Running Title: Peroxisome-chloroplast tethering

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Title: *In vivo* quantification of peroxisome tethering to chloroplasts in tobacco epidermal cells using optical tweezers

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One sentence summary: Optical tweezers shows that peroxisomes are strongly tethered to chloroplasts, and more weakly to other unknown structures.

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Abstract

Peroxisomes are highly motile organelles that display a range of motions within a short time frame. In static snapshots they can be juxtaposed to chloroplasts which has led to the hypothesis that they are physically interacting. Here, using optical tweezers we have tested the dynamic physical interaction *in vivo*. Using near-infrared optical tweezers, combined with TIRF microscopy, we were able to trap peroxisomes and approximate the forces involved in chloroplast association *in vivo*, and observed weaker tethering to additional unknown structures within the cell. We show that chloroplasts and peroxisomes are physically tethered through peroxules, a poorly described structure in plant cells. We suggest peroxules have a novel role in maintaining peroxisome-organelle interactions in the dynamic environment. This could be important for fatty acid mobilisation and photorespiration through interaction with oil bodies and chloroplasts, highlighting a fundamentally important role for organelle interactions for essential biochemistry and physiological processes.

Introduction

A combination of genetically encoded fluorescent probes, advances in light microscopy and interdisciplinary approaches have revolutionised our understanding of organelle transport. Organelle movement in highly vacuolated leaf epidermal cells appears erratic with individual organelles undergoing a range of movements within a relatively short time frame; stop-go, change direction (trajectory) and can move at varying speeds. Use of pharmacological inhibitors indicated a role for actin, and therefore myosins in this process, however myosin-organelle specificity is poorly characterised (Buchnik et al., 2015; Madison and Nebenführ 2013; Tamura et al.,2013). We are therefore still at a relatively rudimentary stage in the understanding of the molecular and physical control, and interaction of, organelles in plant cells compared with that known in other model systems (Prinz 2014; Hammer and Sellers 2012). However, it is clear that organelle movement plays important roles in physiological processes in plants; reduced movement effects growth and development, and movement is correlated with responses to extracellular stresses such as pathogens and heavy metals (see references in Madison and Nebenführ 2013, Buchnik et al., 2015 and Sparkes 2011). Organelle interactions in other systems have

important roles in calcium and lipid exchange setting a precedent for physiologically important roles in plants (Prinz, 2014). However, characterisation of the molecular factors required to physically tether organelles as opposed to those which function in the exchange of molecules at the interaction site is challenging. Monitoring organelle interactions in highly vacuolated plant epidermal cells are further complicated by the constraints imposed by the large central vacuole. Static snapshots provided through electron microscopy of highly vacuolated cells, where the vacuole can effectively 'push' organelles together giving the impression of direct interaction between organelles, is not a suitable method to determine dynamic interactions. Other techniques such as the laser induced shockwave by explosion method used by Oikawa et al., (2015) works globally without directly manipulating the individual organelle. Here, using optical tweezers with sub-micron precision, we provide a means to assess and quantify the dynamic interaction between peroxisomes and chloroplasts *in vivo* in leaf epidermal cells.

Peroxisomes are responsible for several biochemical reactions including the glyoxylate cycle and β-oxidation which provides an energy source for germination in oilseeds. They also produce and scavenge free radicals, synthesise jasmonic acid (JA), IAA and are required for photorespiration (see references in Hu et al. 2012). The photorespiratory pathway spans peroxisomes, chloroplasts and mitochondria where phosphoglycolate produced in the chloroplast is converted back to 3-P-glycerate. It has been suggested that functional connectivity between these organelles accounts for the close association observed in ultrastructural micrographs (Fredericks and Newcomb, 1969). Several Arabidopsis *pex10* (peroxisomal membrane protein) mutants show altered chloroplast-peroxisome juxtaposition with a defect in photorespiration while others do not (Schumann et al., 2007; Prestele et al., 2010). Both Clumped Chloroplasts 1 (CLMP1) and Chloroplast Unusual Positioning 1 (CHUP1) encode for proteins that localise to the chloroplast, with CHUP1 playing a role in cp-actin formation (Yang et al., 2011; Oikawa et al., 2003, 2008; Schmidt von Braun & Schleiff 2008). Whilst CHUP1 and CLMP1 affect chloroplast positioning, they have differential effects on peroxisome and mitochondrial location; *clmp1* causes chloroplast clustering without affecting mitochondria or peroxisome location (Yang et

al., 2011), whereas *chup1* was reported to affect peroxisome location (Oikawa et al., 2003). *In vitro* analysis through density centrifugation highlighted chloroplast sedimentation with peroxisomes under certain conditions (Schnarrenberger and Burkhard, 1977), although this does not necessarily reflect organelle interaction in live cells. Peroxisome proteomics studies have been hampered by difficulties in isolating pure peroxisomal fractions (Bussell et al., 2013). This could be indicative of interaction, where associated membranes are isolated together, or 'sticky' non-specific contaminating chloroplast membranes. The work by Oikawa *et al.* (2015) provides insight into the physiological processes controlling peroxisome-chloroplast interaction (photosynthetic dependent), but they did not determine the effective baseline force required to move peroxisomes that were not next to chloroplasts under control or altered environmental conditions. Comparisons between the relative forces required to move peroxisomes next to chloroplasts versus those which are not next to chloroplasts are critical in understanding and probing the physical interaction between the two organelles; hypothesis being that tethering would increase the force required to move peroxisomes compared to organelles which are not tethered. Since peroxisomes have diverse biochemical roles that affect a wide range of physiological processes throughout the plant life cycle (Hu et al. 2012), then an understanding of if and how peroxisomes may interact with other subcellular structures is likely to be an important consideration for efficient peroxisome function.

