Transport between im/mobile fractions shapes the speed and profile of cargo distribution in neurons

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ABSTRACT Neuronal function requires continuous distribution of ion channels and other proteins throughout large cell morphologies. Protein distribution is complicated by immobilization of freely diffusing subunits such as on lipid rafts or in postsynaptic densities. Here, we infer rates of immobilization for the voltage-gated potassium channel Kv4.2. Fluorescence recovery after photobleaching quantifies protein diffusion kinetics, typically reported as a recovery rate and mobile fraction. We show that, implicit in the fluorescence recovery, are rates of particle transfer between mobile and immobile fractions (im/mobilization). We performed photobleaching of fluorescein-tagged ion channel Kv4.2-sGFP2 in over 450 dendrites of rat hippocampal cells. Using mass-action models, we infer rates of Kv4.2-sGFP2 im/mobilization. Using a realistic neuron morphology, we show how these rates shape the speed and profile of subunit distribution. The experimental protocol and model inference introduced here is widely applicable to other cargo and experimental systems.

INTRODUCTION

Individual cells contain tens of millions of proteins with specialized and site-specific functions.1,2 Transport of these proteins among other cell contents is thus a challenging endeavor, complicated by limited cargo half-lives and the complex intracellular environment. This problem is exacerbated in neurons, which are among the longest, most asymmetric cells.3,4 Delivery and constant replenishment of crucial proteins are integral to the workings of neurons, yet the speed, distribution profile, and mechanisms of protein distribution are not well understood.5 Passive diffusion is one such method of cargo transport that occurs through a number of energy-efficient modalities. Globular proteins can freely diffuse in the cytosol. Integral membrane proteins, including ion channels, diffuse through the cytosol in vesicles composed of a phospholipid bilayer.6–8 The plasma membrane also has fluid-like properties on which ion channels can flow. Originally described as the fluid mosaic model, proteins and other molecules embedded in the plasma membrane undergo lateral diffusion through lipid- and glycoprotein-dense subregions with variable fluidity and elasticity.9,10 In this report, we study the transport dynamics of these lumped modalities in primary neurons using a novel analysis of photobleach recovery.

Fluorescence recovery after photobleaching (FRAP) is an optical microscopy technique for quantifying diffusion kinetics of fluorescent molecules.11 In a typical FRAP experiment, a small region of a fluorescent sample is exposed to high intensity laser such that the molecules of interest no longer emit fluorescence—a process known as photobleaching. After photobleaching, a time series is recorded as bleached fluorophores...
diffuse out of and unbleached fluorophores diffuse into the region of interest. Fluorescence intensity $I$ over time $t$ is normalized and fitted to an exponential curve $I = A(1 - e^{-\tau t})$ to approximate recovery rate $\tau$, which estimates diffusion coefficient. The molecules that contribute to fluorescence recovery comprise the mobile fraction $A$. The immobile fraction $1 - A$ consists of molecules in the bleach region that are immobilized.

The simple model of photobleaching and recovery in standard FRAP analysis makes a few critical assumptions. First, recovering particles are assumed to have only two degrees of mobility. Particles are either mobile or immobile. Mobile particles can diffuse into or out of the bleach region with some measurable $D$. Immobile particles can have a sufficiently low $D$ such that they might not exit the bleach area during an hour-long image acquisition period. Second, the mobility state (mobile or immobile) is assumed persistent and unchanging over the course of an entire experiment. Immobile fluorescent particles cannot mobilize, and mobile particles cannot immobilize. Although these assumptions simplify the analysis, true diffusion on the plasma membrane or in the intracellular environment is more complex.

In this study, we have devised a method of FRAP analysis that considers dynamic particle transitions between mobile and immobile states (im/mobilization). There are well-established physiologic mechanisms observed in experiments to support this model. For instance, integral membrane proteins are constantly trafficked within and between regions of varying mobility. Channels and receptors are shuttled into or out of postsynaptic densities to potentiate or depress synapses. Ion channels in the membrane or in the intracellular environment is more complex.

A suitable candidate cargo for this study is ion channel Kv4.2 in primary hippocampal neurons. Kv4.2 conducts A-type, voltage-gated transient potassium current abundant in dendrites and has a hypothesized role in dendritic integration. Kv4.2 is firstly compelling since its transport is not completely understood, yet its unique subcellular distribution is critical for maintaining cellular excitability. Further, experiments showing Kv4.2 regulation within dendritic spines suggests a potential dynamism between mobile and immobile states. There exists evidence of Kv4.2 interactions with auxiliary subunit KChIP2 and with ion channel Cav2.3, which modulate function and mobility, including interactions with lipid rafts. Lastly, in a study of active Kv4.2 transport, our group has identified vesicles containing Kv4.2 that remain static for over hour-long recordings, suggesting the possibility of a prolonged immobile state.

We designed a model of strongly enhanced green fluorescent protein-tagged Kv4.2 (henceforth referred to as Kv4.2-sGFP2) photobleach and recovery. Rather than studying individual mechanisms, we lump together all subunit interactions and categorize Kv4.2-sGFP2 as mobile or immobile. We crucially allow for interchange between these states, extending this model beyond standard FRAP analysis, allowing us to estimate imm/mobilization rates. We lastly investigate how the inferred rates impact the settling time and localization profile of Kv4.2-sGFP2 by simulating a full neuron morphology. The global intracellular distributions of Kv4.2 and other ion channels are of interest since these proteins are essential to regulating neural functioning and maintaining homeostasis.

