Calcium-dependent signalling processes in *Chlamydomonas*

Glen Wheeler

Marine Biological Association, The Laboratory, Citadel Hill, Plymouth, PL1 2PB

glw@mba.ac.uk

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1) Introduction

All organisms must sense and respond to their environment to ensure their survival. As a motile soil-dwelling photosynthetic green alga, *Chlamydomonas* is likely to experience rapid changes in light, osmotic stress and nutrient availability and possesses diverse signalling mechanisms to help the cell respond to these stimuli. In eukaryotes, Ca^{2+} acts a versatile second messenger, amplifying and propagating intracellular signals in response to diverse environmental stimuli. Eukaryote cells maintain a very low cytosolic concentration of Ca^{2+} (100 nM), resulting in a large inward concentration gradient. The activation of Ca^{2+}-permeable channels localised to the plasma membrane or internal membranes results in rapid Ca^{2+} influx, leading to elevations in cytosolic Ca^{2+} ($[Ca^{2+}]_{cyt}$). Ca^{2+} efflux proteins such as Ca^{2+}-ATPases or Ca^{2+}/H^{+} exchangers return $[Ca^{2+}]_{cyt}$ to its resting level and therefore play an important role in shaping the Ca^{2+} transient (McAinsh and Pittman, 2009). Diverse Ca^{2+} binding proteins, such as calmodulin (CaM), the calcium dependent protein kinases (CDPKs) or the calcineurin B-like (CBL) calcium sensor proteins and their CBL-interacting protein kinases (CIPKs), respond to the changes in cytosolic Ca^{2+} by eliciting a host of downstream responses (Edel and Kudla, 2015; Zhu et al., 2015). As $[Ca^{2+}]_{cyt}$ elevations are generated by a wide variety of stimuli, specificity in Ca^{2+} signalling is conveyed by the spatial and temporal dynamics of each $[Ca^{2+}]_{cyt}$ elevation. For example, $[Ca^{2+}]_{cyt}$ elevations can take the form of brief localised spikes lasting <1 s or large whole cell $[Ca^{2+}]_{cyt}$ elevations that persist for minutes or even hours. In combination with the broad range of downstream responders, the distinct spatiotemporal dynamics of $[Ca^{2+}]_{cyt}$ elevations enable the cell to use Ca^{2+} signalling in response to many different stimuli. Ca^{2+} has been implicated in many different signalling processes in *Chlamydomonas*. Many of these relate to flagella function, reflecting the extensive use of *Chlamydomonas* as a model organism for the study of this organelle, but emerging evidence suggests that Ca^{2+} plays a central role in many other cellular processes. This review aims to highlight the different roles of Ca^{2+} within the cell and identify some of the key recent developments that are now providing insight into the underlying cellular mechanisms.

2) Ca^{2+}signalling in flagella motility

a) Ca^{2+} sensitive elements involved in flagella beat

Ca^{2+} signalling plays a central role in regulating swimming motility in *Chlamydomonas* and this aspect of signalling is the most extensively characterised in this alga. Research into flagella signalling mechanisms has had a significant wider impact, exemplified by the discovery of the novel light-gated ion channel channelrhodopsin. *Chlamydomonas* has two flagella that beat at 50-70 Hz in a breaststroke motion. This results in a helical swimming path, in which the cell moves forward at a

velocity of 100-200 μm s^{-1} whilst the cell body rotates about its longitudinal axis at 2 Hz. Swimming *Chlamydomonas* cells exhibit two very characteristic responses to light, photoshock and phototaxis. In the photoshock response, sudden exposure to high light results in a rapid but transient (duration 500 ms) switch to an undulating flagellar waveform, resulting in a brief period of backwards swimming (Fig 1). In the phototactic response, differential activation of the *cis*- (closest to eyespot) and *trans*- (furthest from eyespot) flagella enables a change in swimming direction towards or away from a directional light source.

Experiments using demembranated cell models (cells permeabilised with a non-ionic detergent) demonstrated that Ca^{2+} plays a direct role in controlling the beat frequency and waveform of the flagella. Changing Ca^{2+} in the medium from $<10^{-6}$ M to 10^{-4} M resulted in a shift from the breaststroke waveform of cell models to the undulating waveform observed during photoshock (Bessen et al., 1980; Hyams and Borisy, 1978). Smaller changes in Ca^{2+} were observed to modulate the breaststroke waveform differently between the two flagella. The *cis*-flagellum beats strongest at low Ca^{2+} ($<10^{-7}$ M) whereas the *trans*-flagellum beats strongest at higher Ca^{2+} concentrations (Kamiya and Witman, 1984). These findings indicated that phototaxis, as well as the photoshock response, are directly mediated by changes in intraflagellar Ca^{2+}. In support of this, a mutant strain was isolated (*ptx1*) which lacks the Ca^{2+}-dependent shift in flagellar dominance and shows very little phototactic ability (Horst and Witman, 1993; Okita et al., 2005).

Ca^{2+} modulates the flagella beat patterns by binding directly to a range of axonemal proteins, although the modes of regulation are likely to be complex. Of the 650 proteins that are known to comprise the flagella axoneme in *Chlamydomonas*, 27 are predicted to bind Ca^{2+} (DiPetrillo and Smith, 2013; Pazour et al., 2005). Flagella beat is driven by the axonemal dyneins, which are arranged in the inner and outer dynein arms. Outer dynein arm mutants exhibit a defective photoshock response, with little or no backwards swimming (Kamiya and Okamoto, 1985). Two Ca^{2+}-binding proteins have been characterised in the outer dynein arm, LC4 and DC3. The docking complex protein DC3 has two consensus EF hands and *oda14* mutants, which lack DC3, show defects in the photoshock response (Casey et al., 2003a). However, this phenotype is most likely due to defects in outer arm assembly in oda14 rather than Ca^{2+}-binding by DC3, as complementation of *oda14* with a modified DC3 protein that does not bind Ca^{2+} restored the assembly of the outer dynein arms and the photoshock response (Casey et al., 2003b). LC4 is also related to calmodulin and undergoes a conformational change on binding Ca^{2+}. The affinity of LC4 for Ca^{2+} and its interactions with other outer dynein arm proteins make it is the prime candidate to act as the major Ca^{2+} sensor in the outer dynein arm and mediate the waveform conversion observed during the photoshock response (Sakato et al., 2007).

Calmodulin (CaM) also plays an important role in modifying flagellar beat, by regulating dynein-driven microtubule sliding through interactions with the central pair of microtubules and the radial spokes (Smith, 2002). CaM-containing complexes associated with either the radial spokes or the central pair have been purified from *Chlamydomonas* flagella, indicating the CaM acts in multiple roles (Dymek and Smith, 2007; Patel-King et al., 2004). A further CaM-containing complex was isolated in high Ca^{2+} buffer, suggesting that changes in intraflagella Ca^{2+} cause CaM to form additional regulatory complexes (DiPetrillo and Smith, 2010, 2013).