Peroxisomes are highly pleomorphic, dynamic organelles bounded by a single membrane (Hu et al., 2012), whose movement is driven by acto-myosin dependent processes (Jedd and Chua, 2002; Mano et al., 2002; Mathur et al., 2002; Avisar et al., 2008; Sparkes et al., 2008). Tubular emanations termed peroxules (Scott et al., 2007) can extend from the main peroxisome body, yet it is unclear what function they may play. Formation is quite frequent in hypocotyl cells (Sinclair et al., 2009; Mano et al., 2002; Cutler et al., 2000), can occur around chloroplasts in cotyledonary leaf pavement cells (Sinclair et al., 2009), and do not always form from the trailing edge of the peroxisome (Sinclair et al., 2009). Exogenous addition of hydroxyl ROS, or exposure to UV light, induces peroxule formation (Sinclair et al., 2009). It has been

suggested that they represent increased surface area for increased biochemical function, or might represent a morphological precursor for peroxisome division (Jedd and Chua 2002). Based on subcellular co-alignment, a retro-flow model for potential exchange of luminal content between the ER and peroxisome through the peroxule has been suggested (Sinclair et al., 2009; Barton et al., 2013). However, these studies, as with many others, interpret the close association between organelles to indicate physical connectivity between organelles, whereas, in fact, in highly vacuolated leaf epidermal cells organelles can be closely packed within the cytoplasm due to mere spatial constrictions generated through the large central vacuole. This is further complicated by the highly motile, and seemingly stochastic nature of acto-myosin driven organelle movement resulting in frequent apparent organelle 'collisions' which may not reflect a functional requirement for organelle interaction.

Optical trapping provides a highly specific and sensitive means to measure physical connectivity between organelles. By focussing an infrared beam, it allows the user to trap objects which have a significantly different refractive index to the surrounding media. Upon trapping, the user can then move the trapped object relative to its original position to gain an understanding of whether the movement affects the position and motion of other structures (such as other organelles) which may be physically attached to the trapped organelle. For example, unlike the ER, Golgi bodies are amenable to trapping. By trapping and micromanipulating (i.e. precisely moving) the Golgi, a physical association between the ER and Golgi was determined in a qualitative manner (Sparkes *et al.* 2009b). Here, we have developed a system to generate quantitative measures for organelle interaction by standardising and automating how far we move the trapped organelle (which we call the translation step) at a defined speed, and assessing how trapping efficiency alters in response to the power of the laser trap itself. By using these parameters we can then model the forces imparted on the organelle providing further insight into the tethering processes. Our results indicate that peroxisomes are amenable to being trapped, that they physically interact with chloroplasts in leaf epidermal cells, and surprisingly that peroxisomes are also tethered to other unknown structures within the cell. This approach therefore highlights that organelle interactions within plant cells are not random but regulated through tethering. In addition we provide a novel role for

peroxules, and a simple biophysical model to describe peroxisome motion during the trapping process.

Results

Peroxisome association with chloroplasts is specific

For organelles to interact they must move and physically 'sit' or reside next to one another in a coordinated manner. To determine how peroxisomes move relative to chloroplasts we observed both peroxisomes, chloroplasts and Golgi bodies within the same tobacco leaf epidermal cell and assessed how long either peroxisomes or Golgi resided next to chloroplasts. As organelles are physically constrained by the large central vacuole and can be 'pushed' together, Golgi were monitored as they are not functionally related to chloroplasts and so act as an inherent control. We observed that the average residency time of peroxisomes was significantly higher than that of Golgi bodies on chloroplasts; 1.46 $^{+}$/$_{-}$ 0.35 minutes (n=17) and 0.42 $^{+}$/$_{-}$ 0.05 minutes (n=51) respectively t-test $p<0.001$ (Supp movie 1). Due to these observations, and functional connectivity through the photorespiratory pathway, we investigated whether peroxisomes physically interact with chloroplasts *in vivo*.

Peroxisomes are associated with chloroplasts in an actin independent manner

In a motile system it is difficult to discriminate between physical tethering processes between two organelles from acto-myosin driven events. We therefore assessed whether interaction characteristics were actin dependent in the first instance. Note, the concentration of latrunculin b used is sufficient to depolymerise actin and cause cessation of organelle movement (Sparkes et al., 2009a; Sparkes et al., 2008).

The average percentage of chloroplasts with a juxtaposed peroxisome in the presence or absence of actin (latrunculin b treated) were not significantly different from one another; 22 $^{+}$/$_{-}$ 5% and 23 $^{+}$/$_{-}$ 3% respectively, t-test $p>0.8$, data taken from 20 images covering 0.4mm^2 leaf epidermal area. Using optical tweezers we then tested whether these results indicated a peroxisome-chloroplast tethering mechanism in both motile and non-motile (latrunculin b treated) samples. By trapping and subsequently moving the peroxisome within the cell (Fig 1A-D) we observed that upon turning the trap off the peroxisome recoiled back towards its place of origin irrespective of chloroplast presence (Supp Movie 2B,C,D). This process has not been previously observed using other techniques. On several occasions peroxules were observed from the trailing

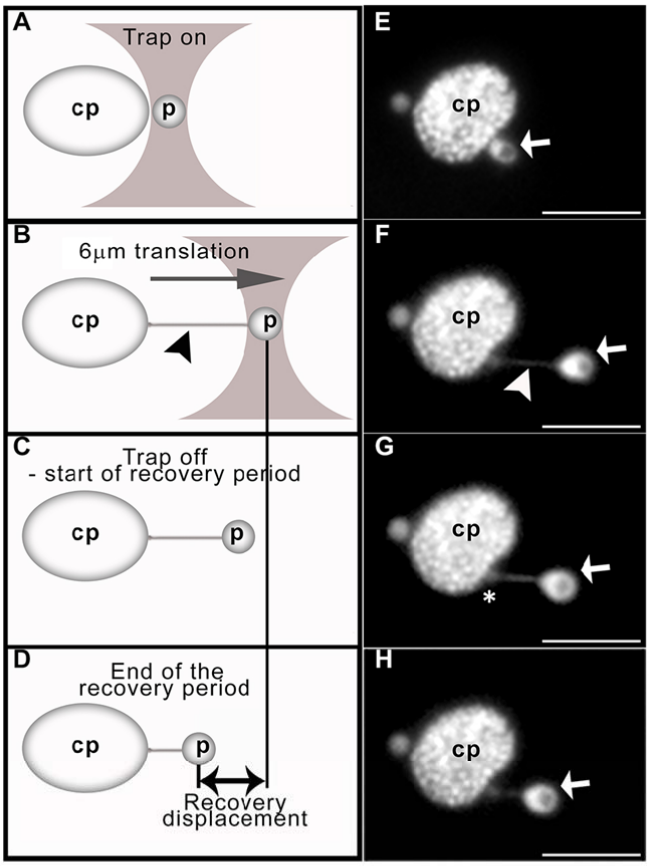

Figure 1. Optical trapping and movement of peroxisomes away from chloroplasts in tobacco leaf epidermal cells.