The described protocol is widely generalizable and applicable to FRAP experiments. This method estimates an otherwise unapparent transport rate without additional constructs or experimental setups.

**MATERIALS AND METHODS**

Experimental and computational methods are described in full elsewhere and are summarized briefly here.

**Animals and cell culture**

Hippocampal cultures were prepared from Sprague-Dawley rats in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals under a protocol approved by the National Institutes of Child Health and Human Development’s Animal Care and Use Committee as described elsewhere and briefly summarized here. Dissection media was composed of 50 mL 10× Hanks’ balanced salt solution (14185-052; Gibco, Gaithersburg, MD, USA), 5 mL penicillin/streptomycin (15140122; Gibco), 5 mL pyruvate (11360070; Gibco), 5 mL HEPES (1 M, 15630080; Gibco), 15 mL 1 M stock solution glucose (from powder; Sigma-Aldrich, St. Louis, MO, USA), and 420 mL Ultra Pure Water (KD Medical, Columbia, MD, USA) and filter sterilized. After mixing with papain (Worthington Biochemical, Lakewood, NJ, USA) for 45 min, tissues were rinsed with dissection media several times before plating in neurobasal media (Thermo Fisher Scientific, Waltham, MA, USA) with 5% fetal bovine serum (HyClone characterized fetal bovine serum, SH30071.03; GE Healthcare LifeSciences, Pittsburgh, PA, USA), 2% GlutaMAX (Thermo Fisher Scientific), and 2% Gibco B-27 supplement (Thermo Fisher Scientific). Cells were incubated in 5% CO$_2$ at 37°C. After 24 h, cells were transferred to neurobasal media containing 2% GlutaMAX and 2% Gibco B-27 supplement, and half the media were replaced every 3 to 4 days for 9 to 13 days until terminal experiments.

Kv4.2-sGFP2 was transfected and incubated for a total of at least 5 h before imaging. pSGFP2-C1 was a gift from Dorus Gadella (plasmid #22881; Addgene, Watertown, MA, USA; http://www.addgene.org/22881; RRID: Addgene_22881).
Lipofectamine 2000 transfection was performed as follows: 2 μL Lipofectamine 2000 Transfection Reagent (Thermo Fisher Scientific) and 2 μg DNA plasmid were each diluted in 200 μL neurobasal media and incubated for 5 min. The two solutions were then combined and incubated for 15–20 min. 100 μL total mixture was added to each well and incubated at 37°C for 4 h before changing media. Cells were then incubated for an additional minimum of 1 h before imaging.23

Microscopy

All imaging was carried out at the National Institutes of Child Health and Human Development Microscopy and Imaging Core using a Zeiss Laser Scanning Microscope 710 confocal microscope (Carl Zeiss Microscopy, White Plains, NY, USA). 18-mm coverslips were removed from wells and placed in a Quick Release Chamber (QRC-41LP, 64-1944, Warner Instruments, Hamden, CT, USA). Cells were immersed in 800 μL imaging buffer consisting of 1× Tyrode’s solution: 135 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 25 mM HEPES, and 10 mM glucose (all Sigma-Aldrich) (pH 7.4). Time series were performed using a 63× oil-immersion objective with with 1.4 numerical aperture, pinhole diameter at 4 Airy units, 495-nm laser line at 100% power for 10 iterations, which reduced baseline intensity by 30% for linear, and unbranching segments of hippocampal dendrites (as described in results).

We then solve for parameters α, ω, v_m, and v_m, using this model and individual bleach recovery curves. We perform the model fit using linear gray-box model estimation tools in MATLAB, as in results and depicted in Fig. 3 B.

We use a similar mass-action model to simulate particle transport in full neuron morphologies, where each compartment denotes a physical location. For each dendrite compartment, such that as depicted in Fig. 5 B, we formulate a differential equation describing all particle input and output (Eq. 4). The cargo transport rates between compartments can be estimated from our experimental data. We first made a one-dimensional assumption, where dendritic cargo is averaged in the radial direction and transport is assumed to occur only in the longitudinal direction. Cargo transport in this model is governed by the drift-diffusion equation

\[
\frac{\partial m(x, t)}{\partial t} = D \frac{\partial^2 m(x, t)}{\partial x^2} + v \frac{\partial m(x, t)}{\partial x} - w m(x, t), \tag{1}
\]

where \(m(x, t)\) is some mass, in this case subunits of Kv4.2-sGFP, as a function of space \(x\) and time \(t\). \(D\) is the diffusion coefficient, \(v\) is the drift coefficient, and \(w\) is the cargo degradation rate. This equation is derived from the random walk master equation using finite difference approximation, as shown in the supporting material of our previous work.23

The diffusion coefficient \(D\) is a fixed constant with units μm²/s representing the rate of particle transport from an area of high concentration to an area of low concentration. \(D\) in this context can be estimated from our FRAP data, as described in results and briefly summarized here. Starting with one-dimensional drift diffusion (Eq. 1), Scott et al. derive a formula for the diffusion of fluorescent particles in neurites based on bleach region length and recovery rate.30 This formula (Eq. 2 in results), is used to obtain realistic estimates of \(D\). A full derivation of Eq. 2 is also found in appendix A.2 of our previous work.23 \(D\) is then discretized to produce transport rates for full-neuron simulations, as described in results and in detail elsewhere.4

RESULTS

Schematic of photobleaching reveals that mobile fraction is dependent on im/mobilization rates

In this study, we address how fluorescence recovery measured during photobleaching reveals rates of cargo im/mobilization. We begin by discussing a conceptual model of our experimental setup to define terms and to illustrate our analysis.