a) Light-dependent signalling pathways

The photoshock and phototactic responses are both mediated by channelrhodopsin, a light-gated ion channel localised to the plasma membrane adjacent to the eyespot. The eyespot is composed of a series of carotenoid-rich lipid globules in the chloroplast and is positioned approximately half way along the longitudinal axis of the cell. The eyespot acts as a quarter-wave stack antenna, reflecting light onto the plasma membrane adjacent to the eyespot where the receptor (channelrhodopsin) is situated (Foster and Smyth, 1980). Electrophysiological approaches have enabled the extensive characterisation of the light-dependent ion currents in *Chlamydomonas*. A flash of light results in an initial inward current (the photocurrent, PC or *I*p) in the region of the eyespot. The photocurrent rises with virtually no delay at saturating light intensities and was initially shown to be carried largely by Ca^{2+} (Harz and Hegemann, 1991; Holland et al., 1996). However, when the driving force is sufficiently large, H^+, Na^+, and K^+ can also contribute significantly to the photocurrent (Ehlenbeck et al., 2002; Holland et al., 1996; Nonnengasser et al., 1996). The lack of a delay in the rise of the photocurrent (<500 µs) suggested that the light receptor and the ion channel were either very tightly linked or represented the same protein. The discovery of two genes (ChR1 and ChR2) encoding a novel light-gated ion channel (channelrhodospsin) demonstrated that the latter hypothesis was correct. When expressed in *Xenopus* oocytes, both ChR1 and ChR2 elicit large light-dependent H^+ conductances (Nagel et al., 2002; Nagel et al., 2003). However, unlike the voltage-gated H^+ channels that exhibit a near perfect selectivity for H^+ (DeCoursey, 2013), channelrhodopsins are much less selective for H^+ (Berthold et al., 2008; Schneider et al., 2013). As the relative external concentrations of other cations is often much greater than that of H^+, particularly at alkaline pH, the photocurrent may be carried by Ca^{2+}, Na^+ or K^+ even though channelrhodopsins exhibit a greater permeability for H^+ (Berthold et al., 2008). The discovery and subsequent optogenetic application of channelrhodopsin has revolutionised the study of neuroscience, by enabling non-invasive activation of transgenic neurons expressing channelrhodopsins.

Studies using anti-sense knockdown of channelrhodospins suggested that ChR1 and ChR2 may perform different roles within the cell, with ChR1 responsible primarily for responses to high light e.g. photoshock and ChR2 responsible for low light responses such as phototaxis (Sineshchekov

et al., 2002). However, a more recent study using gametes of *cw2*, which exhibit very little ChR2 expression, demonstrated that RNAi knockdown of ChR1 almost completely prevented the photocurrent and strongly inhibited the photoshock and the phototactic repsosnes (Berthold et al., 2008). This suggests that ChR1 alone can contribute to both of these processes. The requirement for two functional channelrhodopsins in *Chlamydomonas* therefore remains unclear.

The intensity of the light stimulus determines the amplitude of the photocurrent, which causes depolarisation of the membrane potential. If the extent of depolarisation reaches a certain threshold, voltage-gated Ca^{2+} channels in the flagella membrane are activated, leading to Ca^{2+} influx into the flagella (Harz and Hegemann, 1991). The flagella current is carried by Ca^{2+} and has two clear components; a fast transient current (I_{FF}) lasting a few ms that coincides with the switch to the undulating waveform and a slower sustained flagella current (I_{FS}) that has a much smaller amplitude and lasts around 600 ms, similar to the duration of the backwards swimming (Holland et al., 1997). The fast flagella current represents an action potential, as it is an all-or-nothing response that is triggered when a threshold depolarisation is reached. In the presence of 100 µM Ba^{2+}, the slow flagella current has a much larger amplitude and leads to a sustained and erratic photophobic response (Holland et al., 1996). This suggests that the slow flagella current is normally regulated by feedback inhibition from the intraflagellar Ca^{2+} concentration, allowing maintenance of a similar intraflagellar Ca^{2+} concentration throughout the duration of the photophobic response (Holland et al., 1996). Characterisation of a mutant, *ppr2*, which is defective in the photophobic response but not in phototaxis, revealed that this phenotype was due to a defect in *CAV2*, a homologue of the mammalian four-domain voltage-gated Ca^{2+} channels (Fujiu et al., 2009). CAV2 localises to the distal region of the flagella and appears to play a major role in the mediating the flagella Ca^{2+} influx during the photophobic response.

The electrophysiological characterisation of the photoshock response not only provides important information on the molecular mechanisms of Ca^{2+} signalling in *Chlamydomonas*, but also demonstrates an important environmental aspect of this signalling mechanism. The imperfect H^+ selectivity of ChR1 and ChR2 allows the photocurrent to be carried by other cations, such as Na^+ or Ca^{2+} at alkaline pH environments where the concentration of H^+ is much lower. The feedback inactivation of the resultant flagella current by the intraflagellar Ca^{2+} concentration ensures that the flagella response is very similar even in a wide range of external Ca^{2+} concentrations. Thus, even if the cell encounters aquatic environments with a significantly different ionic composition, which is highly likely for a freshwater/soil organism, the cell is able to generate a robust and reproducible photoshock response.

Whilst is clear that the photoshock response is mediated by light-induced depolarisation of the plasma membrane, the mechanism through which channelrhodopsin directs phototaxis is less clear.

Channelrhodopsin is clearly the sensor for phototaxis, as demonstrated by RNAi knockdown of ChR1 (Berthold et al., 2008). However, phototaxis can be observed at low light intensities where the photocurrent is only sufficient to depolarise the cellular membrane potential by 1 mV, which elicits no measurable flagella current (Hegemann and Berthold, 2009). The *ppr2* mutant, which lacks the flagella-localised voltage-gated Ca^{2+} channel CAV2, exhibits normal phototaxis (Matsuda et al., 1998). These findings suggest that phototaxis does not rely on changes in flagella membrane potential and that additional signalling mechanisms are required in order to transmit information from the sensory region (the eyespot) to the flagella.

b) Other motile responses

Chlamydomonas exhibits a mechanoshock response, where the flagella briefly switch to an undulating waveform on encountering a solid surface, causing a brief period (<1 s) of backward swimming. The swimming response during mechanoshock is similar to that of photoshock, although clearly the sensory mechanisms are different. In addition to the mechanoshock response, *Chlamydomonas* also exhibits a Ca^{2+}-dependent increase in swimming velocity following a mechanical stimulus induced by shear stress induced by vortexing a population of swimming cells (Wakabayashi et al., 2009). By applying suction to flagella using a micropippette, Yoshimura (1996) demonstrated that *Chlamydomonas* flagella are directly mechanosensitive. Suction induced a series of repetitive inward Ca^{2+} currents in flagella, that were sensitive Gd^{3+} and Ba^{2+} (Yoshimura, 1996). *Chlamydomonas* flagella possess a number of candidate mechanosensitive ion channels, most notably a range of transient receptor potential (TRP) channels, which are known to play a role in mechanosensation in animal cells (Huang et al., 2007). A study of the localisation of a range of TRP channels indicated that TRP11 was localised exclusively to the flagella with the greatest abundance at the proximal region (Fujiu et al., 2011). RNAi-mediated knockdown of *TRP11* expression resulted in a greatly reduced mechanoshock response (Fujiu et al., 2011). The mechanoshock response is absent in the *ppr2* mutant, suggesting that activation of TRP11 by mechanical stimulation leads to a depolarisation of the flagella membrane and activation of CAV2 (Fujiu et al., 2009). Mechanoshock responses have been characterised in in other motile green algae such as *Spermatozopsis*, although in this case the alga responds by a very rapid acceleration in swimming velocity (Kreimer and Witman, 1994). Patch clamp recordings using reconstituted liposomes from *Spermatozopsis* flagella membranes indicated the presence of a voltage-gated Ca^{2+} channel that most likely underlies the avoidance response (Hill et al., 2000).

