Size, stoichiometry, and organization of soluble LC3-associated complexes

Lewis J Kraft,1 Tuan A Nguyen,2 Steven S Vogel,1 and Anne K Kenworthy1,*

1Chemical and Physical Biology Program; Department of Molecular Physiology and Biophysics; Department of Cell and Developmental Biology; Vanderbilt University Medical Center; Nashville, TN USA; 2Section on Cellular Biophotonics, Laboratory of Molecular Physiology; National Institute on Alcohol Abuse and Alcoholism; National Institutes of Health; Rockville, MD USA

Keywords: MAP1LC3B, FRAP, FPFA, fluorescence, FRET, diffusion, autophagy, FCS, hydrodynamic radius, ATG8

Introduction

Autophagy is a major pathway for the capture and degradation of cytosolic materials via the lysosome. A distinctive feature of the pathway is the formation of a double-membrane-bound vesicle called the autophagosome.1 Autophagosome membrane expansion, fusion, and substrate selection are performed, in part, by a family of ATG8 proteins.2-10 Despite these proposed major roles for ATG8 proteins in autophagy, there are still many open questions in regard to how ATG8 proteins carry out these tasks.11

ATG8 proteins share high structural (but not sequence) homology with ubiquitin, and thus have been termed ubiquitin-like proteins.12 Interestingly, unlike ubiquitin, which is conjugated to other proteins, ATG8 proteins are conjugated to the head group of the lipid phosphatidylethanolamine.13 In mammals, the best-studied ATG8 family member, MAP1LC3B (LC3B), exists in both soluble (LC3-I) as well as lipid-modified form (LC3-II).14 Under conditions of stress or starvation, LC3 lipid modification becomes upregulated, in turn targeting the protein to phagophore membranes.15 LC3 is released from autophagosome membranes by the cysteine protease activity of ATG4B.16

MAP1LC3B, an ortholog of yeast Atg8 and a member of the family of proteins formerly also known as Atg8 mammals (LC3B henceforth in the text), functions in autophagosome formation and autophagy substrate recruitment. LC3 exists in both a soluble (autophagosome-independent) form as well as a lipid modified form that becomes tightly incorporated into autophagosomal membranes. Although LC3 is known to associate with tens of proteins, relatively little is known about soluble LC3 aside from its interactions with the LC3 lipid conjugation machinery. In previous studies we found autophagosome-independent GFP-LC3B diffuses unusually slowly for a protein of its size, suggesting it may constitutively associate with a high molecular weight complex, form homo-oligomers or aggregates, or reversibly bind microtubules or membranes. To distinguish between these possibilities, we characterized the size, stoichiometry, and organization of autophagosome-independent LC3B in living cells and in cytoplasmic extracts using fluorescence recovery after photobleaching (FRAP) and fluorescence polarization fluctuation analysis (FPFA). We found that the diffusion of LC3B was unaffected by either mutational disruption of its lipid modification or microtubule depolymerization. Brightness and homo-FRET analysis indicate LC3B does not homo-oligomerize. However, mutation of specific residues on LC3B required for binding other proteins and mRNA altered the effective hydrodynamic radius of the protein as well as its stoichiometry. We conclude that when not bound to autophagosomes, LC3B associates with a multicomponent complex with an effective size of ~500 kDa in the cytoplasm. These findings provide new insights into the nature of soluble LC3B and illustrate the power of FRAP and FPFA to investigate the emergent properties of protein complexes in the autophagy pathway.

*Correspondence to: Anne K Kenworthy; Email: Anne.kenworthy@vanderbilt.edu
Submitted: 05/10/2013; Revised: 02/04/2014; Accepted: 02/10/2014
http://dx.doi.org/10.4161/auto.28175
LC3 and other members of the ATG8 protein family have numerous interacting proteins, suggestive of their possible participation in multiprotein complexes. In addition, many LC3-interacting proteins contain a consensus LC3-interacting motif (W/F/Y-XX-L/I/V) for efficient binding to a hydrophobic surface on LC3 in the region of residues F52 and L53, as well as R70. Mutations to these key residues on LC3’s hydrophobic protein interacting surface disrupt binding to tens of proteins, highlighting the importance of this region for engaging other proteins in productive binding. Importantly, it is unknown if soluble LC3 interacts with its protein partners in a binary fashion or if instead it constitutively associates with multiprotein or other multicomponent complexes to accomplish its varied functions in autophagy.

Using confocal FRAP, our group recently showed that the soluble form of LC3B diffuses unusually slowly for a cytoplasmic protein of its size under basal conditions. Because the diffusion coefficient of a soluble, freely diffusing molecule is inversely related to its radius, this slower-than-expected diffusion of LC3B could potentially reflect its association with macromolecular complexes comprised of LC3B and other components of the autophagy pathway. Alternatively, the slow diffusion of soluble LC3B could potentially arise from reversible binding to microtubules in cells or the incorporation of LC3B into homo-oligomers, aggregates, or possibly membranes.

To address these possibilities, in the current study, we investigated the size, stoichiometry, and organization of LC3B-containing protein complexes using 2 complementary fluorescence-based approaches, confocal fluorescence recovery after photobleaching (FRAP) and fluorescence polarization fluctuation analysis (FPFA). The first of these methods, FRAP, is a powerful and versatile tool for measuring the ensemble dynamics of molecules. The instrumentation for FRAP is now widely accessible in many laboratories, and by analyzing FRAP data with recently described models it is now possible to accurately measure diffusion coefficients, as well as quantify reversible interactions. FRAP also has the unique ability to quantify the extent of irreversible interactions and very slow turnover on the timescale of the measurements as reflected in the mobile fraction. The second approach, FPFA, is a newly developed experimental method combining fluorescence correlation spectroscopy (FCS) and time-resolved fluorescence anisotropy (TRFA). Like FRAP, FCS reports on the diffusional mobility of molecules. However, FCS has exceptional sensitivity enabling measurements of the concentrations of fluorescent proteins in the pM to nM range, and is capable of resolving the diffusion coefficients of multiple diffusing species and their molecular brightness. By combining FCS and TRFA into a single measurement, FPFA enables the simultaneous measurement of translational and rotational mobility, concentration, brightness, and homo-FRET. Thus, FPFA is a tool that can be used to simultaneously investigate a protein’s mobility, stoichiometry, and organization.

Our FRAP and FPFA measurements reveal that under basal conditions, soluble Venus-LC3B diffuses as if it associates with a complex with an effective molecular weight of approximately 500 kDa in size, and each complex contains on average a single LC3B. Similar complexes were observed in living cells and in cytoplasmic extracts depleted of intact microtubules. In addition, we show that the size and stoichiometry of complexes containing LC3B are altered by mutational disruption of LC3B’s hydrophobic binding surface, but not mutational disruption of LC3B’s lipid modification. The effective size of the LC3B-associated complexes only modestly changes after perturbations to the autophagy pathway with rapamycin or chloroquine treatments. These findings provide new insights into the nature of soluble LC3B and illustrate the power of confocal FRAP and FPFA to provide novel insights into the emergent properties of protein complexes in the autophagy pathway.

Results

Effect of mutations of the hydrophobic-binding interface and lipid modification site on the subcellular distribution of LC3B

In previous studies, we found GFP-LC3B and Venus-LC3B diffuse more slowly in the cytoplasm of cells than expected for a monomer using confocal FRAP, suggesting LC3B may associate with a high molecular weight complex. Given that many LC3-interacting proteins interact with a hydrophobic interaction surface on LC3 using a W/F/Y-XX-L/I/V motif, we asked whether disruption of specific residues in this region on LC3B will disrupt its association with the putative high molecular weight complexes. To address this question, we focused on 2 sets of mutations, R70A, as well as F52A and L53A. These mutations disrupt binding to at least 18 and 9 binding partners, respectively. Several of the disrupted binding partners are involved in activities ranging from LC3’s lipid modification to autophagy substrate specificity. For comparison, we also examined a G120A mutant of LC3B, which is unable to undergo lipid modification.

