane and indiscriminate internalization of receptors ('Macropinocytosis: ON'). CME is still active in such cells. FEME might be active if the receptors activated also stimulate it.

**Figure 2.** Current understanding of endocytic mechanisms at synapses. **(a)** Full fusion of synaptic vesicles followed by diffusion and clathrin-mediated endocytosis (CME) at distal sites. **(b)** Kiss-and-run: synaptic vesicle fusion pore opening and closing at the active zone. **(c)** Intense stimulation-mediated fusion of multiple synaptic vesicles followed by activity-dependent bulk endocytosis (ABDE) from distal sites. Synaptic vesicle may be reformed from cisternae using clathrin-dependent or -independent mechanisms. **(d)** Full fusion of synaptic vesicles followed by ultrafast endocytosis and reformation of synaptic vesicles by clathrin coats budding from endosomes.

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