Chlamydomonas also demonstrates tactic responses to stimuli other than light. Vegetative cells exhibit chemotaxis towards ammonium and sugars (Ermilova et al., 1998; Sjoblad and Frederikse, 1981) and gametes also exhibit chemotactic responses to peptone and tryptone

(Govorunova and Sineshchekov, 2003, 2005). Chemotaxis towards ammonium is dependent on external Ca^{2+} and chemotactic responses towards sugars can be blocked by Ca^{2+} channel blockers (Ermilova et al., 1998; Sjoblad and Frederikse, 1981). There is some evidence for integration between the chemotaxis and phototaxis signalling pathways, as the addition of tryptone to gametes temporarily inhibits the channelrhodopsin-mediated photocurrent (Govorunova and Sineshchekov, 2003). *Chlamydomonas* also demonstrates negative gravitaxis, i.e. a tendency to swim upwards, in the absence of light and other stimuli. Gravitaxis was found to be normal in the *ptx1* mutant and was unaffected by the removal of Ca^{2+}, suggesting that gravitaxis does not involve Ca^{2+}-dependent signalling mechanisms (Kam et al., 1999; Yoshimura et al., 2003).

3) Other Ca^{2+} signalling processes related to flagella function

a) Mating

Signal transduction during fertilisation has been extensively explored in *Chlamydomonas*. The signalling pathway is initiated by contact between agglutinins on the flagella of gametes of opposite mating types. This results in a signalling cascade that leads to activation of an adenylyl cyclase and a rise of cyclic AMP (cAMP) (Wang and Snell, 2003). The accumulation of cAMP results in shedding of the cell walls and activation of mating structures, followed by cell fusion and formation of the quadriflagellate cell. The elevation of cAMP is central to this signalling pathway, as mating responses can be induced in gametes by the application of cell-permeable cAMP analogue, dibutryl cAMP, in the presence of the phosphodiesterase inhibitor IBMX (Brownlee, 1994; Pan and Snell, 2000). The role of Ca^{2+} signalling during mating is less clear. Inhibition of Ca^{2+} signalling with CaM antagonists (trifluoperazine and W-7) or Ca^{2+} channel blockers (e.g. La^{3+} or Cd^{2+}) had no effect on flagella agglutination but prevented the rise in cAMP and inhibited mating (Goodenough et al., 1993; Pasquale and Goodenough, 1987). Mating could be rescued by the addition db-cAMP + IBMX, suggesting that Ca^{2+} acts upstream of the activation of adenylyl cyclase. Mating efficiency is reduced but not completely inhibited in the absence of external Ca^{2+}, suggesting that a Ca^{2+} influx across the plasma membrane and/or the flagella membrane contributes to the signalling pathway (Goodenough et al., 1993). In combination, these results suggest that flagella agglutination may trigger a flagella Ca^{2+} elevation, which could contribute to the activation of adenylyl cyclase and the rise in cAMP. Support for this hypothesis came from the characterisation of PKD2 in *Chlamydomonas*, a homologue of a mammalian Ca^{2+}-permeable TRP channel. PKD2 is highly expressed in the flagella of gametes and RNAi-mediated knockdown of PKD2 resulted in an inhibition of mating (Huang et al., 2007). The mating defect could be rescued by the addition of db-cAMP and papaverine (a phosphodiesterase inhibitor), suggesting that PKD2 acts upstream of the adenylyl cyclase and may therefore contribute to flagellar Ca^{2+} signalling following flagella adhesion (Huang et al., 2007).

b) Gliding

In addition to swimming, *Chlamydomonas* flagella can also support an alternative form of cellular motility. The flagella membrane of *Chlamydomonas* contains an abundant glycoprotein (FMG-1B), which enables the flagella to adhere to a solid substrate (Bloodgood, 1981; Bloodgood and Workman, 1984). The regulated movement of FMG-1B in the flagella membrane allows the adherent flagella pull the cell body forwards and results in the two flagella becoming orientated at 180º to each other. The movement of FMG-1B in the flagella membrane can also be observed using microspheres (Bloodgood, 1995; Shih et al., 2013). Microspheres move along the flagellum at a rate similar to gliding motility but differs in that the beads move back and forth, whereas gliding flagella only move forwards (Bloodgood, 1981). Adhesion of FMG-1B to a solid substrate, such as a microsphere or a glass surface during gliding motility, results in cross-linking of the flagellar glycoprotein. Bead movement and gliding motility require the presence of micromolar free Ca^{2+} in the medium, suggesting that the movement of FMG-1B in the flagella membrane is directly regulated by Ca^{2+} (Bloodgood, 1995; Bloodgood and Salomonsky, 1990).

As the flagella of gliding cells are arranged at 180º and pull the cell body in opposite directions, there is clearly a need to coordinate the motive force generated by each flagellum. Direct imaging of intraflagella Ca^{2+} ($[Ca^{2+}]_{fla}$) indicated that no $[Ca^{2+}]_{fla}$ elevations were observed in the leading flagellum during gliding motility but a $[Ca^{2+}]_{fla}$ elevation was consistently observed in in the trailing flagellum at the onset of dragging motion (Fig 2) (Collingridge et al., 2013). This was puzzling as it had been previously assumed that Ca^{2+} signalling was linked to the initiation of force generation by the microtubule motor proteins in the leading flagellum (Bloodgood, 2009). However, this discrepancy was resolved by the discovery that the retrograde IFT motor cytoplasmic dynein provides the motive force for gliding motility (Collingridge et al., 2013; Shih et al., 2013). Direct observation of fluorescently-tagged IFT particles during gliding motility demonstrated that retrograde IFT particles accumulate in the leading flagellum and are able to drive the flagellum forward, presumably through a direct interaction with FMG-1B (Collingridge et al., 2013; Shih et al., 2013). In the trailing flagellum, the $[Ca^{2+}]_{fla}$ elevations caused by the dragging motion disrupt the accumulated retrograde IFT particles, which are returned to the cell body by retrograde IFT (Collingridge et al., 2013). This suggests that the interaction between the retrograde IFT particle and FMG-1B is sensitive to Ca^{2+}. The absence of external Ca^{2+} inhibits $[Ca^{2+}]_{fla}$ elevations and leads to a massive accumulation of IFT particles in the regions of the flagellum that are in contact with the solid surface (Collingridge et al., 2013). In contrast, Shih et al (2013) demonstrated that IFT trains pause less frequently in the absence of external Ca^{2+} and proposed that Ca^{2+} was required to initiate the interaction between the

IFT particle and FMG-1B. However, this proposal seems at odds with the direct observations of $[Ca^{2+}]_{fla}$ and IFT.