We first examined the effects of the R70A, G120A, as well as the F52A and L53A mutations on the subcellular distribution of LC3B in HeLa cells. As previously described, Venus-LC3B is found in a diffuse cytoplasmic form as well as in discrete 0.5 to 2 μm puncta in the cytoplasm corresponding to LC3B labeled autophagosomes, autophagosome independent substrates, and aggregates. Some diffuse Venus-LC3B is also observed in the nucleus, consistent with our previous findings quantifying the amount of GFP tagged LC3B in the nucleus (Fig. 1A).

Like wild-type Venus-LC3B, a substantial fraction of diffuse Venus-LC3BF52A,L53A, Venus-LC3BΔ70A, and Venus-LC3BΔ120A was present in both the cytoplasm and nucleoplasm of live HeLa cells (Fig. 1B–D). However, on average about 2-fold fewer puncta were present in cells expressing Venus-LC3BΔ70A than in cells expressing Venus-LC3B (P ≤ 0.008; Bonferroni-corrected t test), and even fewer puncta were positive for Venus-LC3BF52A,L53A or Venus-LC3BG120A (P ≤ 0.008; t tests) (Fig. 1E). These findings are consistent with the idea that LC3B’s hydrophobic binding surface regulates protein-protein interactions, including SQSTM1/p62 (sequestosome 1) binding, which facilitate targeting LC3B to autophagy-independent puncta. In addition, they suggest that the F52A and L53A, as well as the G120A mutants...
are more strongly defective in their ability to bind to cytoplasmic puncta than is the R70A mutant of LC3B.

Since the Venus-LC3B F52A L53A, Venus-LC3BR70A, and Venus-LC3BG120A mutants have defects in the numbers puncta present under steady-state conditions, we wanted to determine if these mutants retain their ability to associate with autophagosomal membranes. To test this, we monitored their accumulation on autophagosomal membranes after inhibition of lysosomal acidification with chloroquine. After treatment with chloroquine, both Venus-LC3BF52A L53A and Venus-LC3B R70A accumulated on autophagosomal membranes, similar to wild-type Venus-LC3B (Fig. 2A–F). This result is consistent with what was previously reported for a GFP-LC3 F52A mutant after treatment with both starvation and bafilomycin A1.28 In contrast, no changes to the localization of Venus-LC3B G120A were observed after treatment with chloroquine (Fig. 2G and H). This is consistent with the inability of the G120A mutant to be targeted to autophagosomes by lipid modification of this residue.33

It was recently shown, using selective photobleaching of GFP-LC3-associated puncta, that LC3 incorporated into autophagosomal membranes turns over much more slowly than GFP-LC3 associated with autophagy substrates.39 We used this method to further characterize the nature of the LC3B-positive puncta present after incubation with chloroquine (Fig. 2I). The FRAP analysis revealed that while Venus-LC3B, Venus-LC3BR70A and Venus-LC3BF52A L53A puncta had very large immobile fractions, Venus-LC3BG120A had a relatively high mobile fraction (Fig. 2J). This is consistent with the notion that the R70A, as well as the F52A and L53A mutants of LC3B retain their ability to become lipid modified and subsequently incorporated into the membranes of autophagosomes, whereas the LC3B G120A mutant does not. In order to further verify that there are no defects in the LC3-I to LC3-II processing of the R70A, as well as the F52A and L53A mutants, we performed SDS-PAGE and western blotted for LC3 (Fig. 2K) and GFP (Fig. 2L) both under basal conditions and after treatment with chloroquine. This experiment showed that there are no defects in the LC3-I to LC3-II processing of Venus-LC3B, Venus-LC3BF52A L53A, or Venus-LC3BR70A in stark contrast with the GFP-LC3BG120A negative control.33

Disruption of LC3B’s hydrophobic-binding interface changes the effective diffusion of LC3B-associated complexes in living cells

Next, we tested the effect of the R70A, as well as the F52A and F53A mutations, on the diffusional mobility of soluble cytoplasmic LC3B in living cells using a quantitative confocal FRAP assay.34,35 As shown in Figure 3A, this experiment was aimed toward quantifying the diffusion of the diffuse pool of LC3B, and excludes any LC3B associated with puncta. As an internal control, we performed FRAP experiments of Venus, a soluble, freely diffusing protein. The Venus FRAP curve was well fit by a single component model for Brownian motion with a diffusion coefficient of 41 ± 4 µm2/s and a mobile fraction of 100% in HEK 293 cells (Fig. 3B and C; Table 1). Next, we performed FRAP experiments on Venus-LC3B in the cytoplasm. Consistent with our previous findings,29,30 we found Venus-LC3B had an approximately 2.5-fold slower D compared with that of Venus, much slower than the predicted diffusion coefficient for monomeric Venus-LC3B (P ≤ 0.008; t test) (dashed line in Fig. 3C).
This corresponds to an effective molecular weight of ~500 kDa (Table 1). We found similar results in HeLa cells ($P \leq 0.008$; $t$-test) (Fig. 3D; Table 2).

After establishing baselines for diffusion comparisons, next, we measured the diffusion of soluble Venus-LC3B$^{52A\,\,L53A}$ and Venus-LC3B$^{70A}$ by confocal FRAP. We found that their mobile
fractions were also both approximately 100% (Tables 1 and 2), similar to that of soluble Venus-LC3B itself (ns; \( P > 0.008; t \) tests). However, their diffusion coefficients differed from that of one another as well as from that of Venus-LC3B. Interestingly, in HEK 293 cells, \( D \) for Venus-LC3B<sup>F52A,L53A</sup> (11.6 ± 0.7 \( \mu \)m²/s) was slower than that of wild-type Venus-LC3B (15 ± 2 \( \mu \)m²/s) (\( P \leq 0.008; t \) test), while \( D \) for Venus-LC3B<sup>R70A</sup> (24 ± 2 \( \mu \)m²/s) was faster than Venus-LC3B, but still not to the level expected.
for a Venus-LC3B monomer \((P \leq 0.008; t\text{ test})\) (Fig. 3). Similar results were obtained in HeLa cells (Fig. 3D). Assuming the slow diffusion of the proteins reflects their association with spherical complexes, their predicted molecular weights would be \(-1.2\) MDa for Venus-LC3B(F52A/L53A) and \(-130\) kDa for Venus-LC3B(R70A) (Tables 1 and 2).

The slow diffusion of LC3B is not due to its lipid modification or association with autophagosome membranes

Although our FRAP experiments were directed toward the soluble pool of LC3B, it is formally possible that LC3B’s slow diffusion is a result of a small fraction of lipid modified LC3B incorporated into sub-diffraction vesicles or autophagosomes. This seemed unlikely, because, theoretically, \(-250\) nm radius vesicles would be expected to diffuse \(-100\) fold slower than Venus (D1/D2 = 2 nm/250 nm), whereas, experimentally, Venus-LC3B diffused only \(-2.5\)-fold slower. However, our FRAP measurements do not have the resolution to adequately test this possibility without additional experimental information. Therefore, to address this question we performed FRAP measurements on Venus-LC3B(G120A), since this mutation of LC3 prevents its lipid modification and subsequent incorporation into autophagosomal membranes. As shown in Figure 3D, Venus-LC3B(G120A) diffuses similarly to wild-type Venus-LC3B (ns; \(P > 0.005; t\text{ test}\)). These data support the notion that the slow diffusion of LC3B is not the result of its association with autophagosomes or interactions with other molecules that depend on lipid modification of the protein. Rather, the slow diffusion could reflect the association of LC3B with other molecules.