Schematic representation of the trapping procedure (A-D) and the corresponding micrographs (E-H) are shown. Upon turning the trap on (A,E) and moving the stage 6μm at a set speed (B,F; referred to as translation period) the trapped peroxisome (p, white arrow) is pulled away from the chloroplast (cp) and a peroxule (arrowhead) is formed. Upon turning the trap off (C,G) the peroxisome recoils backs towards its original position next to the chloroplast (D,H). Peroxisome displacement during the recovery period (referred to as recovery displacement) is measured (double arrowhead). Asterisk denotes the tip of the peroxule. Scale bar 6μm.

214 edge of the peroxisome (Supp Movie 2B,D). Upon actin depolymerisation, trapped
215 peroxisomes displayed similar characteristics; peroxule formation and peroxisome
216 recoil upon turning the trap off (Fig 1E-H, Supp movie 3A,B). These results indicated

that peroxisomes are tethered to chloroplasts and unknown structures in the cell, and that peroxules may represent the site of tethering.

To test the hypothesis that peroxisomes are tethered to chloroplasts we set about quantifying whether the average laser power required to trap and move peroxisomes was dependent on chloroplast positioning and / or actin. The rationale here is that trapping efficiency and movement are dependent on optical trap strength where tethering, which acts as an opposing force, would impede the movement of the trapped organelle causing it to escape the trap. Trapping refers to an organelle which can be trapped and remains in the trap over the 6μm translation distance (Fig 1). Of the fifty organelles from independent cells that underwent the trapping routine (which constituted 5 samples of 10 organelles) there was a clear trend that increasing optical laser power (from 24 to 50mW) resulted in an increase in the number of trapped peroxisomes (20-38% increase) irrespective of actin or chloroplast association. However, peroxisomes which were next to chloroplasts were harder to trap. Significantly fewer chloroplast associated (cp) peroxisomes were trapped when compared to non chloroplast (non-cp) associated peroxisomes under either motile or immotile (latrunculin b treated) conditions at a given laser power; 50mW optical trapping laser power resulted in average trapping of 38 $^{+}/_{-}$ 2% cp and 56 $^{+}/_{-}$ 7% non-cp in the motile system, and 36 $^{+}/_{-}$ 4% cp and 70 $^{+}/_{-}$ 4% non-cp in the immotile system, t-test $p<0.05$ comparing cp to non-cp under a given condition. These results indicate that peroxisomes are tethered to chloroplasts and that this phenomenon is independent of actin. The trapping efficiency of non-cp peroxisomes in the motile system compared to the immotile system was significantly reduced (t-test $p<0.15$) and could be due to a number of reasons; trapped peroxisome being knocked out of the trap by passing organelles, docking the peroxisome onto actin filaments during the translation or moving a trapped organelle into a cytoplasmic stream (Supp movie 2).

Peroxisome tethering can also be quantified by monitoring the recoil of the peroxisome back towards its origin after turning the trap off (Fig 1, termed recovery displacement). However, inherent difficulties of organelles escaping the trap and responding to the acto-myosin driven elements after turning the trap off reduced the number of organelles that could be assessed in this manner; for cp motile, and cp /

non-cp non motile system (latrunculin b treated) between 66-84% were measurable compared with 21% for non-cp motile system. Observations of the small number of organelles which showed recoil back towards the trap origin (recovery displacement, Fig 1), rather than movement in an opposite direction in the motile system, indicated that recoil was significantly larger for cp compared to non-cp in both motile and non-motile conditions: for the motile system cp recovery displacement 3.92 $^{+}/_{-}$ 0.36μm (n=16) compared with non-cp recovery displacement 2.50 $^{+}/_{-}$ 0.29 μm (n=6) $p<0.007$; for the non-motile system cp recovery displacement 3.65 $^{+}/_{-}$ 0.45μm (n=14) compared with non-cp recovery displacement 1.22 $^{+}/_{-}$ 0.20μm (n=23) $p<0.001$. All data were taken using 50mW trapping laser power with a 5.3 second recovery period.

Quantifying the peroxisome-chloroplast tethering process: a novel role for peroxules

The above observations clearly indicate that peroxisomes are tethered to chloroplasts, and that this phenomenon is independent of actin. To further characterise the effects of tethering, the opposing forces generated by the acto-myosin component were removed from the system (latrunculin b treatment). Here, we assessed (1) the relationship between peroxisome behaviour in the trap and trapping laser power over a larger range of laser powers, and (2) behaviour of displaced peroxisomes after turning the trap off (Fig.1,2). All of these observations were carried out under latrunculin b treatment so that any interactions are due to tethering and not the acto-myosin system.

Peroxisomes were either trapped, not trapped or escaped the trap during translation (Fig 2c; movie S3C-E). As expected, the trapping laser power correlated with the observed percentage trapping for both cp and non-cp peroxisomes (Fig.2A, B). However, at laser powers of 37 mW and above there was a significant difference between the trapping of cp and non-cp peroxisomes, with cp peroxisomes escaping the trap more readily and non-cp peroxisomes being trapped and remaining in the trap over the 6μm translation (Fig 2, Supp Fig 1). Taken together this is indicative of more force being required to trap and move peroxisomes away from chloroplasts. Additionally, upon turning the trap off, cp trapped peroxisomes underwent a

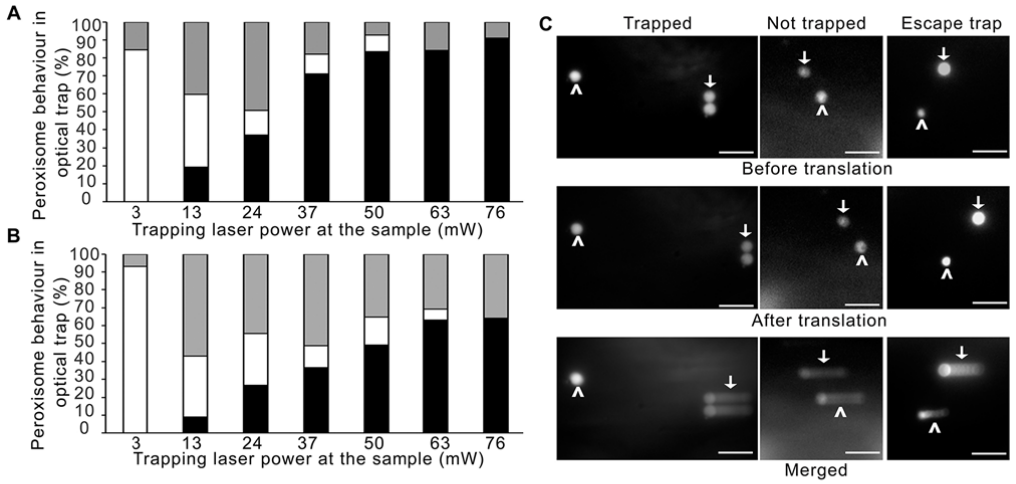

Figure 2. Higher optical trapping laser power is required to trap and move peroxisomes away from chloroplasts.