A region containing proteins of interest (yellow circles) and immobile substrate (blue ribbons) is depicted in Fig. 1 Aii. \(x_{\text{mob}}\) denotes mobile particles that are allowed to diffuse freely with rate \(v_d\). \(x_{\text{imm}}\) denotes immobile particles that are bound to a static structure with restricted diffusion. Mobilization rate \(v_{i,m}\) describes detachment from an immobile structure, i.e., protein transfer from \(x_{\text{imm}}\) to \(x_{\text{mob}}\). It is important to note that these rates are lumped parameters in a coarse-grained model, and they represent the aggregate of several biophysical mechanisms. For an ion channel, this biologically corresponds to exiting a lipid raft or postsynaptic density that was previously restricting mobility. The reverse process is immobilization: \(v_{m,i}\) which

Image analysis

Raw microscope time series were imported into ImageJ for image processing and analysis. Individual dendrites were selected as a segmented line with thickness adjusted to individual dendrite diameter (7–12 pixels), from which a kymogram was generated using the plugin KymoResliceWide. Neurite selection and kymogram generation is outlined and depicted in Fig. S1 of our previous study.23 A sample resultant kymogram is also depicted in Fig. 4 Ci. All kymograms were saved as TIFF files, and all were imported into MATLAB (MathWorks, Natick, MA, USA) for further processing and model fitting.

Modeling

We perform a steady-state analysis for the simplified categorical model in Fig. 3 Aii, as described in results.

The cargo content of each category is defined by a differential equation that sum the quantities of cargo entering and exiting that category. For instance, a generalized rate \(v_{i,r}\) from a donor \(d\) to receiver \(r\) transfers an amount of mass \(x_{i,d} v_{i,r}\). For instance, the complete system of differential equations for the two categories in Fig. 3 Aii is as follows:

\[
x_{\text{mob}}^{\text{int}} = u + x_{\text{mob}}^{\text{int}} v_{i,m} - x_{\text{mob}}^{\text{int}} (v_d + v_{m,i}) \quad \text{and} \quad x_{\text{imm}}^{\text{int}} = x_{\text{mob}}^{\text{int}} v_{i,m} - x_{\text{mob}}^{\text{int}} v_{m,i}.
\]
describes protein transfer from $x_{\text{mob}}$ to $x_{\text{imm}}$, such as an ion channel entering a restrictive region. The depicted region is a neurite subsection (Fig. 1 Aii) in a larger arborization like a dendritic tree (Fig. 1 Aiii). A localized region (Fig. 1 Ai) is bleached by a high intensity laser (yellow trapezoid). During photobleaching, cargo is bleached, which reduces the total fluorescence intensity of cargo (iv). (B) Examples of photobleaching with (i) $v_{m,i} > v_{i,m}$ and (ii) $v_{m,i} < v_{i,m}$ are depicted. Photobleached proteins accumulate on static structures in (i), resulting in a low mobile fraction $A$ at steady state compared with (ii). For illustration purposes, all particles in the photobleach region are bleached. FRAP experiments yield a measurement of fluorescence intensity and recovery, such as that depicted in (iii) for high and low $A$. 

**FIGURE 1** Schematic of recurrent photobleaching reveals increasing mobile fraction with low immobilization rates. (A) Our experimental setup with defined rates and states is depicted (i). The region of interest is part of a dendrite (ii) from a larger dendritic arbor (iii). This region is photobleached, which reduces the total fluorescence intensity of cargo (iv). (B) Examples of photobleaching with (i) $v_{m,i} > v_{i,m}$ and (ii) $v_{m,i} < v_{i,m}$ are depicted. Photobleached proteins accumulate on static structures in (i), resulting in a low mobile fraction $A$ at steady state compared with (ii). For illustration purposes, all particles in the photobleach region are bleached. FRAP experiments yield a measurement of fluorescence intensity and recovery, such as that depicted in (iii) for high and low $A$. 

Schematic of photobleach and recovery with

- **i** $v_{m,i} > v_{i,m}$ → low $A$
- **ii** $v_{m,i} < v_{i,m}$ → high $A$

Measurement of photobleach and recovery

- **iii** Measurement of photobleach and recovery
bleached with rate $v_b$ and fades in color (Fig. 1 Aiv, dark particles). This approximates discrete periods of photobleaching performed experimentally, as described in materials and methods.

Two examples with unequal im/mobilization rates $v_{m,i} > v_{i,m}$ and $v_{m,i} < v_{i,m}$ producing contrasting effects are depicted in Fig. 1 B. Both examples undergo bleaching of all fluorescent cargo in the region of interest. Then, bleached cargo that is mobile, $\lambda_{mob}$, recovers through diffusion.