The effective size of putative LC3B-associated complexes is modestly affected by autophagy modulators

In order to gain further insights into how these complexes are modulated by perturbations to the autophagy pathway, we
extended our analysis of the diffusion of LC3B under basal conditions to 2 additional conditions known to modulate the autophagy pathway. The first condition was to inhibit autophagy by incubating the cells with 100 μM chloroquine for 2 h, and the second condition was to stimulate autophagy by incubating the cells with 200 nM rapamycin for 2 h before performing FRAP measurements. We found that rapamycin and chloroquine treatments produced similar changes in the diffusion of the constructs, as shown in Figure 3. At first glance, the trends between the constructs appear to be maintained. However, after normalization to the diffusion of Venus, using the Stokes Einstein relationship as before, we found that the effective molecular weights for the LC3B-associated complexes were modestly upregulated approximately 2-fold compared with basal conditions ($P \leq 0.005$; $t$ tests; see Table 2 for a summary). These data suggest that the effective sizes of the soluble LC3B-associated complexes are only modestly affected by perturbations to the autophagy pathway with rapamycin and chloroquine in vivo.

Disruption of LC3B’s hydrophobic-binding interface but not of its lipidation site changes the effective diffusion of LC3B-associated complexes in cytoplasmic extracts

In order to test if the complexes observed in cells are intrinsically stable, or if they require cellular components, such as microtubules, we next examined the properties of the LC3B constructs in cytoplasmic extracts. In principle, it is possible to measure the diffusion of LC3 in cytoplasmic extracts using confocal FRAP; however, we wished to obtain additional information about the putative complexes, such as whether multiple types of complexes were present and how many LC3B molecules were present in each complex. Therefore, we turned to another technique that is sensitive to protein mobility and stoichiometry, FPFA, to analyze the LC3B-associated complexes in cytoplasmic extracts. To carry out FPFA measurements on the fluorescently labeled proteins in our cytoplasmic extracts, we utilized a recently described custom-built FPFA instrument with 2-photon excitation to define the focal volume. HEK cells expressing Venus or Venus-LC3B were extracted using passive lysis buffer and clarified by a brief high speed spin before the FPFA measurements were performed.

In order to determine the diffusivity and molecular brightness of the species, FPFA macro-time measurements of orthogonally polarized fluorescence emission were cross-correlated. The cross-correlation curves for both Venus and Venus-LC3B were well fit by a single-component model for Brownian motion suggesting the absence of multiple diffusing species with large differences in their sizes (see Fig. 4A for an example of 1- vs. 2-component models). The diffusion coefficients from the fits to Venus and Venus-LC3B were $67 \pm 2$ and $25 \pm 2 \mu m^2/s$, respectively (Fig. 4B). Thus, the diffusion coefficient for Venus-LC3B in cell extracts was much slower than Venus alone (approximately 2.5-fold) as seen previously using FRAP in live cells, and much slower than predicted for a freely diffusing Venus-LC3B monomer ($P \leq 0.003$; $t$ tests). Making the simplifying assumption that the complexes containing Venus-LC3B are spherical, we calculated their predicted size is about 500 kDa (Table 3).

After establishing baselines for diffusion comparisons, next, we performed FPFA measurements of the Venus-LC3BF52A and Venus-LC3BF52A L53A mutants in cytoplasmic extracts. Interestingly, the cross-correlation curve for Venus-LC3BF52A L53A was not well described by a single component model (Fig. 4A). It was, however, fit well by a 2-component diffusion model where approximately 3/4 of the molecules have a diffusion coefficient of $28 \pm 4 \mu m^2/s$ while the remainder have a diffusion coefficient of $5 \pm 2 \mu m^2/s$ (Fig. 4A and B). The diffusion coefficient for the faster diffusing Venus-LC3BF52A L53A species was similar to that of Venus-LC3B ($P > 0.003$; $t$ test), while the diffusion coefficient for the slower diffusing species was much slower than Venus-LC3B ($P \leq 0.003$; $t$ test). The cross-correlation curve for Venus-LC3BF70A on the other hand was well fit by a single component diffusion model with a diffusion coefficient of $35 \pm 3 \mu m^2/s$, which was statistically faster than Venus-LC3B ($P \leq 0.003$; $t$ test) (Fig. 4B). Assuming the complexes have a spherical geometry, the predicted sizes of the Venus-LC3BF70A L53A-associated complexes are about 300 kDa and 50 MDa (note, however, that we observed relatively high variability in the measurements of the F52A and L53A construct), and the predicted size of Venus-LC3BF70A-associated complexes is about 190 kDa (Table 3). These values are in agreement with those measured by confocal FRAP in live cells, assuming the $D$ measured for Venus-LC3BF70A L53A by FRAP reflects the contributions of 2 diffusing species detected by FPFA.

In order to directly test if LC3B’s incorporation into protein complexes is regulated by its lipid modification we performed FPFA measurements on Venus-LC3BF120A. The diffusion coefficient for Venus-LC3BF120A, $23.4 \pm 0.9 \mu m^2/s$, was identical to wild-type Venus-LC3B (ns; $P > 0.003$; $t$ test), further supporting our conclusion based on the FRAP results that the slow diffusion of Venus-LC3B is not due to its lipid modification (Fig. 4B). In addition, these data further suggest that LC3B’s lipid modification is not a major factor in regulating the effective size of the LC3B-associated complexes.

Our in vivo results revealed the effective sizes of complexes are modestly upregulated by perturbations to the autophagy pathway. We next explored the possibility that the effective sizes of these soluble LC3B-associated complexes under chloroquine conditions are maintained in vitro. We prepared cytoplasmic extracts from cells incubated in 100 μM chloroquine for 2 h before cell lysis. We found that in cells treated with chloroquine, there was little effect on the sizes of the wild-type Venus-LC3B, Venus-LC3BF70A, and Venus-LC3BF120A-associated complexes compared with basal conditions (ns; $P > 0.003$; $t$ test) (Fig. 4C). The diffusion of the Venus-LC3BF52A L53A complex under chloroquine conditions was primarily fit with a single-component model. These results differed slightly from what we observed in living cells, and suggest that some of the components of the soluble LC3B-associated complexes present after treatment with chloroquine were not maintained in vitro. These results further support the conclusion that the complexes we are detecting are independent of lipid modified LC3B incorporated in autophagosomal membranes, as this would have shown up as a component with a diffusion coefficient approximately 2 orders of magnitude slower than Venus.

Interactions of LC3B with polymerized microtubules are not responsible for the slow diffusion of soluble LC3B
It is formally possible that LC3B’s slow diffusion could be due to interactions with microtubules given that LC3B was originally identified as a microtubule-associated protein. To address this possibility we specifically tested for the presence of polymerized microtubules in the cytoplasmic extracts by sedimentation at a relatively high centrifugal force. We were unable to detect significant levels of polymerized microtubules under the conditions of our experiments (Fig. 4D). This suggests that the slow diffusion of Venus-LC3B detected by FPFA is not the result of reversible binding to polymerized microtubules. We further tested this possibility in vivo, by performing FRAP experiments on cells depleted of polymerized microtubules by treatment with nocodazole for 15 min on ice followed by 1 h at 37 °C. We observed that the effective sizes of the complexes were maintained in live cells treated with nocodazole (Table 2), further supporting the notion that reversible interaction with polymerized microtubules is not responsible for LC3B’s slow diffusion.