Non-cp (A) and cp (B) associated peroxisomes underwent the optical trapping protocol using various trapping laser powers and their trapping characteristics were scored; remained in the trap over the 6µm translation (black bar A,B), unable to be trapped (white bar A,B) or escaped the trap during the translation (grey bar A,B). Percentages displayed are based on weighted means from a set of independent experiments. Supp Figure 1 compares cp with non-cp for all three trapping categories and indicates significant differences between peroxisomes that are trapped or escape from the trap for cp versus non-cp. Relationship between optical laser trap power and peroxule formation are given in Supp table 1.Stills from Supp movie 3C-E representing before and after translation events for peroxisomes which are trapped, not trapped or escape the trap during the translation event are displayed (C, arrowhead). Note, peroxisomes not subjected to trapping in the same cell are shown for comparison (arrow). The translation event is based on movement of the stage and not the trap. Composite image of frames captured during the translation event show that the trapped peroxisome does not appear to move whereas organelles that escape the trap or are not trapped result in comet like tails (merged panels). Scale bar 6µm.

significantly larger recovery displacement (i.e. recoil, Fig 1D) than non-cp trapped organelles: cp recovery displacement $4.39^{+}/.0.17$µm (n=94), non-cp recovery displacement $2.93^{+}/.0.17$µm (n=91) using a laser power of 37mW, which has a t test probability value of $p<0.001$. This proves that peroxisomes are tethered to chloroplasts *in vivo* in tobacco leaf epidermal cells. The above data were generated under a long recovery period (21.5 seconds rather than 5.3 seconds) to allow organelles to reach their equilibrium position, which improves the accuracy of the force determination discussed later.

Peroxule formation can occur upon exposure to the trapping laser prior to and during the translation, however the frequency of formation is independent of the power of the optical trapping laser indicating that formation is not solely due to exposure to the trapping laser (Supp. table 1). Interestingly, both cp (38% n=170) and non-cp (37%

n=183) peroxisomes had a similar propensity to form peroxules, but the relative percentages between formation in response to exposure to optical trap versus translation differed (Supp table 1). It is unclear why more peroxules would form in the absence of chloroplast positioning prior to translation (2.9% compared with 12%), but we speculate that formation may occur in response to stress which is ameliorated by the antioxidant properties of the chloroplast (Sinclair et al., 2009; Asada 2006), or non-cp peroxisomes are tethered to structures whose positioning alters in response to trapping the peroxisome.

During peroxisome translation peroxule formation is more frequent in cp versus non-cp associated peroxisomes (28.2% compared with 15.3%, table S1), correlative with peroxules being the visible manifestation of the tether to chloroplasts. The tip (point of origin; see Fig 1G asterisk) of peroxules moved less during the translation process in cp versus non-cp association, indicative of an anchored tether; cp $1.85^{+}/.0.3\mu m$ (n=37), non-cp $2.35^{+}/.0.2\mu m$ (n=46). In comparison, during the recovery period peroxule tip displacement was much smaller, with values for cp and non-cp being similar; cp $0.89\ ^{+}/.\ 0.2\mu m$, non-cp $1.13^{+}/.0.1\mu m$. If the base of the tether (i.e. peroxule tip; see Fig 1G asterisk) is anchored, one would expect a higher level of peroxisome movement (i.e. recoil) during the recovery period to correspond with a lower level of peroxule tip movement during the translation; unlike non-cp samples, there is a cluster of cp samples indicative of such behaviour suggesting strongly anchored tether bases (Fig. 3).

Biophysical modelling of peroxisome recoil indicates differences in relative forces for peroxisome interactions

Since both cp and non-cp peroxisomes exhibit different trapping (Fig. 2) and recovery (Fig. 3) behaviours, we sought to understand the forces involved in this process; specifically is it only the recoil distance which changes or are there changes in the tether properties between cp and non-cp peroxisomes? To allow us to distinguish between tether properties and changes in recoil distance we used a simple viscously damped spring model to estimate the tether stiffness (i.e. spring constant) and tether tension forces (i.e. initial recovery force) involved in the recovery process (Fig 4,

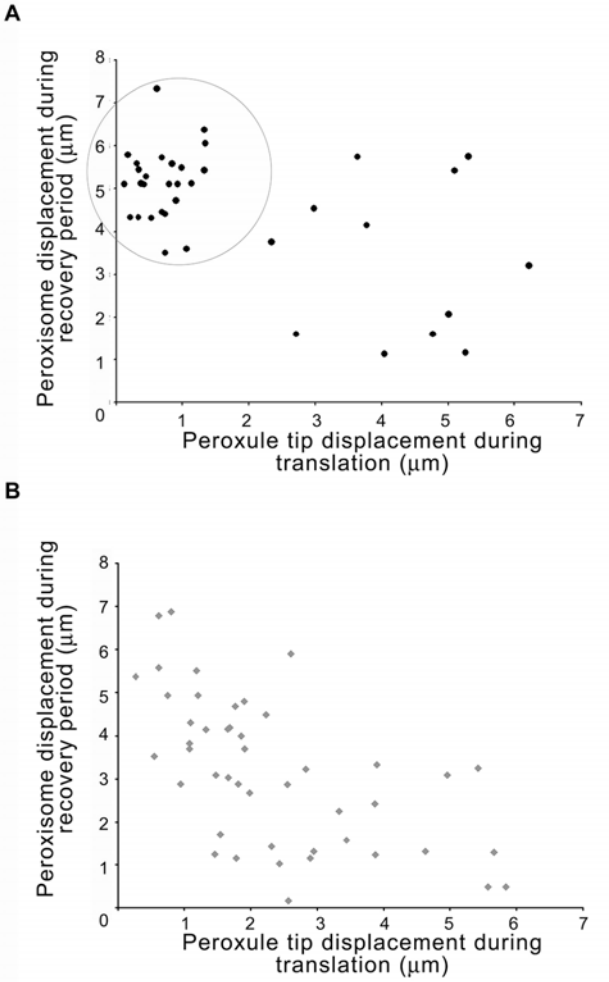

Figure 3. Correlation between peroxisome displacement during the recovery period and peroxule tip displacement during translation indicates anchored tethering between chloroplasts and peroxisomes.