The ability to directly observe $[Ca^{2+}]_{fla}$ in *Chlamydomonas* has highlighted some important properties relating to these signalling pathways. Firstly, $[Ca^{2+}]_{fla}$ elevations are very rapid (often <1 s) and they occur simultaneously along the length of the flagellum, suggesting that changes in membrane potential and activation of voltage-gated Ca^{2+} channels are likely to be involved in their generation. Secondly, the two flagella are able to act as independent Ca^{2+} signalling entities, which is essential for the coordination of gliding motility. Thirdly, $[Ca^{2+}]_{fla}$ elevations are directly linked to the movement of microtubule motor proteins, which has interesting parallels with other cellular processes, such as the Ca^{2+}-dependent movement of mitochondria along microtubules (Saotome et al., 2008). Although the molecular identity of the Ca^{2+}-channels involved in the flagellar signalling pathway have not yet been confirmed, it is likely that the dragging motion of the trailing flagellum is sensed initially by a mechanosensitive ion channel, which leads to membrane depolarisation and activation of voltage-gated Ca^{2+} channels along the length of the flagellum (Collingridge et al., 2013). Further characterisation of the signal transduction pathway regulating gliding motility is likely to yield important broader insights into the nature of ciliary signalling.

c) Deflagellation

The process of flagellar excision is another flagella-related signalling pathway that has been extensively characterised in *Chlamydomonas*. Cells exposed to various stressors including chemicals (such as dibucaine and mastoparan), pH shock, elevated temperature and mechanical shear rapidly shed both flagella by precise severing of the nine outer doublet microtubules at the distal end of the flagella transition zone (Quarmby, 2004). This process has been much exploited by *Chlamydomonas* researchers in order to obtain large quantities of flagella for biochemical characterisation of their component proteins. It is presumed that deflagellation contributes to survival in unfavourable conditions, e.g. by reducing the surface area of exposed cellular membranes, although there is little experimental evidence to support this. The microtubule severing activity is directly dependent on Ca^{2+}, as axonemal severing can be induced in purified axonemes/basal bodies exposed to 1 μM Ca^{2+} (Lohret et al., 1998). How exactly Ca^{2+} triggers the severing process is not yet clear. The Ca^{2+}-binding protein centrin may contribute to flagella excision through contraction of the stellate fibers in the distal flagella transition zone. However, the centrin mutant *vfl2* deflagellates normally suggesting that the activity of centrin alone does not induce microtubule severing (Quarmby, 2004). Katanin is a likely candidate for the microtubule severing protein, although katanin likely plays further roles in the cell and conclusive evidence supporting a role for katanin in deflagellation is lacking (Lohret et al., 1998; Rasi et al., 2009).

Treatment of *Chlamydomonas* cells with organic acids, such as benzoate or acetate, results in cytosolic acidification, which rapidly leads to deflagellation (Braun and Hegemann, 1999; Quarmby, 1996). Quarmby and Hartzell (1994) demonstrated that acid-induced deflagellation is completely dependent on a Ca^{2+} influx across the plasma membrane and does not occur in the absence of external Ca^{2+}. Cytosolic acidification due to organic acids also causes activation of phospholipase C and an accumulation of inositol triphosphate (IP_3), which may play a role in the deflagellation signalling pathway (Quarmby et al., 1992). Mastoparan, a G-protein agonist that stimulates intracellular Ca^{2+} release through elevation of IP_3, deflagellates cells in the absence of external Ca^{2+}, suggesting that deflagellation can also result from the release Ca^{2+} from internal stores (Quarmby and Hartzell, 1994). However, the evidence for the role of IP_3 in the signalling pathway remains inconclusive (Quarmby, 2004).

Direct imaging of cytosolic Ca^{2+} demonstrates that organic acids cause a rapid and sustained elevation in $[Ca^{2+}]_{cyt}$ throughout the cytosol, which coincides with the timing of flagellar excision (Braun and Hegemann, 1999; Wheeler et al., 2008). The *adf1* mutant, which is defective in acid-induced deflagellation but deflagellates normally in response to other stimuli, does not exhibit a Ca^{2+} influx in response to acid or demonstrate the $[Ca^{2+}]_{cyt}$ elevation (Finst et al., 2000; Quarmby and Hartzell, 1994; Wheeler et al., 2008). The absence of a Ca^{2+} influx led to proposals that *ADF1* encodes a H^+-stimulated Ca^{2+} channel localised to the apical region of the cell, although the molecular identify of the gene is not yet known (Quarmby and Hartzell, 1994). Closer examination of the *adf1* mutant revealed that a small percentage of cells do deflagellate during benzoate addition and in each of these instances a localised transient $[Ca^{2+}]_{cyt}$ elevation was observed in the apical region of the cell coinciding with deflagellation (Wheeler et al., 2008). Assuming Adf1 is a Ca^{2+}-permeable ion channel, its reduced activity in the *adf1-1* mutant may result in greatly reduced Ca^{2+} influx in the apical region, leading to a transient localised $[Ca^{2+}]_{cyt}$ elevation that is sufficient to cause flagellar excision but does not trigger further Ca^{2+} release from intracellular stores. This indicates that deflagellation can be induced solely by a localised $[Ca^{2+}]_{cyt}$ elevation in the region around the basal body and does not require the whole cell $[Ca^{2+}]_{cyt}$ elevation normally observed in wild type cells. It is likely that additional inputs are required in the signalling pathway before Ca^{2+} can act to trigger flagellar excision. Rapid addition of 20 mM external Ca^{2+} to *Chlamydomonas* causes a series of repetitive $[Ca^{2+}]_{cyt}$ elevations and leads to deflagellation (Fig 3) (Wheeler et al., 2008). However, whilst each deflagellation event was observed to coincide with a $[Ca^{2+}]_{cyt}$ elevation, many cells exhibited multiple $[Ca^{2+}]_{cyt}$ elevations prior to flagellar excision indicating that $[Ca^{2+}]_{cyt}$ elevations alone do not induce deflagellation in an intact cell. One additional component of the deflagellation signalling pathway is the Aurora protein kinase CALK, which has a role in the regulating flagellar length and assembly. CALK is phosphorylated during acid-induced deflagellation and RNAi-mediated knockdown of CALK led to defects in deflagellation (Pan et al., 2004), although how

CALK interacts with the Ca^{2+} signalling pathway is not known. As $[Ca^{2+}]_{cyt}$ elevations are linked to many different stress responses, understanding their interactions with other signalling components is critical in determining the specificity of Ca^{2+} signalling.

d) The role of Ca^{2+} in flagella length

In addition to its central role in the process of flagellar excision, Ca^{2+} also contributes to the regulation of gene expression during flagellar regrowth (Cheshire and Keller, 1991). Acid-induced deflagellation is followed by the rapid accumulation of transcripts for many flagella-related genes and the increase is dependent on external Ca^{2+} (Evans 1997). The induction of gene expression is also greatly reduced in the presence of inhibitors of Ca^{2+} channels and in the *adf1* mutant, although these also exhibit decreased deflagellation (Evans and Keller, 1997). However, deflagellation of *Chlamydomonas* cells by mechanical shear in the absence of Ca^{2+} prevents the upregulation of flagella-related gene transcripts, indicating that Ca^{2+} entry is required for the transcriptional response (Cheshire et al., 1994; Cheshire and Keller, 1991).

There is also a direct requirement for Ca^{2+} in flagella regrowth and length control. In low external Ca^{2+} (<0.15 µM), deflagellated cells do not regrow flagella at all until Ca^{2+} is restored to c. >0.5 µM (Cheshire and Keller, 1991; Liang and Pan, 2013). Ca^{2+} also appears to be required for the maintenance of flagellar length, as cells placed in very low Ca^{2+} media containing 25 mM Na^{+} or K^{+} will resorb their flagella (Lefebvre et al., 1978). *Chlamydomonas pf18* (paralysed flagella) cells have longer flagella when grown in elevated Ca^{2+} (2 mM), but they also produce longer flagella in the presence of Ca^{2+} channel blockers (La^{3+} and Cd^{2+}), which makes this result difficult to interpret (Tuxhorn et al., 1998). However, mammalian IMCD (immortalised kidney collecting duct) cells treated with 30 µM Gd^{3+} (a non-specific blocker of Ca^{2+} channels) also show elongated cilia, suggesting that the effect of lanthanides on ciliary length may be conserved in eukaryotes (Besschetnova et al., 2010). Whilst it is not clear exactly how intraflagellar Ca^{2+} acts to regulate flagella length in *Chlamydomonas*, significant recent progress has been made in identifying a requirement for Ca^{2+} sensor kinases. *Chlamydomonas* contains an expanded family of 14 Ca^{2+} dependent protein kinases (CDPK), which possess an N-terminal serine/threonine kinase and 4 Ca^{2+}-binding EF-hands at the C-terminus (Edel and Kudla, 2015). Three CDPKs (CDPK1, CDPK3 and CDPK11) were identified in the flagellar proteome, suggesting a probable role in flagella function (Liang and Pan, 2013; Pazour et al., 2005). RNAi knockdown strains for CDPK3 showed slow regrowth of flagella at low concentrations of external Ca^{2+}, but flagella length, motility and mating were unaffected (Liang and Pan, 2013). In contrast, RNAi knockdown of CDPK1 led to significantly reduced flagella length and defects in IFT (Liang et al., 2014). CDPK1 acts to phosphorylate the FLA8 subunit of the microtubule motor protein kinesin-II, which disrupts the interaction between