There is no evidence of homo-FRET between Venus-LC3B, Venus-LC3BF52A L53A, Venus-LC3BR70A, or Venus-LC3BG120A. Our diffusion measurements indicate soluble LC3B is constitutively associated with a high molecular weight complex; however, it is possible the complexes we detect by diffusion may correspond to small aggregates of LC3B. We previously tested for oligomerization of puncta-independent LC3B in vivo using Cerulean-LC3B and Venus-LC3B as FRET donors and acceptors. FRET microscopy failed to detect significant levels of energy transfer, suggesting soluble LC3B is unlikely to homo-oligomerize or aggregate. However, unfavorable dipole-dipole orientation may have prevented energy transfer, even between fluorophores in close proximity. Thus, LC3B’s oligomerization state remains unclear.

In order to test if there were multiple LC3B molecules within FRET proximity in our cytoplasmic extracts, next we examined our FPFA measurements for evidence of homo-FRET. Homo-FRET analysis provides information about the proximity of Venus fluorophores, and thus may be able to detect homo-oligomerization of Venus-LC3B or the Venus-LC3B mutants. For the purpose of performing an analysis of homo-FRET, our FPFA micro-time measurements of orthogonally polarized fluorescence emission were used to calculate time-resolved fluorescence anisotropy decay curves. The decay of fluorescence anisotropy...
can be due to several factors including rotational diffusion and the presence or absence of FRET.43 In the absence of FRET, we expect the fluorescence anisotropy curve will exhibit a single exponential decay as was previously measured for Venus, and in the presence of FRET we expect the fluorescence anisotropy curves will exhibit a multicomponent exponential decay as has been previously measured for a series of positive controls consisting of Venus molecules separated by short linkers.37

We found the fluorescence anisotropy decay curves for both Venus and Venus-LC3B were well fit using a single exponential decay model, indicating little, if any, homo-FRET was occurring in these samples (Fig. 5A). This result is consistent with a previous study in which we have found that no significant FRET occurs between soluble Cerulean-LC3B and Venus-LC3B in the cytoplasm of living cells.50 The single exponential decay rates of the fluorescence anisotropies for Venus and Venus-LC3B are related to the rotational diffusion of the Venus fluorophore attached to LC3B by a flexible linker (Table 3).45 However, because of the flexible nature of the linker it is not possible to use these values to calculate the size of the Venus-LC3B-associated complexes based on their rotational correlation times alone.

Next, we examined our FPFA measurements of Venus-LC3BFG52A, LF53A, Venus-LC3BR70A, and Venus-LC3BG120A for evidence of homo-FRET in order to determine if these mutations resulted in a change in the proximity of multiple LC3B molecules. In both cases, the anisotropy decay curves were fit by a single exponential, with similar rotational correlation times to that of wild-type Venus-LC3B (Fig. 5A; Table 3). This suggests the Venus proteins attached to the LC3B mutants are also, to some extent, free to rotate independently of LC3B, and that no detectable homo-FRET occurs in either case.

There is on average only one soluble Venus-tagged LC3B protein per complex, and disruption of LC3B’s hydrophobic binding interface changes the stoichiometry of LC3B-associated complexes

A negative FRET result cannot conclusively rule out the presence of multiple Venus-LC3B’s per complex, as the Venus fluorophores may be positioned such that their proximity or their average relative dipole orientations does not satisfy the FRET requirements.43 Fortunately, FPFA measurements provide an additional opportunity to gain insights into the stoichiometry of a protein by quantifying its molecular brightness. Fluorescence brightness is extracted from the statistics of fluorescence intensity fluctuations using a method that has been termed brightness analysis.38 The principle behind the interpretation of our data lies in the idea that a Venus labeled molecule with a stoichiometry of 2 will have twice the brightness compared with Venus alone, and it follows that higher order oligomerization states will have quantized brightness values of Venus as was previously measured using a series of positive controls.37

After establishing the Venus baseline for brightness comparisons, next, we investigated the brightness of Venus-LC3B using our FPFA measurements. We found Venus-LC3B has a normalized brightness of 1.16 ± 0.09, which is approximately 16% brighter than Venus (P ≤ 0.008; t test) (Fig. 5B). These data strongly suggest the majority of Venus-LC3B does not extensively oligomerize or aggregate in solution. Instead, they suggest Venus-LC3B primarily has a stoichiometry of close to one in cytoplasmic complexes.

Finally, in order to gain additional information about the stoichiometry of the mutant LC3B proteins, we performed brightness analysis using our FPFA measurements of Venus-LC3BFG52A, LF53A, Venus-LC3BR70A, and Venus-LC3BG120A. Assuming the brightness values of the 2 Venus-LC3BFG52A, LF53A species with distinct diffusion times are the same, the normalized brightness of the Venus-LC3BFG52A, LF53A complexes is about 1.8 ± 0.1. This brightness value was approximately twice that of Venus, suggesting either one or both of the Venus-LC3BFG52A, LF53A complexes has a stoichiometry of 2 (P ≤ 0.008; t test) (Fig. 5B). On the other hand, the brightness values obtained from the Venus-LC3BR70A, and the Venus-LC3BG120A FPFA data, were both similar to Venus-LC3B. Venus-LC3BR70A had a normalized brightness of 1.1 ± 0.1, or about 10% brighter than Venus (P > 0.008; t test), and Venus-LC3BG120A had a normalized brightness of 1.0 ± 0.1 which was indistinguishable from that of Venus (ns; P > 0.008; t test) (Fig. 5B). Thus, like wild-type Venus-LC3B, these data suggest

| Construct       | Monomer MW (kDa) | Basal predicted MW (kDa)* | CQ predicted MW (kDa)* | Basal percent species (%) | Basal rotation correlation time (ns) | CQ rotation correlation time (ns) |
|-----------------|------------------|---------------------------|------------------------|----------------------------|--------------------------------------|-----------------------------------|
| Venus-LC3B      | 45               | 500 ± 100 (10)            | 480 ± 90 (6)           | N/A                        | 24.9 ± 0.6 (10)                     | 25.0 ± 0.3 (6)                    |
| Venus-LC3B     | 45               | 300 ± 100 (7)             | 190 ± 70 (3)           | 70 ± 4 (4)                 | 25.6 ± 0.3 (7)                      | 25.5 ± 0.1 (4)                    |
| Venus-LC3B     | 45               | 5 × 10^4 ± 5 × 10^4 (7)   | N/A                    | 30 ± 4 (4)                 | N/A                                 | N/A                               |
| Venus-LC3B     | 45               | 190 ± 50 (7)              | 260 ± 30 (3)           | N/A                        | 24.3 ± 0.6 (7)                      | 23.9 ± 0.1 (3)                    |
| Venus-LC3B     | 45               | 600 ± 90 (7)              | 330 ± 40 (7)           | N/A                        | 24.8 ± 0.3 (7)                      | 25.3 ± 0.5 (7)                    |

*Calculated assuming diffusing species has a spherical shape; mean ± 95% CI (N, # extracts); CQ, chloroquine.
only one Venus-LC3B\textsuperscript{F52A,L53A} and only one Venus-LC3B\textsuperscript{G120A} molecule is associated with a given complex.