Peroxule tip displacement during the translation period was plotted against the peroxisome displacement during the recovery period for chloroplast associated (A; n=37) and non associated peroxisomes (B; n=46). Peroxisomes were trapped with 37mW optical trap laser power followed by a 21.5 second recovery period. The behaviour of cp samples is indicative of anchored tethers where the peroxule tip represents the base of the tether: small peroxule tip displacement combined with large peroxisome recovery displacement (circle). Note, the sample sizes are different to those in supplementary table 1 as displacement could only be measured if the peroxule was observable for the entire period.

Supp. note, Supp Fig 2-4). This first approximation indicates that tether stiffness values are similar for non-cp and cp (Fig 4B,C) and that differences in the recovery forces are solely due to the more rigid anchoring of cp associated peroxisome tethers, which leads to greater tether extension and subsequently greater recovery

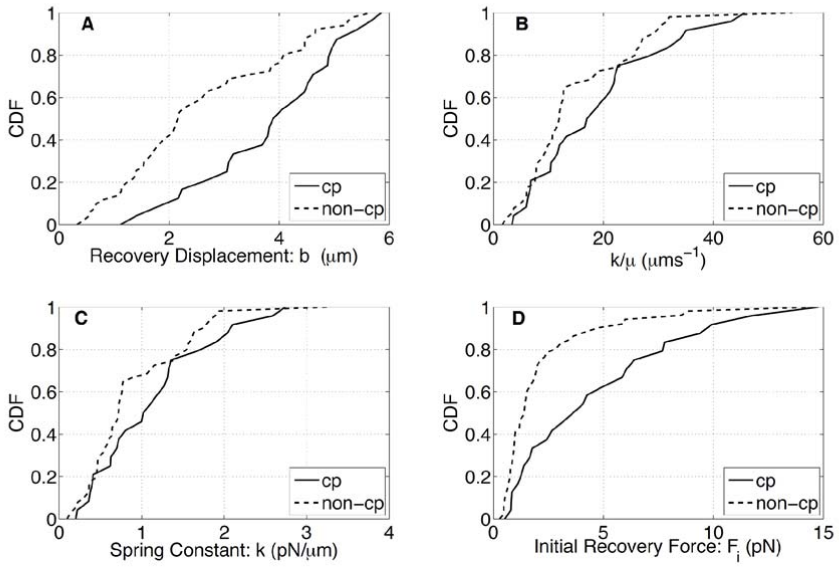

Figure 4. Cumulative distribution functions of model parameters for cp and non-cp associated peroxisomes.

Recovery displacement b is larger for cp than non-cp peroxisomes (A), whereas k/μ values, indicative of the tether stiffness, are broadly similar (B). Also, shown are the derived spring constants (C) and initial recovery forces (D) calculated assuming a viscosity of 0.06 Pa s for the cytoplasm. Values are derived using the spring model described in the supplementary information alongside Supp Fig 2-4.

displacement and initial recovery forces (Fig 4). In other words the biological structure that forms the tether between cp and non-cp peroxisomes behaves in a similar manner (i.e. similar stiffness), but the base of the tether (i.e. anchor point) moves less for cp peroxisomes thus generating more tension during translation and resulting in greater recoil. Here, non-cp peroxisomes are tethered to a structure which has greater mobility than chloroplasts during the trapping routine, so that upon moving non-cp peroxisomes, the tethered structure is also able to move to a certain extent resulting in lower tension 'build up' during the translation process. As we cannot independently estimate cytoplasmic viscosity in our system, this approach can only be used to determine relative differences in forces between cp and non-cp associated peroxisomes. However, using a reasonable value of 0.06 Pa s (Scherp and Hasenstein 2007) gives tether stiffnesses of ~1pN/μm and initial recovery forces of ~1-4pN (median values from Fig 4).

Discussion

By using optical tweezers we clearly show that peroxisomes can be tethered to chloroplasts, and that relative differences in tethering strength highlight additional subcellular interactions. Moreover, these tethers can be observed in several instances as peroxules (Supp movie 2, 3). Such tethers are not solely restricted to chloroplast interaction, but are also prevalent on non-cp peroxisomes (Supp movie 2, 3). In the latter case, the tether interaction is either unstable or the structure it is tethered to is more readily motile, accounting for the movement of the peroxule tip base during translation. The mechanism of peroxule formation and extension is unclear, but the rapid rate of extension makes *de novo* synthesis unlikely. Alternatives could be that the bounding membrane itself is deformable, or that peroxules are tightly coiled around the peroxisome and indistinguishable from the fluorescence signal arising from the lumen of the main peroxisome body. It is unclear if the connectivity between peroxisomes and chloroplasts is direct or indirect as positioning could be mediated through interaction with the ER. The ER forms a basket around the chloroplasts (Schattat et al., 2011), and *in vitro* optical trapping data inferred a chloroplast-ER connection in Arabidopsis and pea leaf cells (Andersson et al., 2007). Peroxisomal membrane protein Pex3p has been implicated in acting as a direct tether between the ER and peroxisomes in *S. cerevisiae* (Knoblach et al., 2013). However, the complex biogenetic link between peroxisomes and the ER has been, and continues to be, debated within the community (Hu et al., 2012). Our previous observations of ER responses upon trapping and moving Golgi highlight that a large percentage of the ER is freely mobile, however chloroplast-ER interactions were not investigated (Sparkes et al., 2009b). Therefore, if chloroplast-peroxisome connectivity is mediated by an ER bridge, then perhaps the ER is highly constrained around chloroplasts which could lead to greater recoil of trapped cp peroxisomes compared with non-cp cases. This is an area of future study requiring further development of the imaging system. Using the approaches developed here, future studies will enable the molecular and physiological consequences of peroxisome-organelle interaction to be studied, and could also be used to study the formation of membrane extensions.