kinesin and the IFT-B subunit of the IFT particle. The phosphorylation of FLA8 by CDPK1 appears to block entry of kinesin into the flagella, but also promotes dissociation of kinesin from the IFT-B particle at the flagellar tip. As a consequence, CDPK1 RNAi strains accumulate FLA8 and components of the IFT particle at the flagella tip (Liang et al., 2014). These findings indicate that Ca^{2+} sensor kinases play an important role in flagellar length assembly and length control. As Ca^{2+} plays a direct role in the regulation of IFT during gliding motility (Collingridge et al., 2013), it will be interesting to determine whether the directed movement of IFT particles also contributes to flagellar length control.

4) Cytosolic Ca^{2+} signalling during stress responses

Compared to the wealth of evidence examining the diverse roles of Ca^{2+} in flagella function, there is relatively little information on the nature of the Ca^{2+}-dependent signalling processes in the cell body. However, there has been some important recent progress, most notably in examining the link between Ca^{2+} and photosynthesis. In land plants, Ca^{2+} signalling plays a central role in the response to many different environmental stimuli, including osmotic stress, oxidative stress, temperature and biotic stimuli, and the dynamics of the $[Ca^{2+}]_{cyt}$ elevations associated with each stimulus have been characterised in detail (Kiegle et al., 2000; Knight et al., 1997; Takahashi et al., 1997). In contrast, there have been very few studies involving direct imaging of $[Ca^{2+}]_{cyt}$ in green algae and our understanding of the nature of $[Ca^{2+}]_{cyt}$ elevations in *Chlamydomonas* remains in its infancy (Bauer et al., 1997; Braun and Hegemann, 1999; Thompson et al., 2007). A recent study demonstrated that different osmotic stimuli give rise to distinct $[Ca^{2+}]_{cyt}$ elevations in *Chlamydomonas*, although $[Ca^{2+}]_{cyt}$ elevations were not observed in response to oxidative stress from hydrogen peroxide (Bickerton et al., in press). Hyperosmotic shock through the addition of NaCl resulted in a single $[Ca^{2+}]_{cyt}$ elevation that originated in the apex of the cell and rapidly propagated to rest of the cytoplasm, resembling the well-characterised Ca^{2+} waves observed in animal cells. The timing and amplitude of the $[Ca^{2+}]_{cyt}$ elevation was dependent on the intensity of the stimulus. In contrast, hypoosmotic shocks induced by very dilute media or deionised water resulted in a series of repetitive $[Ca^{2+}]_{cyt}$ elevations (Bickerton et al., in press). Hypoosmotic shock also induced repetitive $[Ca^{2+}]_{fla}$ elevations, but these did not coincide with the $[Ca^{2+}]_{cyt}$ elevations, suggesting that the flagella and the cytosol can respond independently to this stimulus (Bickerton et al., in press). The requirement for Ca^{2+} in the osmotic signalling responses in *Chlamydomonas* that appears to be conserved with land plants, although the spatial and temporal nature of the resultant $[Ca^{2+}]_{cyt}$ elevations is distinct.

The $[Ca^{2+}]_{cyt}$ elevations observed in response to NaCl-stress and hypo-osmotic shock were both severely inhibited in the absence of external Ca^{2+}, suggesting an important role for Ca^{2+} entry across the plasma membrane in the initiation of the signalling response. However, it is likely that

these $[Ca^{2+}]_{cyt}$ elevations involve further release of Ca^{2+} from intracellular stores following the initial Ca^{2+} entry (e.g. during propagation of the Ca^{2+} wave). A major internal store of Ca^{2+} in *Chlamydomonas* is likely to be the acidocalciosomes, which play a direct role in Ca^{2+} signalling in a range of other protists (Docampo and Huang, 2016; Ruiz et al., 2001). Acidocalciosomes are numerous small low pH vesicles that are rich in polyphosphate, Ca^{2+} and a range of other metal ions. Recent evidence in *Chlamydomonas* suggests that the acidocalciosomes play an important role in cell survival during nutrient deficiency (sulphate, nitrate, phosphate) and are also used for the storage of metals such as zinc and copper (Aksoy et al., 2014; Hong-Hermesdorf et al., 2014). In trypanosomes and apicomplexan parasites, Ca^{2+} entry into acidocalciosomes is mediated by the action of Ca^{2+}-ATPases, although it is likely that Ca^{2+}/H^{+} antiporters also contribute to Ca^{2+} uptake (Docampo and Huang, 2016). *Chlamydomonas* possesses three CAX Ca^{2+}/H^{+} antiporters (Emery et al., 2012), although it is not known whether any of these localise to the acidocalciosomes. Characterisation of *Chlamydomonas* CAX1 identified some unusual properties, as CAX1 exhibits Na^{+}/H^{+} exchange activity in addition to Ca^{2+}/H^{+} exchange (Pittman et al., 2009). CAX1 may therefore contribute to Na^{+} efflux during salt stress, in addition to its likely role in Ca^{2+} homeostasis. However, CAX1 expression was repressed by high concentrations of Ca^{2+} or Na^{+}, suggesting that it is unlikely to function primarily in the sequestration of excess Na^{+} or Ca^{2+} (Pittman et al., 2009).

5) Ca^{2+} signalling and photosynthesis

One emerging area of significant interest in *Chlamydomonas* is the role of Ca^{2+} in regulating photosynthesis. An early report suggested that Ca^{2+} and CaM played a role in regulating the biosynthesis of chlorophyll by contributing to the transcriptional regulation of glutamate-1-semialdehyde aminomutase by blue-light (Im et al., 1996). More recently, a direct link to Ca^{2+}-dependent regulation of photosynthesis has emerged through studies of the Ca^{2+}-sensor protein, CAS. CAS was initially identified in land plants as a sensor of external Ca^{2+}, but was subsequently shown to localise to the thylakoid membranes and play a major role in the modulation of $[Ca^{2+}]_{cyt}$ elevations in stomatal guard cells (Nomura et al., 2008; Weinl et al., 2008). CAS also localises to the thylakoid membranes in *Chlamydomonas* and plays an important role in photoprotection through its role in the accumulation of LHCSR3 in high light (Petroutsos et al., 2011). LHCSR3 is a light harvesting protein that is required for the dissipation of excess light energy through non-photochemical quenching (NPQ). CAS RNAi knockdown strains exhibit low levels of LHCSR3 and defects in NPQ, although both of these phenotypes can be rescued by the addition of 3 mM Ca^{2+} in the external media (Petroutsos et al., 2011). LHCSR3 is encoded by two genes *LHCSR3.1* and *LHCSR3.2* that produce an identical amino acid product and both genes appear to be transcriptionally regulated by Ca^{2+} (Maruyama et al., 2014). Under anoxic conditions, CAS RNAi knockdown strains show a strong