Given the effective sizes of complexes appear to change upon treatment with chloroquine, we reasoned there may also be changes in LC3B's stoichiometry or organization under these conditions. We tested for these changes by examining the time-resolved anisotropy and the molecular brightness from our FPFA measurements of the LC3B constructs, after treatment with chloroquine, in cytoplasmic extracts. However, we found the anisotropy decays for the constructs were similar to basal conditions (Fig. 5C), and the molecular brightness values were also similar to basal conditions (Fig. 5D). On the other hand, the Venus-LC3B\textsuperscript{F52A,L53A} construct's brightness became more similar to Venus at 1.0 ± 0.2. These data suggest soluble LC3B does not have a propensity to aggregate or homo-oligomerize either under basal conditions or after treatment with chloroquine.

**Discussion**

In this study, we analyzed the size and stoichiometry of puncta-independent LC3B in the cytoplasm of living cells and in cytoplasmic extracts and examined how these properties are regulated by specific residues on LC3B's hydrophobic protein binding surface as well its G120 residue required for lipid modification. On the basis of our findings, we suggest the following working model for the basal organization of LC3B (Fig. 6). A single soluble Venus-LC3B protein associates with a cytoplasmic complex with a molecular weight about an order of magnitude greater than expected for a spherical monomer. Similarly slow diffusion for puncta-independent LC3B was detected in HeLa, COS7, and HEK 293 cells (29,30 and current study), suggesting this is a general feature of LC3B in the cytoplasm.

We also find that disruption of specific residues on LC3B's hydrophobic protein binding surface alters both the hydrodynamic radius and stoichiometry of LC3B-associated complexes (Fig. 6). We studied 2 mutants known to disrupt the interaction of multiple proteins with LC3. The first is an F52A and L53A double mutation to LC3B that has been reported to disrupt the interaction of LC3B with several important protein binding partners including SQSTM1 and NBR1 (neighbor of BRCA1 gene 1), cargo adaptors involved in targeting substrates for autophagic degradation.\textsuperscript{17,18,25,27,28} The second, an R70A mutation to LC3B, is also known to disrupt interactions with important binding partners such as SQSTM1 and NBR1, but also additional proteins including FYCO1, a RAB7 effector mediating microtubule plus-end directed vesicular transport.\textsuperscript{17,19,25}

Both Venus-LC3B\textsuperscript{F52A,L53A} and Venus-LC3B\textsuperscript{G120A} were poorly targeted to puncta in the cytoplasm under steady-state conditions,
but showed no dramatic defects in their accumulation on autophagosomal membranes, consistent with the idea that these residues are important for the interactions of LC3 with cargo adaptors, but not autophagosomal membranes.\textsuperscript{21,28} Remarkably, however, the R70A, as well as the F52A and L53A mutations had dramatically different effects on LC3B’s rate of diffusion. Venus-LC3B\textsuperscript{F52A,L53A} diffused more slowly than Venus-LC3B, indicating the complexes it associates with become larger, rather than smaller. The predicted size of the larger Venus-LC3B\textsuperscript{F52A,L53A} associated complex is exceptionally large, with a predicted molecular weight on the order of 50 MDa. This corresponds to an effective Stokes radius of ~25 nm, although we note there is large variability in this estimate. For comparison, the measured hydrodynamic radius of the 70S ribosome is between ~12 to 15 nm.\textsuperscript{46,47} Furthermore, the Venus-LC3B\textsuperscript{F52A,L53A} mutant appears to associate with at least 2 discrete complexes, in which one or both contain multiple copies of LC3B\textsuperscript{F52A,L53A}. The Venus-LC3B\textsuperscript{F52A,L53A} data provide an excellent example of how FPFA can overcome the limitations of FRET by simultaneously providing information about molecular brightness. In particular, we found no evidence of homo-FRET, but the brightness indicated a stoichiometry of approximately 2 suggesting the 2 Venus-LC3B\textsuperscript{F52A,L53A} proteins are part of the same complex, but further than 10 nm apart. Although it is appealing to imagine the larger Venus-LC3B\textsuperscript{F52A,L53A} complex revealed by FPFA might serve as a sink for many LC3B proteins, this is not supported by the molecular brightness data.

In contrast to the F52A and L53A double mutation, the R70A mutant largely disrupted LC3B’s slow diffusion. In addition, the measured stoichiometry of LC3B in complexes containing the R70A mutant is one, similar to Venus and wild-type Venus-LC3B. Thus, the R70A mutation decreases the size of LC3B-associated complexes, while the F52A and L53A mutations increase the size of LC3B-associated complexes as well as the number of LC3B molecules in the complexes (Fig. 6). This was unexpected, since both mutants are less efficient at binding to numerous important LC3 interacting proteins compared with wild type.\textsuperscript{25} If the residues on LC3B’s hydrophobic protein interaction surface were all required for its association with a high molecular weight complex, we would have predicted that both mutants would have smaller hydrodynamic radii compared with wild-type LC3B. The data taken together raise the possibility that the F52A and L53A mutations to LC3B may change the properties of the protein such that it is sequestered in an entirely new complex, whereas the R70A mutation to LC3B nearly completely disrupts its ability to associate with the complex altogether. Furthermore, the complexes appeared to change upon treatment of cells with rapamycin or chloroquine, suggesting they could be modulated by perturbations to the autophagy pathway.

The identity of the components of these soluble LC3B-associated cytoplasmic complexes and their function(s) in the regulation of autophagy or autophagy-independent processes remain to be determined. Given that the F52A and L53A, as well as the R70A mutations interfere with the binding of LC3 to SQSTM1 and NBR1, it seems unlikely that binding to cytoplasmic cargo adaptors, and thus sub-diffraction aggregates could account for the composition of the complexes. Instead, we speculate that these complexes may consist of other LC3B interacting proteins or LC3B interacting protein complexes that may be disrupted due to the R70A mutation. At first glance, the LC3 lipid conjugation machinery is an obvious candidate given that ATG7 is known to homo-oligomerize and form a complex with ATG3 to carry out E1- and E2-like LC3 processing reactions.\textsuperscript{48} The ATG12, ATG5, and ATG16L1 proteins form complex as large as ~800 kDa,\textsuperscript{49} which is thought to function as an E3-like ubiquitin ligase for LC3 lipid modification\textsuperscript{50} and direct LC3 to the site of autophagosome formation.\textsuperscript{51} However, our observation that the diffusion of the LC3B\textsuperscript{G120A} mutant is similar to the wild-type protein, despite the fact that it fails to interact with the LC3 ubiquitin-like conjugation machinery,\textsuperscript{51} suggests the possibility that puncta-independent LC3B constitutively associates with the ATG12–ATG5–ATG16L1 complex is unlikely. There are, however, several other proteins whose binding to LC3B is completely disrupted by mutation of LC3B’s residue R70 that would make for prime candidates.\textsuperscript{25} Future work will be required to determine whether the ~500 kDa LC3B-associated complexes consist of these other known autophagy-related proteins, or perhaps include others of the recently identified LC3 interacting proteins.\textsuperscript{25} One approach that can be used in the future to test for the presence of
specific multiprotein complexes in cells is through the combined use of FRET and FRAP, as we have illustrated previously for the case of complexes containing LC3B and ATG4B\(^{74A}\).\(^{30}\) The study of the interaction of LC3B and ATG4B\(^{74A}\) validated these approaches as a useful means for characterizing the properties of protein complexes under physiological conditions. Intriguingly, LC3 has also been reported to associate with the 3' UTR of FN1 (fibronectin) mRNA,\(^{52}\) and this interaction depends on a triple arginine motif (residues 68 to 70) which overlaps with the R70A mutant analyzed in our current study. Thus, LC3B-associated complexes detected in our experiments could also potentially represent mRNA-containing complexes.