Interactions between organelles are likely required for communication and transport. Examples in yeast and mammals infer a requirement for lipid and calcium exchange

385 (Prinz 2014). In plants reports for ER-Golgi (Sparkes et al., 2009b), nucleus-plastid
386 (Higa et al., 2014), ER-chloroplast (Andersson et al., 2007; Mehrshahi et al., 2013),
387 peroxisome-oil body (Thazar-Poulot et al., 2015) interactions have been made, along
388 with a recent report from Oikawa *et al.* (2015) inferring a chloroplast-peroxisome
389 interaction in Arabidopsis mesophyll cells. This study, along with previous reports,
390 indicates peroxisomes undergo light dependent morphological changes (Desai and Hu
391 2008;Oikawa et al., 2015). Furthermore, by effectively inducing a localised
392 intracellular shock wave, Oikawa *et al.* inferred light, and photosynthesis, dependent
393 connections between peroxisomes and chloroplasts. Here, using a complementary
394 approach we trap individual peroxisomes in tobacco leaf epidermal cells, and
395 additionally compare the responses between chloroplast associated and non-associated
396 peroxisomes. Our results provide a clear indication of interaction of peroxisomes with
397 chloroplasts, and other unknown structures, and we provide a biophysical model for
398 the forces involved in the tethering process. We have also visualised the tethering
399 process through peroxule production, observations which were not made in the work
400 of Oikawa *et al*. and therefore suggest a novel role for peroxules in maintaining
401 physical connectivity between peroxisomes and the structure(s) to which it is tethered
402 to. The two techniques infer forces for the peroxisome-chloroplast interaction, but by
403 the very nature of the techniques the forces relate to different biological aspects of the
404 interaction; Oikawa *et al.* models the force required to push the two organelles apart
405 (23-61 fN nm^{-2}), whereas here we model the forces imparted on the organelle after
406 they have been separated. It is important to note that the speed used to separate the
407 organelles using optical tweezers is within the range of reported peroxisome speeds in
408 an unperturbed system (Sparkes *et al.* 2008), and so cytoplasmic viscosity will affect
409 interactions in a way in which reflects the native motile system. Whereas the force
410 imparted on peroxisomes using the focused femtosecond laser technique was reported
411 to be so large that the effects of cytoplasmic viscosity would not hinder free
412 peroxisome motion, and are therefore negligible in their system. We do not infer a
413 precise force for trapping and moving the organelle (as viscosity values are currently
414 unknown for the system) and so compare the trapping profiles of chloroplast
415 associated and non-associated peroxisomes in response to the trapping laser power.
416 Both systems therefore provide different force components and have different
417 strengths and weaknesses in assessing the peroxisome-chloroplast interaction.
418 Furthermore, the basic spring model provides a baseline for interactions and will be

useful in testing how effective tension and stiffness change under altered environmental conditions that may regulate the interaction between peroxisomes and chloroplasts. For example, as photorespiration and photosynthesis may affect interaction, does the rate of recoil of a trapped peroxisome change indicating a 'tighter' tethering process between peroxisomes and chloroplasts or other structures within the cell, and does this altered response affect tether stiffness rather than tension?

Several organelles in plant cells produce tubular emanations; stromules, matrixules and peroxules extend from chloroplasts, mitochondria and peroxisomes respectively (Scott et al., 2007; Mathur et al., 2012; Hanson and Sattarzadeh 2013). Mapping stromule dynamics, and the movement of protein and small molecules lend support to a role in communication. However, contradictory data from different groups on molecular exchange between stromules makes this an interesting and contentious area of research (Hanson and Sattarzadeh 2013; Mathur et al., 2013). Here, our results infer a similar role in communication, and we propose that tubular emanations are a consequence of organelles attempting to maintain connections in the highly dynamic intracellular environment. Peroxule formation occurs in response to hydroxyl reactive oxygen species (ROS) with a concomitant reduction in peroxisome speed (Sinclair et al., 2009). This could be interpreted as a response to maintain connections between peroxisomes and another organelle whose motility has not been affected, or has been increased during this treatment, effectively increasing the spatial separation between the two organelles. Whilst the biophysical model provided herein reveals pN force measurements imparted on the organelle during the recovery process, it also gives an indication of the force required to pull the organelle micron distances. Here, the motor force to separate organelles is expected to be the same or greater than the force required for the organelles to be 'pulled' back towards their resting position (i.e. referred to as the restorative force in the biophysical model). This approach could therefore determine how motor regulation is controlled in order to maintain peroxisome movement under conditions where interaction with chloroplasts is up / down regulated.

Organelle movement plays important roles in growth, development and in response to (a)biotic stresses (see references in Madison and Nebenführ 2013, and Buchnik et al.,

2015). In a wider context, the results presented herein allow us to start to bridge the interface between organelle movement and interaction, and the forces involved in these processes. Whist future studies are required to validate the force measurements with known cellular viscosities, in broader terms, these studies demonstrate that interactions between organelles such as peroxisomes and chloroplasts in plant cells are not random, but are controlled through tethering mechanisms which can be quantified using optical tweezers. Regulation of organelle interaction / association will be controlled by motor driven movement to position organelles next to one another to allow tethering processes to occur. The force balance between these two processes therefore needs to be viewed in conjunction to describe organelle motion and positioning. Organelle interactions in plants could be required for communication and so future studies pinpointing the tethering and motor components could provide a novel way in which to control subcellular communication.

An interdisciplinary approach will be needed to fully characterise the molecular and physiological role(s) of peroxisome-chloroplast interactions, and interactions with other unknown organelles which could include lipid bodies. Current evidence points towards photosynthetic dependent processes and a role for PEX10 in peroxisome-chloroplast interactions (Oikawa et al., 2015; Schumann et al., 2007; Prestele et al., 2010). It will also be interesting to assess what role ROS signalling may play in these interactions (Sandalio and Romero-Puertas, 2015), and whether the exchange of additional small molecules such as IAA and JA may be facilitated by organelle interaction. Future genetic screens and proteomic approaches will pinpoint the complex of proteins necessary for interaction. The essential domains required for tethering will be mapped using biophysical means, such as optical tweezers, to quantify effects on peroxisome-chloroplast / organelle interaction. Ultimately, the analysis of resulting lines deficient in the tethering process will provide both molecular, biochemical and physiological evidence for the role of peroxisome-chloroplast / organelle interaction.

Methods

Plant material and sample generation

Nicotiana tabacum plants were grown and transiently transformed according to Sparkes *et al.* (2006) GFP-SKL (Sparkes et al., 2003), YFPSKL (Mathur et al., 2002) and StCFP (Brandizzi et al., 2002) constructs were infiltrated at 0.04 optical density. Leaf samples (~5mm^2) were taken from plants after 3-4 days expression and incubated in 25μM latrunculin b for 60 minutes prior to imaging.