In the case of high immobilization ($v_{m,i} > v_{i,m}$, Fig. 1 Bii), most cargo is static and does not freely diffuse into/out of the bleach region. The majority of cargo after recovery is bleached and immobilized, resulting in a observed low mobile fraction $A$ during FRAP.

Now, consider the example with low immobilization ($v_{m,i} < v_{i,m}$, Fig. 1 Bii). During recovery, most cargo is mobile and can freely diffuse into/out of the bleach region. The observed mobile fraction $A$ for proteins with high mobilization is higher. These quantities can be measured using FRAP microscopy, and sample traces for cases with low and high $A$ (corresponding to Fig. 1 Bi and Bii, respectively), are depicted in Fig. 1 Biii.

Rates of im/mobilization and diffusion determine the speed $\tau$ and degree $A$ of fluorescence recovery.
FIGURE 3  Im/mobilization rates are inferred from mass-action model of recurrent photobleach recovery. (A) A mass-action model of photo-bleaching categorizes cargo according to mobility and fluorescence (i). A variant of the model (ii) is fitted to experimentally observed fluorescence for constraining rates. (B) The results of model fitting to >bin1,000 bleaches are depicted as distributions $u$ and $v_d$ in (i) and $v_{m,i}$ and $v_{m,i}$ in (ii). A comparison of rate fits for first to third bleaches are depicted in (iii)–(vi). (C) $v_{m,i}$ and $v_{m,i}$ fits from individual recoveries are paired, showing $v_{m,i} > v_{m,i}$ with significant variability. (D) Model fits with at least 70% accuracy are used as rate constraints.
Pursuing this principle, we design a model to infer $v_{m,i}$ and $v_{i,m}$ from FRAP recording.

**Mobile fraction of Kv4.2 increases with subsequent photobleaching**

To establish reliable estimates of FRAP mobile fraction and im/mobilization rates, we performed hour-long recordings with recurrent photobleaching in over 450 dendrites of rat hippocampal cells. Each dendrite was bleached multiple times, and each photobleach diminished fluorescence intensity by 30%–70%, with bleach intervals ranging from 5 to 20 min. Example traces of fluorescence intensity during these time series are depicted in Fig. 2, A and B (with more detail in Fig. S1), where regions of dendrites were photobleached every 1,000 s. Individual recoveries resemble prototypical FRAP curves,\textsuperscript{13,14} to which single exponential curves were fitted to obtain recovery rates $\tau_i$ and mobile fractions $A_i$ for each bleach iteration indexed with $i$.

Of 308 dendrite photobleached at least thrice, 193 recordings (62%) exhibited a monotonic increase in successive $A_i$. One such example is depicted in Fig. 2 A, with more detail in Fig. S1 A. This trend is consistent with low cargo immobilization (Fig. 1 Bii). However, this is not always the case. 113 recordings (37%) showed no definitive increase or decrease in successive $A_i$, such as in Fig. 2, B and S1 B, which indicates high cargo cimmobilization (Fig. 1 Bi). Lastly, two recordings (<1%) exhibited a monotonic decrease in successive $A_i$, which can likely be attributed to experimental error and/or noise. The aggregate data (Fig. 2...
have a general trend of increasing $A_i$ with moderate variability. The median ± standard deviations for $A_i$ for $i = 1$ to 4 are $0.30 ± 0.12, 0.49 ± 0.17, 0.58 ± 0.20$, and $0.69 ± 0.16$.

The aggregate trend for $\tau_i$ differs, as depicted in Fig. 2 D. The median ± standard deviations for $\tau_i$ for $i = 1$ to 4 are $0.0049 ± 0.0028, 0.0035 ± 0.0019, 0.0035 ± 0.0017$, and $0.0031 ± 0.0014$ s$^{-1}$. The initial photobleach results in faster recovery than successive photobleaches: $\tau_1 > \tau_2$. This is possibly explained by the close proximity of a high concentration of fluorescent protein outside the photobleached region. During subsequent bleaches, fluorescent protein outside the bleached region is depleted. Subsequent bleaches
have similar $\tau$, with decreasing variance, which might indicate the cell achieving a steady state of fluorescent protein flow into the photobleached region.

Given the hour-long recording periods with extensive exposure to laser both for imaging acquisition and and photobleaching, it is reasonable to suspect that dendrites might be photodamaged during experiments. In order to control for this possibility, we perform additional extended experiments during which dendrites are exposed to a four- to fivefold increase in laser energy. Six dendrites were imaged for over 10 h at 0.1 Hz with photobleaching every 50 min; all other imaging parameters were held constant. The resultant parameters $A$ and $\tau$ for each bleach iteration are depicted in Fig. S2 and are consistent with those in Fig. 2, C and D. $A$ remains stable or increases slightly for the first few bleaches before remaining stable, and $\tau$ is not significantly changed. These recovery parameters do not significantly change for all dendrites undergoing the extended experiment, as might be expected if imaging exposure caused photodamage that affected passive diffusion. Further, our previous study with the same experimental setup found no significant difference in active transport during extended imaging acquisition. These results demonstrate that photodamage to the dendrite affecting diffusion is unlikely.