Our findings also allow us to rule out several alternative models that could account for the slow diffusion of Venus-LC3B. First, we found that the measured diffusion of Venus-LC3B\(^{G120A}\) was indistinguishable from wild-type LC3B. This implies that neither lipidation of LC3B nor its ability to bind to autophagosomal membranes contribute to its slow diffusion. Second, we compared the diffusion of Venus-LC3B in live cells under control conditions and following microtubule disruption, as well as in cytoplasmic extracts under conditions that are unfavorable for the maintenance of polymerized microtubules in order to determine if its diffusion is slowed as the result of reversible binding to microtubules. We observed no difference in LC3B’s diffusion in cells following microtubule disruption, and very close agreement between our in vivo measurements and our in vitro measurements for the diffusion of LC3B. This suggests that microtubule binding is not responsible for the slow diffusion of LC3B, at least under basal conditions. Finally, our homo-FRET and brightness results strongly suggest that the slowly diffusing complexes of LC3B do not represent either homo-oligomers\(^{59}\) or aggregates\(^{59}\) of LC3B. These findings imply that if LC3B homo-oligomerization is involved in contributing energy to membrane fusion events in autophagy,\(^{7}\) this event must be limited to autophagosomal membranes.

In this study we utilized 2 complementary fluorescence-based approaches, FRAP and FPFA, to characterize the properties of soluble LC3B-associated complexes. The FPFA and FRAP results were in excellent agreement, lending confidence in the conclusions of our studies. However, like any method, each approach has some limitations. First, both FRAP and FPFA rely on measurements of exogenous, fluorescently tagged versions of the protein of interest. This could have several possible consequences on the interpretation of our findings. Our experiments were performed under conditions where Venus-LC3B is overexpressed, and thus other LC3B-binding partners could potentially become limiting for complex formation. Under these conditions one might expect to find excess monomeric Venus-LC3B, but our data suggest this is unlikely given the combination of our FRAP and FCS measurements failed to show any evidence for such a scenario. On the other hand, one advantage of overexpressing Venus-LC3B is that it is in excess over the endogenous protein, and thus it is likely that the complexes we detect contain primarily the tagged form of the protein. This increases the likelihood that our measurements accurately report the effective size, stoichiometry, and organization of LC3B-associated complexes.

Second, both FRAP and FPFA interpretation requires the fitting of models which carry with them certain a priori assumptions. Often, several competing models can fit a particular data set equally well, e.g., a small percentage of LC3B associating with membranes or a fraction of LC3B reversibly binding to a microtubule network. Therefore, additional experiments are required to distinguish between competing models, such as the use of mutants, drug treatments, or comparisons of in vivo vs. in vitro results as we have done here. Even with such information in hand, there are still circumstances where different models cannot be resolved by an analysis of diffusion alone. For example, although our FRAP and FPFA data were well fit by single-component diffusion models, we cannot conclusively rule out the possibility that there are multiple distinct complexes of similar size present. Similar assumptions are inherent in our modeling of the FPFA brightness measurements; in the case of a mixture of complexes, as we detected for F52A and L53A, we cannot rule out the possibility that the 2-fold increased brightness may have originated from one of the complexes in particular and not both.

Third, although FPFA can potentially provide information about rotational diffusion, and thus yield insights into the size and shape of the diffusing species, our current data cannot be quantitatively analyzed in this way. This is because in our experiments, the Venus label is not rigidly attached to LC3B allowing it to undergo rotational motion independently of LC3B or the LC3B-associated complexes. In addition, the lifetime of the Venus fluorophore (on the order of a few nanoseconds) is not long enough to allow accurate quantitation of rotational diffusion for slowly rotating complexes.\(^{45}\) Thus, our rotational diffusion measurements should not be expected to match our translational diffusion measurements. A rigidly attached fluorophore with a much longer lifetime would be a better choice for measuring the rotational mobility of large complexes.

Lastly, the details of our experimental design should be considered carefully as they also constrain our conclusions in several ways. Our FRAP measurements were specifically designed to avoid LC3B in puncta, and instead focus on the diffuse pool of LC3B independent of bright puncta. Under certain conditions, upregulation of the autophagy pathway for example, the cytoplasm may become full with large numbers of puncta making it difficult or impossible to avoid puncta with a 1 \(\mu\)m radius bleach region. Although this was not a problem under the conditions examined in our study, in the future, methods of analysis may need to be devised in order to extend the range of conditions which can be analyzed by FRAP, for example, the application of reaction and diffusion models\(^{56}\) to accurately quantify autophagy protein turnover rates on puncta. Similarly, our method of cellular extract preparation should be taken into consideration when interpreting our FPFA findings; we prepared them in such a way that LC3B-II incorporated into autophagosomal membranes was either solubilized by detergent and/or excluded from the extract by centrifugation. In the future, FPFA measurements could be performed on LC3-II in autophagosomes using an alternative extraction procedure or using an in vivo configuration in order to test if lipid modified LC3 homo-oligomerizes on autophagosomal
membranes and to follow up on previous stoichiometry determinations for ATG proteins on autophagosomes.

In summary, our data suggest that in the cytoplasm, individual soluble LC3B molecules associate with a ~500-kDa complex and that residues on LC3B’s hydrophobic protein interaction surface are important for regulating its association with these complexes. In the future, we anticipate FRAP and FPFA will become valuable methods for uncovering the emergent properties of protein complexes in the autophagy pathway.

Materials and Methods

cDNA constructs

GFP-LC3B was the kind gift of T Yoshimori. Cerulean and Venus tagged versions of LC3B were as previously described. Venus-LC3B<sup>B70A</sup>, Venus-LC3B<sup>F52A</sup> <sup>153A</sup>, and Venus-LC3B<sup>G120A</sup> mutations were constructed using Stratagene’s QuikChange site-directed mutagenesis kit (Agilent Technologies, Inc., 200523). For LC3B<sup>B70A</sup> the forward and reverse primers were 5’-CATCAA-GATA ATTAGAAGGGC CCTGCAAGCTCA ATGCTAAC-3’ and 5’-GGTACATTG AGCTGCAGGG CCCCCTTCTAAT TATCTGTATG-3’. For LC3B<sup>F52A</sup> <sup>153A</sup> the forward and reverse primers were 5’-GTCTTGGACA AGACCAAGGC CTTGTACCT GATCAGT-3’ and 5’-ACGTGATCAG GTACAGGGC CTTGGTCTTG TTCAAGGAC-3’ respectively. For LC3B<sup>G120A</sup> the forward and reverse primers were 5’-CAGGACAGT TCGCCAGACG ACTGGCT-3’ and 5’-AGCCAGTGCT GTGCGGAACGT CTCTCTG-3’ respectively.

Cell culture and transfections

HeLa cells and HEK 293T cells (American Type Culture Collection, CCL-2 and CRL-1573) were maintained in Dulbecco Modified Eagle’s Medium (DMEM) containing 10% fetal bovine serum, 10% fetal calf serum, 1% PenStrep, and 25 mM HEPES. The cells (24 h after transfection) cell culture medium was rinsed and lysed with passive lysis buffer (Promega, E1941) containing 1% Protease and Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific Inc., 78440). The homogenates were centrifuged at 20,000 RCF for 15 min, and the supernatants were diluted -20-fold with purified water for FPFA to yield a photon count rate between -25 kcps and 100 kcps (> 30× the dark count rate) to avoid TCSPC pile-up artifacts. The clarified homogenates were then loaded into 35 mm glass bottom dishes for measurements at 25 °C.