inhibition of cyclic electron flow (CEF) in photosystem I, which in wild-type cells is required for the generation of the pH gradient across the thylakoid membrane during NPQ (Terashima et al., 2012). CAS was shown to form a complex with a novel protein ANR1 and with PGR5-Like 1 (PGRL1), a protein that is required for normal CEF in land plants (Terashima et al., 2012). Recently, a novel class of Ca^{2+}-sensor proteins has also been described in *Chlamydomonas*. Calredoxin (CRX) possesses a unique combination of four Ca^{2+}-binding EF-hands at the N-terminus and a C-terminal thioredoxin domain and was localised to the chloroplast stroma (Hochmal et al., 2016). CRX interacts with a 2-cys peroxidredoxin in a Ca^{2+}-dependent manner in order to drive the detoxification of hydrogen peroxide. CRX mutant and RNAi knockdown strains are not defective in the induction of *LHCSR3* expression under high light, but exhibit increased CEF (Hochmal et al., 2016). Together, these findings illustrate that *Chlamydomonas* is a very useful model to study the Ca^{2+}-dependent regulation of photosynthesis and its associated photoprotective mechanisms.

6) Evolution of the Ca^{2+} signalling toolkit

The sequencing of the *Chlamydomonas* genome and further advances in algal genomics have provided important insight into the evolution of the Ca^{2+} signalling in the green algae. Land plant genomes lack clear homologues of several classes of ion channel that are central to Ca^{2+} signalling in animal cells, including the four-domain voltage-dependent Ca^{2+} channels (VDCC), transient receptor potential (TRP) channels and the inositol triphosphate receptor (IP_3R) (Edel and Kudla, 2015; Verret et al., 2010; Wheeler and Brownlee, 2008). Instead, land plants possess an extended family of cyclic-nucleotide gated channels and ionotropic glutamate receptors (Swarbreck et al., 2013). In contrast, green algae possess multiple homologues of the well-characterised 'animal-like' Ca^{2+} channels that are absent from land plants and experimental evidence indicates that members of the VDCC and TRP classes of ion channel play important roles in signal transduction in *Chlamydomonas* (Arias-Darraz et al., 2015; Fujiu et al., 2011; Fujiu et al., 2009; Huang et al., 2007; Merchant et al., 2007; Wheeler and Brownlee, 2008). The broad distribution of these channels in the green algae and other eukaryote lineages suggests that they have been specifically lost in the land plants. Identification of their functional roles in *Chlamydomonas* and other green algae will be important in understanding the evolutionary processes leading to the loss of these Ca^{2+} channels in the land plants.

The mechanisms through which Ca^{2+} signals are generated in green algae are therefore very different from those found in land plants. Significant differences in the mechanisms that sense $Ca^{2+}$are also found between these lineages. Land plants have an expanded family of CaMs and CaM-like proteins (CML), with a total of 58 proteins present in *Arabidopsis*. Whilst CaM is highly conserved amongst eukaryotes, CML is absent from animals and this family has become greatly expanded in the land plants. *Chlamydomonas* possesses a CaM homologue and 8 CMLs belonging to 1c and III (Zhu

et al., 2015). This complement is similar to the charophyte *Klebsormidium* suggesting that the major diversification of the CMLs into multiple sub-groups occurred after this lineage diverged from the land plants (Zhu et al., 2015). Land plants also possess an expanded repertoire of Ca^{2+} sensor kinases. For example, *Arabidopsis* has 34 CDPKs, 26 CIPKs and 10 CBLs (Edel and Kudla, 2015). Whilst *Chlamydomonas* has 15 CDPKs, the CIPKs and their binding partner the CBLs appear to be absent, along with the CCaMKs. CIPKs/CBLs are not specific to the land plants though, as a single CIPK and a CBL are present in *Ostreococcus* (Edel and Kudla, 2015). This suggests that the CIPKs may have been lost in an ancestor of *Chlamydomonas*, although greater taxonomic sampling of other green algal lineages is required to resolve this. One of the *Chlamydomonas* CDPKs has unique domain architecture, possessing a C2 domain at the N-terminus (Hamel et al., 2014). C2 domains are often involved in Ca^{2+}-dependent membrane interactions and this unique C2-CDPK was only found in other members of the Volvocales, suggesting that a single gene fusion event gave rise to this novel protein (Hamel et al., 2014).

7) Technical considerations for the study of Ca^{2+} signalling in *Chlamydomonas*

The studies listed above illustrate just how many diverse processes are regulated by Ca^{2+} in *Chlamydomonas*. Whilst significant progress has now been made in characterising these signalling pathways, a fuller understanding of the role of Ca^{2+} in these signalling pathways has been hampered by the technical issues associated with Ca^{2+} imaging in *Chlamydomonas*. As with many plant and algal cells, loading of Ca^{2+}-responsive fluorescent dyes via membrane-permeant acetoxymethyl (AM) esters is problematic, due to unequal loading and dye compartmentalisation (Braun and Hegemann, 1999). For example, the AM-ester of the Ca^{2+}-responsive dye Fluo-4 was found to localise to the acidocalciosomes in *Chlamydomonas* (Hong-Hermesdorf et al., 2014). Because of these problems, the use of ester-loaded dyes for the examination of $[Ca^{2+}]_{cyt}$ dynamics, particularly over longer timescales or in fluorimetric approaches (rather than direct single cell imaging), is not recommended. Dextran-conjugated dyes are not subject to compartmentalisation as they are membrane impermeable, but they are require specialised techniques in order to load them into intact cells. Microinjection is clearly not appropriate for small algal cells, but a biolistic delivery method for dextran-conjugated dyes into *Chlamydomonas* has been developed that provides robust and reproducible cytoplasmic dye loading (Bothwell et al., 2006; Wheeler and Brownlee, 2008). Although this technique has proven to be reliable, there are clearly some disadvantages associated with this delivery method, such as the equipment and technical expertise required and the low proportion of cells that are loaded. Therefore, the future development of genetically encoded calcium indicators in *Chlamydomonas* is of top priority in order to advance this field. GECIs not only allow for simple routine imaging of $[Ca^{2+}]_{cyt}$, they may also be targeted to specific cellular locations (e.g. the mitochondria or the chloroplast) to enable the

study of localised or intra-organellar $[Ca^{2+}]_{cyt}$ elvations. It is important to note that GECIs will be an important additional tool to be used alongside Ca^{2+}-responsive fluorescent dyes in *Chlamydomonas*, as the properties (e.g. Ca^{2+} dissociation kinetics, pH sensitivity) of certain dyes may make them a preferred choice for certain applications, such as the observation of the very rapid flagella Ca^{2+} elevations (Chen et al., 2013; Hendel et al., 2008). Whilst the development of further robust protocols for Ca^{2+} imaging are likely to be of greatest benefit in this field, the ability to perform electrophysiological studies in *Chlamydomonas* should not be overlooked. Much of our knowledge on flagella signalling in *Chlamydomonas* originates from these approaches and their application led to the discovery of channelrhodopsin.