We have amassed a sizable dataset of fluorescence intensity during recurrent photobleaching using ion channel Kv4.2-sGFP2 as candidate cargo (Fig. 2). We also have a working theory on how these data might reflect im/mobilization rates that are otherwise difficult to measure (Fig. 1). We next develop a mathematical model to make sense of this data and theory.

**Im/mobilization rates and im/mobile fractions are inferred from a mass-action fluorescence recovery model**

We have thus far presented a conceptual model of cargo im/mobilization with photobleaching (Fig. 1) and experimental data of Kv4.2 subunits undergoing this scheme in hippocampal dendrites (Fig. 2). We now formalize these results in a mass-action model constrained to our empirical observations. We subsequently infer rates of Kv4.2 im/mobilization that are otherwise difficult to measure (Fig. 1). We next develop a mathematical model to make sense of this data and theory.

In a region of interest, proteins are categorized by fluorescence and mobility into four categories: $x_{\text{mob}}^{\text{unbl}}, x_{\text{imm}}^{\text{unbl}}, x_{\text{mob}}^{\text{blea}}, \text{ and } x_{\text{imm}}^{\text{blea}}$, depicted in Fig. 3 Ai. Superscripts $\text{unbl}$ and $\text{imm}$ correspond to unbleached and bleached proteins, respectively. Subscripts $\text{mob}$ and $\text{imm}$ indicate mobile and immobile proteins, respectively. As in the conceptual model, proteins are mobilized ($x_{\text{imm}} \rightarrow x_{\text{mob}}$) and immobi-

lized ($x_{\text{mob}} \rightarrow x_{\text{imm}}$) with rates $v_{im,j}$ and $v_{mi,j}$, respectively. Mobile proteins diffuse freely with rate $v_d$ and can disperse out of the region of interest into the external region—that is, the rest of the neuron. $x_{\text{mob}}^{\text{unbl}}$ enters the region of interest with rate $u$.

Photobleaching ($x_{\text{mob}}^{\text{unbl}} \rightarrow x_{\text{mob}}^{\text{blea}}$) occurs in discrete events with high-intensity laser power. We therefore model photobleaching rate $v_b$ as a series of impulses ($\delta$ function), between which fluorescence recovery $f$ is modeled as an impulse response. We therefore use a variant of the model without photobleaching (Fig. 3 Aii) for fitting to individual fluorescence recoveries.

The quality of model fits is depicted in Fig. 3 D, where the percent fit is defined as the complement of normalized root mean-squared error (NRMSE) expressed as a percentage: percent fit $= (1 - \text{NRMSE}) \times 100\%$, which provides a measure of model fit accuracy. This analysis shows that the vast majority of fluorescence recoveries had reasonably accurate fits, suggesting that our model sufficiently captures this trafficking phenomena. We used parameter fits with $>70\%$ accuracy for all further analysis.

The results of these fits are depicted as distributions in Fig. 3, Bi and Bii. Mean rates were $u = 0.0021$ units/s, $v_d = 0.0063$ s$^{-1}$, $v_{im} = 0.00066$ s$^{-1}$, and $v_{mi} = 0.0016$ s$^{-1}$. Median rates were $u = 0.0018$ units/s, $v_d = 0.0044$ s$^{-1}$, $v_{im} = 0.00073$ s$^{-1}$, and $v_{mi} = 1.7 \times 10^{-7}$ s$^{-1}$. We also compare rate estimates from first, second, and third bleaches (Fig. 3, Biii–Bvi), which revealed no significant differences between photobleach iterations. This supports our modeling as a linear, time-invariant system.

It is important to note that although we report medians and means for these parameters, there is substantial overlap between their distributions of model fits. None of these rates are substantially larger than the others. We further depict the amount of variability between $v_{im}$ and $v_{mi}$ in Fig. 3 C to illustrate this point. We later use these data to show that even minimal differences in these parameters, when spread across a large neuron, can have global differences in the distribution of particles.

**Intersample variability does not strongly correlate with fluorescence recovery parameters**

We now focus on photobleach recovery parameters in the context of variable experimental and biological parameters.

Cultured neurons are highly variable in structure, size, and—in transfected expression systems—fluorescence. It is reasonable to assume that the variability in our data (Fig. 2, C and D) is attributable to fluctuations in these metrics.
To address this, we query whether this data variability is explained by intrinsic variability in our experimental setup. We plot recovery parameters \( \tau \) and \( A \) against measured experimental parameters in Fig. 4 A. Bleach region length (Fig. 4 A, first column) is the total length of dendrite over which fluorescence recovery is measured. Fluorescence intensity (second column) is the total unbleached content prior to the first bleach. Distance from the soma (third column) is measured from the proximal end of the bleach region to base of the dendritic trunk. Branch degree (fourth column) refers to the number of branches from the soma, where primary is the apical dendrite, secondary is a branch of the primary, etc.

Each of these experimental parameters vary greatly in the data set, but all seem to have weak correlations with \( \tau \) and \( A \). The coefficients of determination \( R^2 \) for linear fits between these parameters (bleach length, prebleach intensity, distance from soma, branching degree, and drift slope) and \( \tau \) are 0.0129, 0.0254, 0.0058, 0.0015, and 0.00070, respectively. \( R^2 \) for linear fits with \( A \) are 0.0656, 0.0366, 0.0231, 0.0225, and 0.0072, respectively. This indicates that bleach recovery parameters do not correlate strongly with experimental parameters, indicating that reducing this experimental variability in our experimental setup would not have significantly altered the derived \( A \) and \( \tau \).