Analysis of LC3-I to LC3-II processing

The LC3-I to LC3-II processing assay was performed by lysing cells plated in 6-well culture dishes using Celllytic M buffer (Sigma-Aldrich Co. LLC, C2978) as per the manufacturer’s instructions, spinning down at 13,000 × RCF for 20 min, followed by SDS-PAGE of the supernatant fraction. We blotted using antibodies against TUBB (β-tubulin) (Developmental Studies Hybridoma Bank, E7), GFP (JL-8) (Clontech, 632380), and LC3 (Novus Biologicals, NB100-2220).

Drug treatments

In order to block lysosomal degradation of LC3 or upregulate the autophagy pathway, we incubated the cells with either 100 µM chloroquine (Sigma-Aldrich Co. LLC, C6628) or 200 nM rapamycin (Sigma-Aldrich, R8781) for 2 h at 37 °C before performing live-cell imaging or preparing cell extracts. In order to disrupt microtubules in live cells, we incubated the cells on ice with 5 µg/ml nocodazole (Sigma-Aldrich Co. LLC, M1404) for 15 min before transferring to 37 °C for an additional 1 h. The cells were imaged in the continued presence of nocodazole.

Microtubule pelleting assay

We examined the extent of polymerized microtubules in our cytoplasmic extracts using a microtubule pelleting assay. The cytoplasmic extracts were loaded onto a 40% sucrose cushion and spun at 200,000 × RCF for 20 min at 25 °C. Fractions from the pellet and supernatant were analyzed by SDS-PAGE. Although the conditions of the passive lysis buffer are not suitable for maintenance of polymerized microtubules in vitro, we attempted to further depolymerize microtubules or repolymerize tubulin by incubating our extracts with nocodazole (5 µg/ml) for 15 min on ice or with paclitaxel (20 µM) at 37 °C for 15 min before the pelleting assay.

Laser-scanning confocal microscopy and quantification of LC3-associated puncta

Cells were imaged live using a Zeiss LSM 510 confocal microscope (Carl Zeiss Microscopy LLC) equipped with an Argon/2 30 mW laser (458, 488, 514 nm) using an oil immersion 40 × 1.3 N.A. Zeiss Plan-Neofluar objective. The total numbers of bright, Venus-LC3B labeled spots (~0.5–2 µm) obtained in single confocal sections (1 Airy unit) were manually counted in the cytoplasm of each cell and reported as the mean number of cytoplasmic spots per cell.

FRAP methods

Confocal FRAP measurements were performed using a Zeiss LSM 510 confocal microscope (Carl Zeiss MicroImaging Inc.) equipped with an Argon/2 30 mW laser (458, 488, 514 nm) using an oil immersion 40 × 1.3 N.A. Zeiss Plan-Neofluar objective.
Confocal FRAP data were obtained using 512 × 45 pixel images at 4× digital zoom using a 1 Airy unit pinhole at a rate of 42 frames per second. Venus was irreversibly photobleached by iteratively scanning a 514 nm laser (30 mW nominal power) 10 times in a circular ROI with a nominal radius $r_n$ of 0.99 µm, centered in the imaging window. Imaging of the prebleach steady-state fluorescence and the postbleach recovery was performed using a much lower nominal laser power of 0.06 mW. In order to separate excitation and emission light sources we employed several standard filter sets for the Zeiss LSM 510 (HFT 458/514 and LP 530). A total of 600 images were acquired for each recovery (~15 s), and the bleaching event required 129 ms before acquisition of the postbleach image under our conditions.

To analyze the confocal FRAP data we utilized a recently developed analytical equation for simple Brownian motion to extract an instrument-independent diffusion coefficient. The first step in the approach is to plot the mean fluorescence intensity within the bleach region as a function of time to yield a FRAP curve, $I(t)$. We next normalize $I(t)$ by dividing by the prebleach steady-state fluorescence and correct for any unintentional photobleaching encountered during normal imaging. To do this we fit the last 50 data points, at a time sufficiently long enough after the bleach to reach diffusional steady-state, of $I(t)$ to a first order decay process,

$$I(t) = \exp(-k_{\text{decay}} t)$$

(1)

where $t$ is time, and $k_{\text{decay}}$ is the unintentional photobleaching rate constant. $I(t)$ is corrected by dividing by the exponential decay.$^{55}$ Next, the diffusion coefficient $D$ and mobile fraction $Mf$ were found by fitting the data to a 1-component FRAP model for Brownian motion with infinite boundary conditions,

$$I(t) = I_0 \sum_{m=0}^{m=299} \frac{-K^n r_n^2}{m[(r_n^2 + m(8Dt + r_n^2)]} Mf + (1-Mf)I(0)$$

(2)

where $I_0$ is 1 for a normalized FRAP curve, and $r_n$ is the nominal radius of the bleaching ROI.$^{35}$ This is a modified form of the Axelrod equation$^{56}$ where the laser is assumed to be a Gaussian, and the parameters $r_n$ and $K$ take into account the initial conditions for the solution of the diffusion equation. The FRAP model assumes a homogenous distribution of molecules were bleached along the Z-axis resulting in diffusional exchange along the X and Y axes. We determined $r_n$ and $K$ by fitting the normalized radial post-bleach profile, $I(x; t = 0)$, to an analytical approximation,

$$I(x; t = 0) = I_0 \exp\left(-\frac{2x^2}{r_n^2}\right)$$

(3)

where $I_0$ is 1 for a normalized post-bleach profile, and $x$ is the radial distance from the center of the bleaching ROI.$^{57}$

For comparison, we also analyzed a subset of the data using a newly described simplified FRAP equation for Brownian motion.$^{54}$ In this approach, $I(x; t = 0)$ was further approximated as,

$$I(x; t = 0) = 1 - K \exp\left(-\frac{2x^2}{r_n^2}\right)$$

(4)

resulting in the simplified FRAP model,

$$I(t) = I_0 \left[1 - K \left(1 + \frac{r_n^2}{r_e^2} + \frac{8Dt}{r_e^2}\right)^{-\frac{1}{2}}\right] Mf + (1-Mf)I(0)$$

(5)

A comparative analysis of these 2 analytical FRAP models (Eqns. 2 and 5) revealed no significant differences in $D$ or $Mf$ with the level of noise present in our data. This means using Equation 5 is the preferred method of extracting diffusion coefficients and mobile fractions, as it greatly reduces the complexity in programming a nonlinear fitting routine. Furthermore, if there is a priori knowledge that the fluorescence recovery is dominated by Brownian motion rather than reaction kinetics, and the system was allowed to relax back to a steady-state one could use linear interpolation to find the half time of the recovery ($\tau_{1/2}$) and resultant $D$ without any fitting required using

$$D = \frac{r_n^2 + r_e^2}{8r_{1/2}}$$

(6)

Where indicated, $D$ values were used to calculate the Stokes radius using the Stokes-Einstein equation:

$$D = \frac{k_BT}{6\pi\eta r}$$

(7)

where $k_B$ is Boltzmann constant, $T$ is temperature, $r$ is the Stokes radius, and $\eta$ is viscosity. The apparent molecular weight $MW$ was then calculated as follows (assuming a spherical geometry):

$$MW = MW_{\text{GFP}} \left(\frac{D_{\text{GFP}}}{D}\right)^3$$

(8)

where $MW_{\text{GFP}}$ is the molecular weight of GFP (27 kDa) and $D_{\text{GFP}}$ is the measured diffusion coefficient of the GFP control.