8) Concluding remarks

The broad scope of this review demonstrates the wide reaching influence of Ca^{2+}-dependent signalling processes on the cell biology of *Chlamydomonas*. Significant progress has now been made in identifying the molecular mechanisms and physiological processes associated with diverse signalling pathways. However, there is clearly much to be learnt and our knowledge of Ca^{2+} signalling in *Chlamydomonas* remains limited. In particular, whilst individual components of some signalling pathways have now been identified, e.g. the ion channels, the Ca^{2+} elevations or the Ca^{2+} sensors, there are very few examples where all of these aspects have been characterised and compiled into working knowledge of the whole pathway. To some extent, this is partially true for the land plants, where the molecular identification of the specific Ca^{2+} channels responsible for well-characterised ion currents and Ca^{2+} elevations has proven to be problematic (Swarbreck et al., 2013). The reduced complexity of the Ca^{2+} signalling toolkit in *Chlamydomonas* may allow for simpler resolution of these issues. *Chlamydomonas* has already proven to be an important model for the study of signalling processes relating to flagella and photosynthesis. Studies in *Chlamydomonas* have also contributed important information on the evolution of Ca^{2+} signalling. Together these studies have revealed many surprising and novel aspects of the Ca^{2+}-dependent signalling pathways in *Chlamydomonas* and there are likely to be many more exciting discoveries in the future.

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Figure Legends:

Figure 1: Cellular mechanisms underlying the phobic motile responses

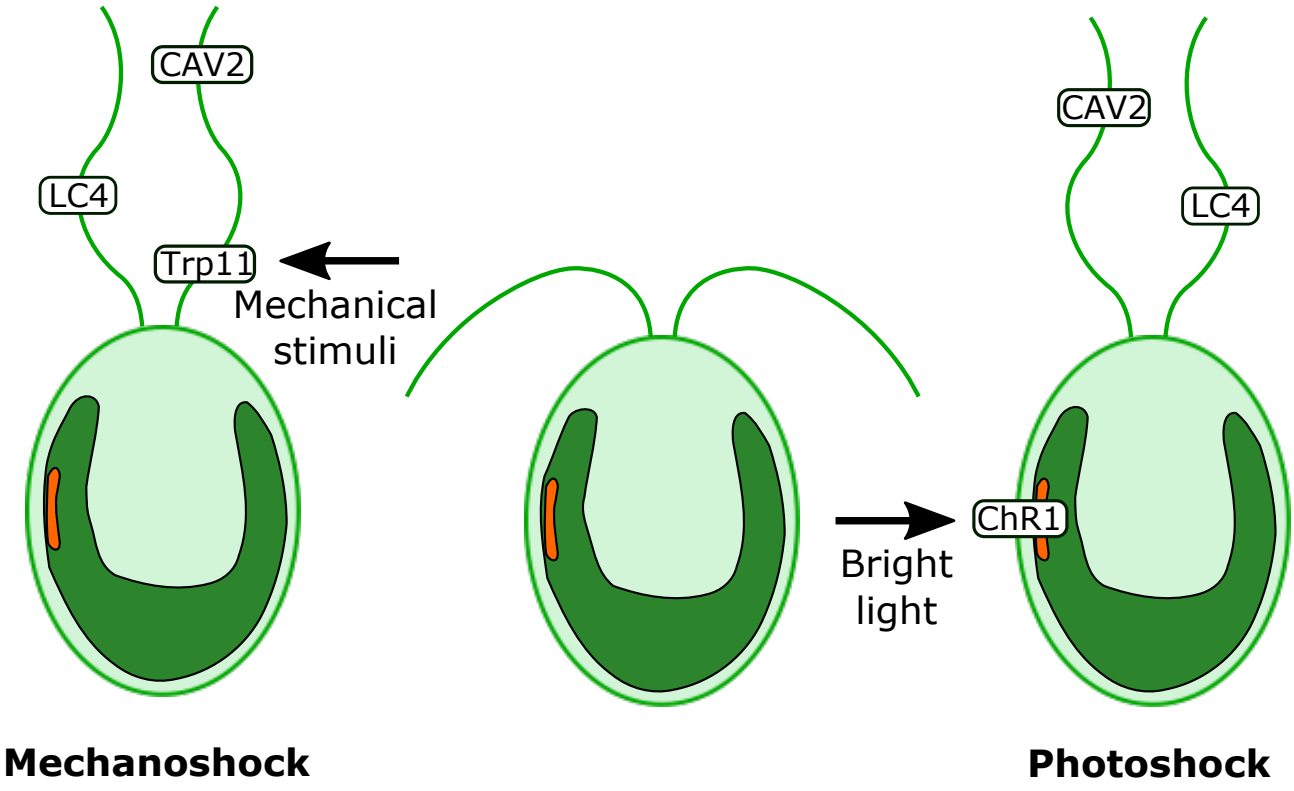
The scheme illustrates the major proteins identified in phobic swimming responses of *Chlamydomonas* cells. In the mechanoshock response, the flagella-localised mechanosensitive ion channel TRP11 is proposed to activate the voltage-gated Ca^{2+} channel CAV2. In the photoshock response, bright light activates channelrhodospin (ChR1), leading to a whole cell depolarisation and activation of CAV2 in the flagella membrane. In both responses, the resultant intraflagellar Ca^{2+} elevations are likely sensed by LC4 in the outer dynein arm, which causes the switch to the undulating waveform and the onset of backwards swimming.

Figure 2: Flagella Ca^{2+} signalling during gliding

Chlamydomonas cells adhere to solid substrates via their flagella and glide along the surface due to the movement of adherent flagella membrane glycoproteins. (i) Retrograde IFT particles accumulate in adherent flagella and provide the motive force to pull the cell body forwards. (ii) As the leading flagellum begins to move forwards, a intraflagellar Ca^{2+} elevation occurs in the trailing flagellum only, which disrupts the interaction between the IFT particles and the flagella membrane in the trailing flagellum. (iii) This prevents a futile tug-of-war between the two flagella and allows the leading flagellum to pull the cell body forwards.