We do find moderate variability between \( A \) and each bleach region length and distance from soma using a reciprocal (multiplicative inverse) fit. These fits are depicted in Fig. S3 and have \( R^2 \) of 0.0983 and 0.0925, respectively. An increasing \( A \) with a narrower bleach region length is intuitive since a narrower bleach length contains less Kv4.2-sGFP2 and is thus replenished more quickly and fully. A decreasing \( A \) with distance from the soma might result from the bulk of mobile Kv4.2-sGFP2 particles being produced in the soma.

The strongest correlation was found between the recovery parameters \( \tau \) and \( A \) themselves, depicted in Fig. 4 B with a reciprocal fit. The equation depicted is \( A = 0.144 / \tau^{0.433} \) with \( R^2 \) of 0.2837, stronger than all previous fits. This suggests that dendrites with high \( A \) have slower recovery (smaller \( \tau \)). One possible explanation is that cargo mobility is dependent on fluorescence recovery source. Dendrites with small fluorescence recovery fractions (low \( A \)) might recover protein only from a single rapid source (e.g., cytosol diffusion). Dendrites with large recovery fractions (high \( A \)) might recover particles from multiple sources (e.g., membrane, microtubule, and cytosol transport) as discussed in the introduction. Since some transport is slower, these dendrites may have smaller \( \tau \). It might be possible to differentiate between these transport rates by fitting bleach recovery to double exponential curves. Nonetheless, this demonstrates that the measured variables are more strongly correlated with each other than with experimental parameters, suggesting a potential intrinsic relationship between \( A \) and \( \tau \).

We next analyzed fluorescence recovery profiles along the length of the bleach region to quantify directional bias. Each photobleach region was binned by length (Fig. 4 Bii), and recovery rates were computed as a function of distance (Fig. 4 Cii). The slope of a linear fit corresponds to the directional bias in fluorescence recovery—we term this measurement the drift index. If recovery rates on the proximal side of the bleach length exceed those on the distal side, there is a drift directed away from the soma. This is the case in Fig. 4 Cii: the computed slope is directed away from the soma, consistent with the observed drift in the kymogram (Fig. 4 Ci).

The resultant drift indices for \( N = 252 \) dendrites are depicted as a histogram in Fig. 4 Cii. The median of the drift indices is negative, directed away from the soma. Given that the majority of Kv4.2-sGFP2 subunits are synthesized in the soma, it is possible that this directional bias is a result of cargo disseminating throughout the cell following production. Neuronal volume proximal to the photobleached regions is larger than distal neuronal volume, which also potentially contributes to the measured protein drift. Protein drift also showed weak correlation with recovery parameters (Fig. 4 A, last column).

Inferred parameters markedly affect protein distribution in neurons

We next assess how im/mobilization rates and mobile fractions—estimated using model inference (Fig. 3)—impact intracellular distribution of Kv4.2-sGFP2. We begin by adapting the measured kinetics of Kv4.2-sGFP2 (Fig. 2) to a full neuron morphology.

Our experiments revealed a distribution of fluorescence recovery rates \( \tau \) (Fig. 2 D), from which we derive the diffusion coefficient \( D \) of Kv4.2 subunits. Dendrites in our FRAP experiment were modeled as small-diameter cylinders, which reduces complexity to diffusion only along the longitudinal axis. The one-dimensional diffusion equation was solved for \( D \), as described in the supporting text of Scott et al.:

\[
D = 0.231 \frac{W^2}{t_{1/2}},
\]

where \( W \) is the length of the bleached region and \( t_{1/2} \) is the time to half recovery: \( t_{1/2} = \ln(2) / \tau \). Using fitted \( \tau \) (Fig. 2 D), measured \( W \) (Fig. 4, first column), and Eq. 2, we obtain a distribution of \( D \), depicted in Fig. 5 A.
Estimated median $D$ was 1.2 $\mu m^2/s$ with first and third quartiles at 0.69 and 2.0 $\mu m^2/s$.

These estimates are generally reasonable for GFP-tagged Kv4.2 subunits that have a molecular weight, mol wt, of approximately 100 kDa. Previous studies have suggested that $D$ inversely scales with the third power of molecular weight. Other studies have estimated $D$ of GFP alone—a protein with a molecular weight of 27 kDa—to be 27 $\mu m^2/s$ in eukaryotic cytosol. We theorize that our protein, Kv4.2-sGFP2 (100 kDa), is approximately three- to fourfold larger and therefore has a theoretical diffusion coefficient approximately 27- to 64-fold smaller than GFP alone: 0.422 to 1 $\mu m^2/s$. Our empirical estimation of $D = 1.2$ $\mu m^2/s$ is close to this range. This deviation from the theoretical estimate might result from experimental limitations. Our methods did not isolate passive diffusion from active microtubule-based transport, which we have found transports Kv4.2-sGFP2 faster.