In principle, a freely diffusing control such as Venus should be 100% mobile on the time scale of our experiments as indicated by $Mf$. However, under the conditions of our experiments a significant fraction of molecules in the cytoplasm were irreversibly destroyed during the experiment resulting in $Mf$ values less than 1. In order to correct for the significant loss of fluorescence in the compartment due to the bleaching event, we calculated the true mobile fraction ($Mf_{\text{correct}}$) by,
\[ M_{\text{correct}} = 1 - (I_{\text{adjacent}}(t) - I(t)) \]  

where \( I_{\text{adjacent}}(t) \) is the intensity inside an ROI placed adjacent to the bleaching ROI.

**FPFA methods**

The experimental setup and validation are described elsewhere. Briefly, an 80-MHz, 200-fs mode locked Ti:sapphire laser (Coherent Chameleon Ultra-2, Santa Clara, CA) operated at 950 nm provided pulsed 2-photon excitation. The excitation beam, passed through a power attenuator, a spatial filter system (Thorlabs, KT310), a near-IR linear polarizer (100,000:1 extinction ratio, Thorlabs), and finally a multiphoton short-pass dichroic beamsplitter (Semrock, FF670-SDi01-25x36) was used to reflect the excitation beam to a Zeiss 63X-1.2NA water objective (back aperture slightly overfilled). This setup focused the beam to a diffraction-limited spot (~0.4 \( \mu \text{m} \) in diameter). The excitation power was kept low (typically ~10.2 mW at the focal point) to avoid bleaching during acquisition (150 to 200 s).

Fluorescence collected from the observation volume in the sample was guided through a BG39 filter (to block residual near-IR photons), a high throughput band-pass filter (Semrock, FF01-540/50-25), and finally a polarizing beam splitter (Thorlabs) augmented with 2 orthogonally oriented linear polarizers (Thorlabs) to increase the extinction ratio. At the beam splitter, parallel and perpendicular emitted photons were separated and focused onto 2 HPM-100-40 hybrid detectors (Becker and Hickle GmbH, Berlin, Germany). The dark count rate for these detectors was typically 200 to 750 cps at 25 °C. Photons detected by each detector were processed by a SPC-132 TCSPC card (Becker and Hickle). For synchronization between excitation pulses and detected photons, a small fraction of the excitation beam was extracted and focused onto a high-speed photodetector (DET10A, Thorlabs) powered by a battery to avoid crosstalk. Note that all optics used in the excitation pathway were selected to minimize group delay dispersion.

For each homogenate, 3 to 5 replicate measurements were performed and these were averaged for each point. All measurements were performed at room temperature.

SPCM software (Becker and Hickle) running in FIFO mode was used to calculate time-resolved fluorescence and cross-correlation functions. Time-resolved anisotropy was calculated based on fluorescence decay of parallel and perpendicular channels using the following equation:

\[ r(t) = \frac{I_p(t) - g \cdot I_{\perp}(t)}{I_{\parallel}(t) - 2g \cdot I_{\perp}(t)} \]  

where \( I_{\parallel}(t) \) and \( I_{\perp}(t) \) are the fluorescence intensity of parallel and perpendicular channels (dark noise subtracted) respectively, and \( g \) is an instrument correction factor which for our microscope had a value of 0.96 as determined by tail fitting calibration measurements of fluorescein.

For FPFA, independently measured emission events detected in the parallel and perpendicular light paths were cross-correlated, \( G(\tau) \). The time dependence of these curves were initially fit using a single-component 3-dimensional (3D) diffusion equation to estimate the value of \( <N> \), the average number of fluorescent molecules in the excitation volume, and \( \tau_D \), the correlation time (the average time that a molecule spends in the detection volume) for each sample:

\[ G(\tau) = \frac{1}{\langle N \rangle} \left[ \frac{1}{1 + \left( \frac{\tau}{\tau_D} \right) \left[ 1 + \left( \frac{\omega}{z} \right)^2 \left( \frac{\tau}{\tau_D} \right) \right] \right] \]  

where \( \omega \) and \( z \) are the radial and axial beam waists respectively calculated from calibration experiments, and the constant \( \gamma \) has a value of 0.35 for a 3-dimensional Gaussian PSF.

The molecular brightness \( b \) is the average number of photon emitted per second per molecule (cpsm)

\[ b = \langle k \rangle / \langle N \rangle \]  

where \( <k> \) is the average photon count rate recorded during data acquisition.

To model a mixture of 2 species having the same brightness but independent diffusion times:

\[ G(\tau) = \frac{\gamma}{\left( \langle N_1 \rangle + \langle N_2 \rangle \right)^2} \left( \frac{1}{1 + \left( \frac{\tau}{\tau_{D1}} \right) \left[ 1 + \left( \frac{\omega}{z} \right)^2 \left( \frac{\tau}{\tau_{D1}} \right) \right]} + \left( \frac{1}{1 + \left( \frac{\tau}{\tau_{D2}} \right) \left[ 1 + \left( \frac{\omega}{z} \right)^2 \left( \frac{\tau}{\tau_{D2}} \right) \right]} \right) \]  

In this case,

\[ b_1 = b_2 = \frac{\langle k \rangle}{\langle N_1 \rangle + \langle N_2 \rangle} \]  

With 2-photon excitation the relationship between correlation time \( \tau_D \) and \( D \) is given by:

\[ \tau_D = \frac{\omega^2}{8D} \]  

**FPFA calibrations**

The instrument correction factor \( g \) for calculating time-resolved anisotropy (Eqn. 10) was measured using tail-fitting
of fluorescein samples and found to be 0.96. The value of $\omega/z$ for our microscope (used in Eqs. 11 and 13) was 0.1 at an excitation power of 10.2 mW (at 950 nm) using calibration standards of fluorescein in water at pH 9.5. Assuming fluorescein has a diffusion coefficient of 300 $\mu$m$^2$/s, using the measured correlation time of 75 ± 9 $\mu$s, the value of $\omega$ was 424 ± 30 nm. The validity of this calibration procedure was confirmed by measuring the diffusion coefficient of Venus monomers under identical conditions. Using $\omega/z = 0.1$, the measured correlation time for Venus monomers with 10.2 mW excitation power was 343 ± 17 $\mu$s (n = 3). This corresponds to a diffusion coefficient for Venus monomers in solution of 65.5 ± 8.6 $\mu$m$^2$/s (n = 3), in good agreement with the diffusion coefficient measured for GFP.\(^\text{10}\)

**Curve fitting and statistics**

IGOR Pro software (Version 6.22) was used for fitting of time-resolved anisotropy, cross-correlation curves, and for global fitting of calibration standards. FRAP data was fit using custom routines written in MATLAB based on the trust-region-reflective algorithm (TheMathWorks; R2010B). The errors reported throughout the text are 95% confidence intervals unless otherwise stated. To test the hypothesis that 2 group means are not different, under circumstances where there were multiple groups being compared, we used the Bonferroni method to control the overall type-I error rate such that for all pairs of comparisons the familywise significance level was 0.05. For example, if 4 groups are being compared the total number of comparisons is 6, thus the $P$ value cutoff is 0.05/6 = 0.008.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Acknowledgments**

We are grateful for the constructive criticism and advice from the other members of the Kenworthy laboratory and to Dr Christian Behrends for helpful discussions. This work was funded by grant NSF/DMS 0970008 from the National Science Foundation, by the intramural program of the National Institutes of Health, National Institute on Alcohol Abuse and Alcoholism, Bethesda, MD, and utilized the core(s) of the Vanderbilt Diabetes Research and Training Center funded by grant DK020593 from the National Institute of Diabetes and Digestive and Kidney Disease. The funding sources had no role in the study design, collection, analysis or interpretation of data, writing the report, or the decision to submit the paper for publication.

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