Confocal imaging and determination of organelle association and residency time with chloroplasts

Triple imaging of peroxisomes (YFPSKL), Golgi (StCFP) and chloroplasts (autofluorescence) in live tobacco epidermal pavement cells was done using multi-tracking in line switching mode on a Zeiss LSM510 Meta confocal microscope. CFP was excited with a 458-nm argon laser and YFP/ chloroplast autofluorescence with a 514nm laser, their emissions passed through a HFT 458/514 main dichroic beam splitter and NFT 490 and NFT 595 secondary dichroic beam splitter, and detected using 470-500nm, 530-600nm and 647-690nm filters respectively. All imaging was carried out using a 63 x 1.4 Numerical Aperture (NA) oil immersion objective with a scan speed of 1.94 frames per second. Peroxisomes / Golgi which were up to 1μm from the chloroplasts (as monitored by the autofluorescent signal) were categorised as residing next to chloroplasts. Residency time of peroxisomes and Golgi on chloroplasts were analysed manually. Only those which resided next too (and could move laterally over the surface of) the chloroplast for more than 3 seconds were included in the statistical analysis.

Dual imaging of peroxisomes (GFPSKL) and chloroplasts (autofluorescence) was carried out using multi-tracking in line switching mode on a Zeiss LSM510 Meta confocal microscope. GFP was excited with a 488-nm argon laser and autofluorescence with a 514nm laser, their emissions passed through a HFT 488/543 main dichroic beam splitter and NFT 515 and NFT 545 secondary dichroic beam splitter, and detected using 505-530nm and 636-690nm filters respectively. All imaging was carried out using a 63 x 1.4 NA oil immersion objective. Twenty single

519 scans of a 143 x 143 μm area were taken, and the number of chloroplasts with a
520 juxtaposed peroxisome in each image was counted.
521
522 ***Optical trapping setup and data generation***
523 Optical trapping was performed using a cw 1090 nm laser (SPI) focused using a x100,
524 oil immersion, NA 1.49 TIRF objective lens (Nikon). Here we assume the effective
525 NA of the objective lens for optical trapping approaches a value of 1.0. The
526 assumption is based upon comparison of escape force measurements made on 1.0 μm
527 diameter polystyrene beads to theoretical values calculated using an optical tweezers
528 computational toolbox (Nieminen et al., 2007). TIRF objectives are not commonly
529 used for optical tweezers. Mahamdeh *et al.* (2011) also indicate that spherical
530 aberrations arising from trapping in aqueous media will reduce the effective numerical
531 aperture.
532 TIRF used an excitation laser with 473 nm wavelength (Becker and Hickl) with a
533 maximum output power of 5 mW, coupled by an optical fibre to a Nikon TIRF
534 adapter system and attenuated by neutral density filters (2 and / or 8 dependent on the
535 level of GFP-SKL expression). Emitted fluorescent light was filtered using a long
536 pass filter for wavelength transmission above 505 nm and imaged using an electron-
537 multiplier charge-coupled device (Andor Ixon EMCCD). This allowed visualisation
538 of the excited GFPSKL probe and detection of chloroplast autofluorescence. Note,
539 that whilst the TIRF technique was employed to give significant improvement of
540 signal to noise, it is also likely that we are operating in a highly inclined illumination.
541 Custom LabVIEW® software (National Instruments) was used to control the EMCCD
542 camera (Andor), microscope stage (Marshauser) and a shutter, which blocked the
543 laser beam used for trapping. A LabVIEW® interface was used to synchronise the
544 timing of peroxisome capture, stage translation and peroxisome release over 110 or
545 229 frame videos; peroxisomes were monitored for 10 frames prior to trap activation,
546 40 frames upon trap activation prior to movement, 10 frames for the 6μm translation,
547 10 frames after the translation, and 40 or 159 frames after the trap was deactivated
548 (relating to 5.3second or 21.5second recovery periods respectively). Stage translation
549 was measured to be 5.74μm in 1 second with the EMCCD cycle time of 0.135
550 seconds giving approximately 7.5 frames per second. The video sequences were
551 stored as 16-bit stacked "tagged image file format" (tiff) files for subsequent analysis

of peroxisome behaviour. Note, the data sets generated for figure 2 are a combination of the above trapping routine and an earlier version where trap shuttering was manually controlled over a 70 frame video.

The minimal force (i.e. the escape force) required to trap peroxisomes, in a non-cp environment, were measured by application of a viscous drag force (Supp Fig. 2). The laser trap strength and viscous properties were investigated using a set of controlled experiments where the stage velocity was varied. For each stage velocity, the laser power required to keep 50% of the captured peroxisomes in the optical trap was determined over a fixed 6μm translation distance (Supp Fig. 2). The fluorescent organelles were observed under TIRF illumination. Due to variability in peroxisome diameter it was necessary to measure 30-80 peroxisomes at each stage velocity to obtain a representative laser power. Thus, the reported laser power is for an "average" peroxisome (with plotted error bars indicating S.E uncertainty in laser power). The viscous drag force for each stage velocity was calculated using Stokes' law with an assumed viscosity value of 0.06 Pa s (Scherp and Hasenstein, 2007) and the average measured peroxisome diameter. Error bars for viscous drag force calculations used the S.E. variation of peroxisome diameter. As a control, the same procedure was applied to 1 μm diameter polystyrene beads in water (0.00089 Pa s).

Analysis of optical trapping data

Trapping data from each repetition was normalised against differences in sample size to determine the percentages of peroxisomes that were either trapped, untrapped or which escaped the trap per leaf sample. The weighted mean values were taken of these percentages for whole datasets and plotted. Between 36 and 62 peroxisomes in total underwent the trapping protocol at any given laser trapping power resulting in n=338 for chloroplast and n=381 for non-chloroplast associated total sample sizes. These totals represent between 5 and 9 repetitions, where each repetition is from 1 leaf sample taken from 6 – 9 independent plants. Trapping was only attempted once per peroxisome, repeated trapping of the same peroxisome was not undertaken.

Displacement values for peroxisome and peroxule dynamics were carried out using ImageJ (NIH).

In order to gather statistically significant peroxisome motion data, we developed a customized detection and tracking algorithm using a combination of python (scipy) and custom written scripts and algorithms. The data were first filtered using the Laplace-of-Gaussian scale-space method (Lindenberg 1994) to selectively filter for objects in a given size range. Next, robust image statistics based thresholding (Median Absolute Deviation) selected only salient objects in the resulting filtered data as outlined in Murtagh *et al.* (2000). Object tracking was performed using a Global Nearest Neighbours point registration approach, implemented as a modified version of the Jonker-Volgenant linear assignment problem algorithm, altered to allow rectangular cost matrices and cost cut-offs. In addition, sub-pixel peroxisome positions were calculated using a filtered intensity weighted centroid function. Tracking validation was performed by manual verification. The resulting trajectories were then analysed to determine the peroxisome motion between the moment that the optical trap was disengaged and the end of the recovery period.