Further, our methods do not differentiate cytoplasmic and membrane-bound diffusion. Indeed, prior studies have found that surface-bound proteins have a greater propensity for transit toward distal sites in branching arbors compared with soluble proteins. Integral membrane proteins the size of Kv4.2, which ranges in structural dimensions according to the number of subunits and auxiliary proteins, have been found to diffuse on lipid membranes on the order of magnitude measured here ($D = 1.2$ $\mu m^2/s$). We therefore suspect that our measured $D$ represents a combination of mechanisms possibly including cytosolic diffusion, membranous diffusion, and, perhaps, active transport.

With estimates of $D$, we built a compartmental model of a full neuron morphology to quantify intracellular distribution of Kv4.2-sGFP2 subunits. The drift-diffusion equation describes transport rates, where diffusion $D$ and drift $v$ coefficients describe the bulk flow of a population of particles. When discretized (Fig. 5 B), $D$ and $v$ relate to the forward $v_f$ and backward $v_b$ rates between compartments as follows:

$$D = \frac{v_f + v_b}{2} \quad \text{and} \quad v = v_f - v_b,$$

as derived in Williams et al. Since $D >> (Fig. 4 Ciiii)$, we assume $v = 0$ and use the distribution of $D$ (Fig. 5 A) to solve for $v_f = v_b$, which are then scaled to a compartment size of 100 $\mu m$. We simulate this in a full-size pyramidal cell from the human L3 neocortex (NeuroMorpho.org ID: NMO_86957), as depicted in Fig. 5 C. All cargo is produced in the soma (yellow circle) and degrades with rate $w = 0.000048$ s$^{-1}$, consistent with the observed half-life of Kv4.2. In simulation, generalized rate $v$ from a donor $d$ to receiver $r$ transfers an amount of mass $vd$. As an example, consider the differential equation for the central compartment $x_{mob}$ in Fig. 5 B:

$$x_{mob}^{i'j} = v_i x_{mob}^{i-1} + v_j x_{mob}^{i+1} + v_{imi} x_{imm}^{i}$$

$$- v_b x_{mob}^{i} - v_a x_{mob}^{i} - v_{imi} x_{mob}^{i} - w x_{mob}^{i}$$

where each term corresponds to mass entering or exiting $x_{mob}^{i}$. Branch points have additional terms and terminal compartments have fewer terms. Such a system of ordinary differential equations—one for each compartment—describes the distribution of cargo.

With all parameters defined, we now simulate intracellular distribution of Kv4.2-sGFP2. In our simulations, we use a range of parameter values to best understand how said parameters affect our full morphology distribution. We sweep through $D$ ranging across the first to third quartiles (Fig. 5 A) and $v_{imi}$ and $v_{imi}$ ranging from second (median) to third quartiles (Fig 3 Bii). As a baseline, we begin with median $D$, $v_{imi}$, and $v_{imi}$ plotted in Fig. 5 D, where each line corresponds to an individual compartment. Mean $x_{imm}$ is approximately 15-fold larger than mean $x_{mob}$, consistent with experimental fits. $x_{mob}$ reach 95% of their steady states with a settling time of 1 to 2 h, whereas $x_{imm}$ require 17 to 19 h.

Varying $D$ primarily changes the cargo distribution profile (Fig. 3 E). With high $D$ (Fig. 3 Eii), cargo has a flatter profile, with little deviation between proximal and distal compartments. Low $D$ results in a larger spread of cargo densities (Fig. 5 Ei). Mean $x_{mob}$ and $x_{imm}$ remain roughly the same.

Varying $v_{imi}$ and $v_{imi}$ changes the steady states of mean $x_{mob}$ and $x_{imm}$ (Fig. 5 F). Simulation with median $v_{imi}$ and upper quartiles $v_{imi}$ results in significantly increased $x_{imm}$, averaging 50-fold larger than mean $x_{mob}$ but with similar settling times (Fig. 5 Fii). Upper quartile $v_{imi}$ and median $v_{imi}$ drastically changes the distribution profile, with mean $x_{imm}$ now 25% less than mean $x_{mob}$ at steady state. Both $x_{mob}$ and $x_{imm}$ now have settling times between 16 and 20 h, varying with distance from the soma.

In the context of intracellular cargo distribution, these results are interpreted in a few ways. The mobile fraction $x_{mob}$ is a finite-capacity transport channel. Cargo diffuses in this channel with rate $D$. The immobile fraction $x_{imm}$ behaves like a sink in which cargo becomes confined during dissemination. Inferred parameters $v_{imi}$ and $v_{imi}$ approximate which fraction of the dendrite behaves as a transport channel or a sink, which in turn shapes the speed and profile of cargo localization. Larger $x_{mob}$ are achieved more
rapidly with high $v_{m}$ and low $v_{m}$. Further, diffusion coefficient is the speed at which cargo disseminates. High $D$ allows more cargo into distal compartments, whereas low $D$ produces a decreasing profile with distance from the soma.

**DISCUSSION**

In this study, we leverage common FRAP analysis to infer a biological transport rate that is not directly quantifiable in our experimental setup. Using a mathematical model of un/bleached and im/mobile particles, we deduce rates of cargo im/mobilization.

The method described here avoids a problem innate to many experiments with customized constructs. It is difficult to ascertain whether modified proteins are transported and function identically to wild-type variants. Protein sequestration and triggered release requires large chemical moieties that can alter native intracellular distribution. Kv4.2-sGFP2, the protein fusion used here, was compared and found to be similar to endogenous Kv4.2 in function and regulation.