Figure 3: Imaging $[Ca^{2+}]_{cyt}$ elevations in *Chlamydomonas*

A) Confocal microscopy images of a *Chlamydomonas* cell loaded with the Ca2+-responsive dye Oregon Green BAPTA dextran. The dextran-conjugated dye demonstrates diffuse cytoplasmic loading, with no compartmentalisation. Chlorophyll autofluorescence is also shown. Bar = 10 µm. B) An example of the $[Ca^{2+}]_{cyt}$ elevations induced by adding 20 mM external Ca^{2+} to a *Chlamydomonas* cell. The upper images show changes in Oregon Green BAPTA fluorescence viewed by epifluorescent microscopy. Images are generated by dividing each image by a rolling median image (n=20 images) and are pseudo-coloured to illustrate relative changes in fluorescence. The lower graph shows the corresponding changes in the fluorescence ratio of Oregon Green BAPTA (OG) and a non-responsive dye, Texas Red (TR) for the same cell. Perfusion of the cell with 20 mM external Ca^{2+} for 30 s results in a series of $[Ca^{2+}]_{cyt}$ elevations that persist after the cell is returned to the initial external Ca^{2+} (300 µM). Bar = 10 µm.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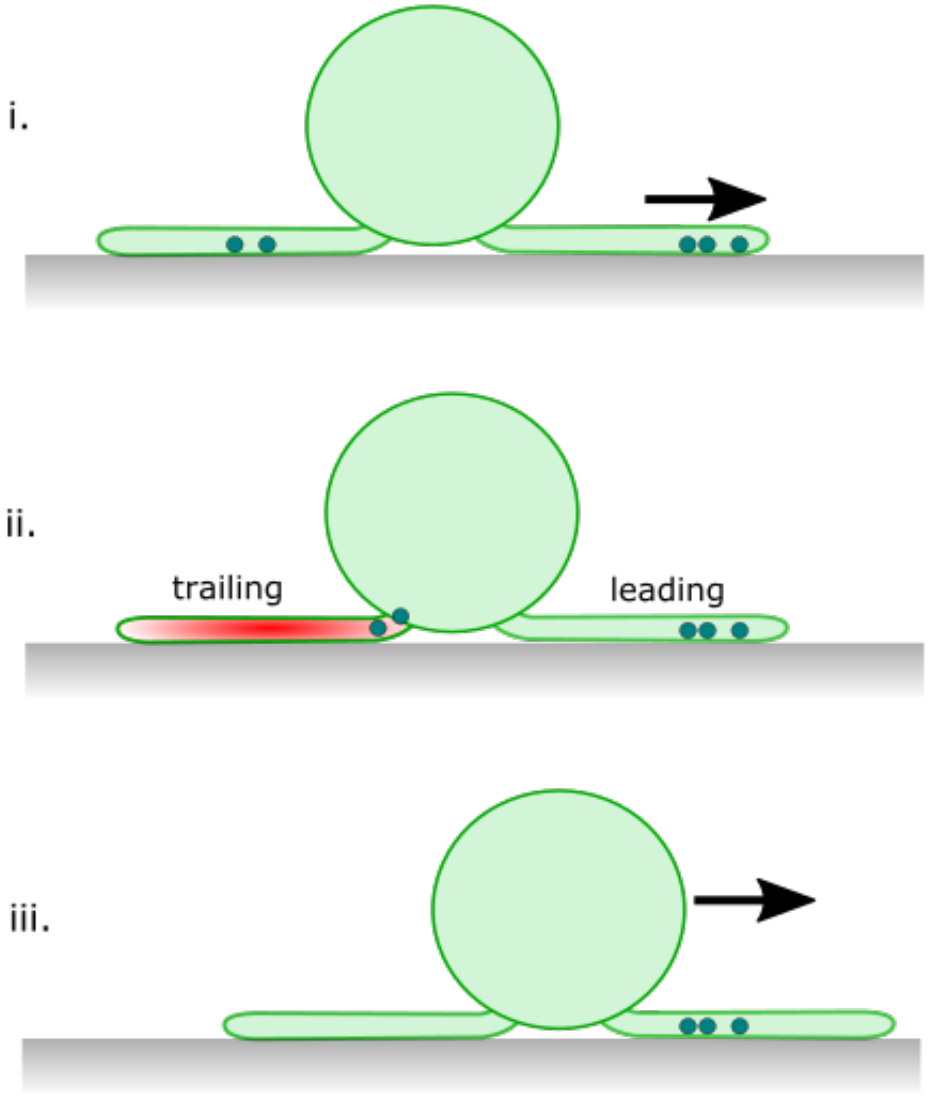
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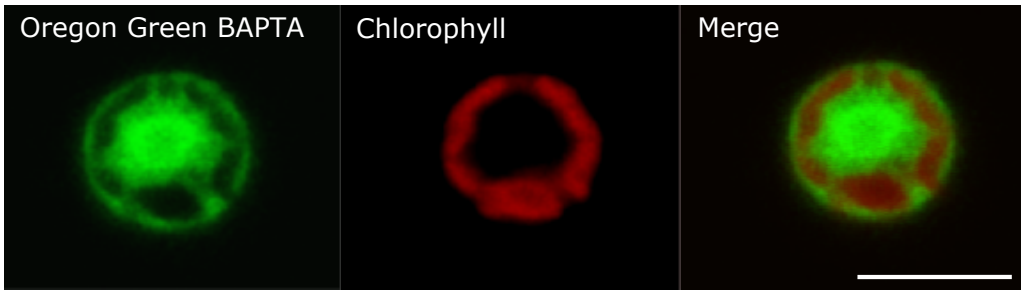
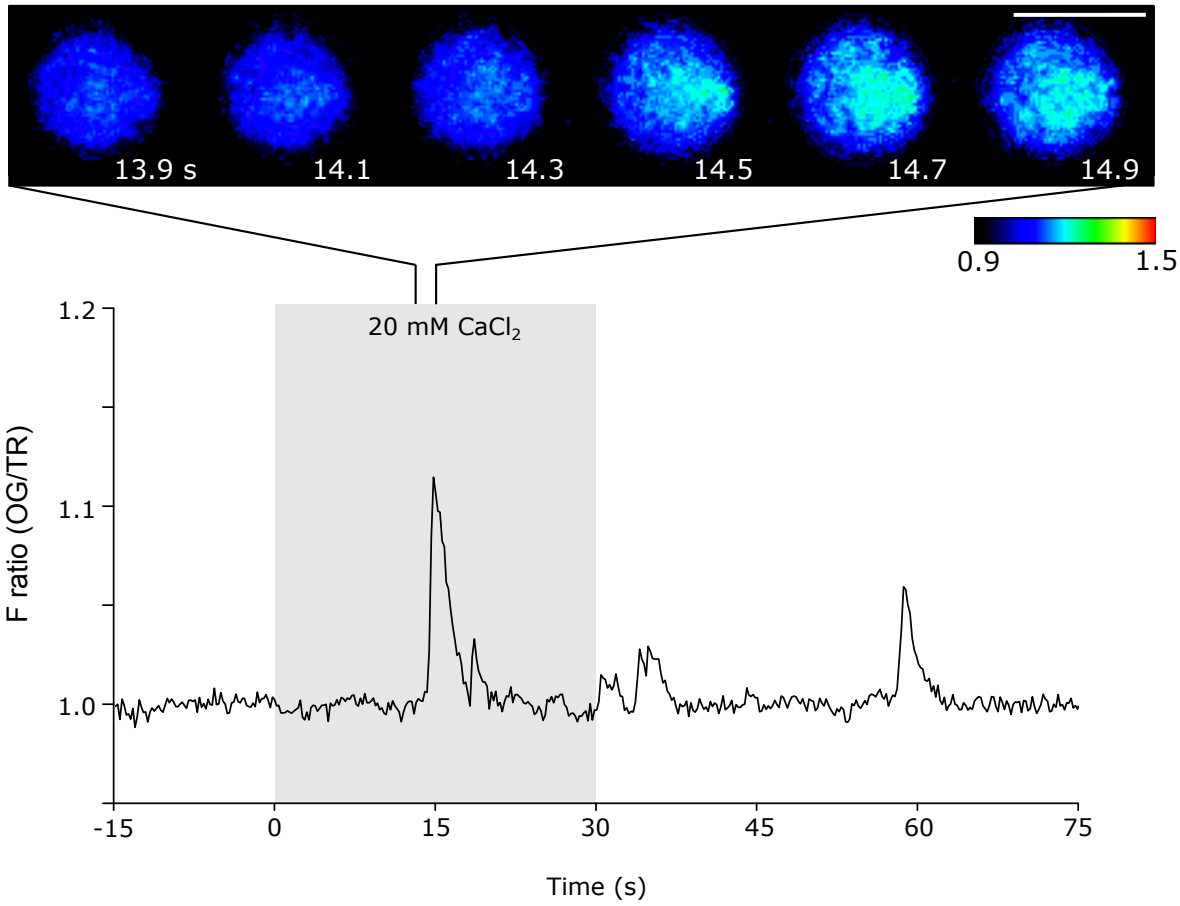

Oregon Green BAPTA
Chlorophyll
Merge

B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0
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60
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Time (s)