Force calculations are described in the spring model (see supplementary note).

Supplementary data

Supplementary Figure 1. Relationship between cp and non-cp peroxisomal behaviour in the optical trap.

Supplementary Figure 2: Laser power required for trapping peroxisomes and polystyrene beads at different stage velocities.

Supplementary Figure 3: Spring model definition.

Supplementary Figure 4: Example fits to the data using the simple spring model.

Supplementary Table 1: Relationship between optical laser trap power and peroxule formation characteristics from cp and non-cp peroxisomes.

Supplementary Movie 1. Peroxisome association with chloroplasts

Supplementary Movie 2. Peroxisomes can be trapped and moved laterally within tobacco leaf epidermal cells.

Supplementary Movie 3. Peroxisome behaviour in the optical trap under actin depolymerisation.

Supplementary Note. Spring Model of Peroxisome Motion

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Author contributions

Experiments were conceived by IS and experimental data generated by HBG and IS. ADW, SWB, BC and MRP built, customised, maintained and facilitated the use of the optical trap-TIRF system. Experimental design was discussed with HBG, ADW, SWB and JM. ADW calibrated the system and performed bead trapping experiments. HBG performed all Image J analysis. JM wrote the tracking algorithm to generate the displacement values for NAT. Visual confirmation of tracking was carried out by JM, HBG and IS. NAT applied the simple model of a viscously-damped sphere on a spring to determine the forces involved in the system. IS wrote the manuscript with comments from all authors. NAT wrote the supplementary note section. NAT and ADW were involved in writing the section relating to forces.

Figure legends

Figure 1. Optical trapping and movement of peroxisomes away from chloroplasts in tobacco leaf epidermal cells.

Schematic representation of the trapping procedure (A-D) and the corresponding micrographs (E-H) are shown. Upon turning the trap on (A,E) and moving the stage 6µm at a set speed (B,F; referred to as translation period) the trapped peroxisome (p, white arrow) is pulled away from the chloroplast (cp) and a peroxule (arrowhead) is formed. Upon turning the trap off (C,G) the peroxisome recoils backs towards its original position next to the chloroplast (D,H). Peroxisome displacement during the recovery period (referred to as recovery displacement) is measured (double arrowhead). Asterisk denotes the tip of the peroxule. Scale bar 6µm.

Figure 2. Higher optical trapping laser power is required to trap and move peroxisomes away from chloroplasts.

Non-cp (A) and Cp (B) associated peroxisomes underwent the optical trapping protocol using various trapping laser powers and their trapping characteristics were scored; remained in the trap over the 6µm translation (black bar A,B), unable to be trapped (white bar A,B) or escaped the trap during the translation (grey bar A,B). Percentages displayed are based on weighted means from a set of independent experiments. Supp Figure 1 compares cp with non-cp for all three trapping categories and indicates significant differences between peroxisomes that are trapped or escape from the trap for cp versus non-cp. Relationship between optical laser trap power and peroxule formation are given in Supp table 1.

Stills from Supp movie 3C-E representing before and after translation events for peroxisomes which are trapped, not trapped or escape the trap during the translation event are displayed (C, arrowhead). Note, peroxisomes not subjected to trapping in the same cell are shown for comparison (arrow). The translation event is based on movement of the stage and not the trap. Composite image of frames captured during the translation event show that the trapped peroxisome does not appear to move whereas organelles that escape the trap or are not trapped result in comet like tails (merged panels). Scale bar 6µm.

Figure 3. Correlation between peroxisome displacement during the recovery period and peroxule tip displacement during translation indicates anchored tethering between chloroplasts and peroxisomes.

Peroxule tip displacement during the translation period was plotted against the peroxisome displacement during the recovery period for chloroplast associated (A; n=37) and non associated peroxisomes (B; n=46). Peroxisomes were trapped with 37mW optical trap laser power followed by a 21.5 second recovery period. The behaviour of cp samples is indicative of anchored tethers where the peroxule tip represents the base of the tether: small peroxule tip displacement combined with large peroxisome recovery displacement (circle). Note, the sample sizes are different to those in supplementary table 1 as displacement could only be measured if the peroxule was observable for the entire period.

Figure 4. Cumulative distribution functions of model parameters for cp and non-cp associated peroxisomes.

Recovery displacement *b* is larger for cp than non-cp peroxisomes (A), whereas k/μ values, indicative of the tether stiffness, are broadly similar (B). Also, shown are the derived spring constants (C) and initial recovery forces (D) calculated assuming a viscosity of 0.06 Pa s for the cytoplasm. Values are derived using the spring model described in the supplementary information alongside Supp Fig 2-4.

Supplementary material

Supplementary Movie 1. Peroxisome association with chloroplasts.

Time lapse images were taken of peroxisomes (green), Golgi (cyan) and chloroplasts (magenta) in tobacco leaf epidermal cells. Organelles were visualised through transient expression of fluorescent fusions (YFPSKL for peroxisomes and STCFP for Golgi bodies) or autofluorescence (chloroplasts). Compared to Golgi, peroxisomes spend longer periods of time associated with chloroplasts. The peroxisome appears tethered to a fixed zone on the surface of the chloroplast as the chloroplast moves (A), and in some cases the peroxisomes can also move laterally over the surface (B). Scale bar 5 μm.

Supplementary Movie 2. Peroxisomes can be trapped and moved laterally within tobacco leaf epidermal cells.

Peroxisomes were trapped (arrowhead) and the stage moved 6 μm horizontally. During the translation peroxisomes either escaped the trap (A,C) or were moved 6 μm (B,D). Upon turning the trap off the peroxisomes moved back towards their original position (B,D). Peroxisomes juxtaposed to chloroplasts (C,D) behaved similarly to peroxisomes which were not (A,B). In both cases, peroxules were observed (B,D) Scale bar 6 μm.

Supplementary Movie 3. Peroxisome behaviour in the optical trap under actin depolymerisation.

A trapped non-cp (A) and cp (B) peroxisome undergoes the 6 μm translation resulting in peroxule formation. Upon turning the trap off the peroxisome moves back along the length of the peroxule. Movies highlighting examples of non-cp peroxisomes in tobacco leaf epidermal cells which are either (C) trapped, (D) not trapped (E) or escape the trap over the translation period. The samples were treated with latrunculin B and so any subsequent motion upon turning the trap off is independent of acto-myosin. Scale Bar 6 μm. Arrow head denotes peroxisome undergoing the trapping routine.

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