Precise reporting of im/mobilization rates for a particle of interest can be supplemented with quantitative controls. Such control particles ideally have known im/mobilization rates obtained by direct measurement. If controls are repeatedly bleached and im/mobilization rates are inferred as described here, one can create a standard or calibration curve for a particular experimental setup.

The accuracy of this method is limited by the model and its assumptions, some of which warrant discussion. This framework firstly assumes that the total population of nonphotobleached florescent protein (in the entire neuron) is not depleted with multiple photobleaching iterations. This assumption is sensible for our experiments since we observed no trend in fitted $u$ (Fig. 3 Bi) and found no correlation between prephotobleach fluorescence intensity and recovery (Fig. 4 A, second column). Since a subregion of a dendritic branch was photobleached, the impact of bleaching on total fluorescent protein is likely negligible. However, if FRAP recovery was limited by total protein concentration, we could not assume that all incoming cargo is unbleached, which complicates the analysis.

We also consider the implications of im/mobilization rates in the context of intracellular distribution. For mass-action transport as in the sushi belt model, a large immobilization rate results in frequent protein sequestration in immobile structures. High immobilization rates can significantly decrease mobile protein densities. In expansive cell morphologies, such as the pyramidal cells studied here, this can result in dysregulation of protein transport, which can disrupt cell homeostasis. Further, the presence of two distinct mobile and immobile cargo states potentially benefits cell function. Some cargo, like ion channels in postsynaptic densities, are functionally active in a relatively fixed position. To model the transport of such cargo, distinct mobile and immobile species are required. An inactive, mobile state can also provide a local pool from which to replenish active cargo. We explore this interplay between mobile and immobile and inactive and active states elsewhere.5

A major limitation of our methods is our inability to differentiate between modes of transport. Our estimated $D$ therefore likely represents a combination of diffusion in the cytosol, diffusion in the plasma membrane, and active microtubule-based transit. More sensitive or powerful imaging techniques, such as single-particle tracking or total internal reflection fluorescence microscopy, might be required to differentiate between these modes of transit. Alternatively, advanced methods can inactivate or otherwise isolate individual transport mechanisms.

We lastly consider how undirected diffusion might contribute to intracellular distribution compared with directed, active transport. Measured transport appeared largely undirected or weakly directed away from the soma (Fig. 4 Ci). The setting times for cargo distribution (to 95% steady state) estimated here all fall within 24 h, which is well within the range of observable global changes in complex neurons, such as in synaptic scaling or regulation of intrinsic excitability.4,45,46 This brings to question the relative roles of directed versus undirected (or active versus passive) transport mechanisms in models of intracellular distribution. For instance, a model with diffusion alone, such as in these simulations, is sufficient to capture the regulatory behavior of synaptic receptors or ion channels on the timescales observed in vivo. What then is the specific contribution of microtubule-based transit, an energy-intensive process, versus passive diffusion? The relative roles of such transport mechanisms are not fully understood.

**SUPPORTING MATERIAL**

Supporting material can be found online at https://doi.org/10.1016/j.bpr.2022.100082.

**AUTHOR CONTRIBUTIONS**

A.A.B. performed all experiments, analyses, and modeling. All authors discussed the results and commented on versions of the manuscript.

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DECLARATION OF INTERESTS

We have no interests to declare.

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Supplemental information

Transport between im/mobile fractions shapes the speed and profile of cargo distribution in neurons

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Supplementary materials

Transport between mobile and immobile fractions, inferred from photobleach recovery, shapes the speed and profile of protein distribution in neurons

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Figure S1: Demonstration of exponential fits for recurrent photobleaching for dendrites with increasing mobile fraction (A) and stable mobile fraction (B). Recovery graphs from Figure 2A,B are depicted in enhanced quality. Also shown are normalizations and exponential fits for each individual bleaches, with labeled quality of fit and estimated mobile fraction (A).
Figure S2: Extended recordings in which six dendrites were imaged at 0.1 Hz with bleaching every 50 min over ~10 hours (a 4-5-fold increase over standard-length (~1 hour) recordings) demonstrate consistent recovery parameters (mobile fraction [left] and recovery rate [right]). Mobile fraction remains stable or increases slightly for the first few bleaches before remaining stable, consistent with Figure 2Ci. Recovery rate is unchanged, consistent with Figure 2Di. Recovery parameters do not significantly change, suggesting minimal laser-induced photodamage affecting passive diffusion.
Figure S3: Moderate correlation between mobile fraction (A) and experimental parameters bleach region length (left, $R^2=0.0983$) and distance from soma (right, $R^2=0.0925$). These scatter plots are fitted with exponential decay curves and show moderate reciprocal fits with equations $A = 1.471/x^{0.4385}$ (left) and $A = 0.5265/x^{0.1259}$ (right). A moderately increasing mobile fraction with a narrower bleach region length is intuitive since a narrower bleach length requires less Kv4.2-sGFP2 and thus replenishes more fully. A moderately decreasing mobile fraction (A) with distance from the soma might result from the bulk of mobile Kv4.2-sGFP2 particles being produced in the soma and/or the dendrite having a larger diameter toward the soma.