Imaging methodologies for systems biology
Investigations of cell polarity

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Systems biology has recently achieved significant success in the understanding of complex interconnected phenomena such as cell polarity and migration. In this context, the definition of systems biology has come to encompass the integration of quantitative measurements with sophisticated modeling approaches. This article will review recent progress in live cell imaging technologies that have expanded the possibilities of quantitative in vivo measurements, particularly in regards to molecule counting and quantitative measurements of protein concentration and dynamics. These methods have gained and continue to gain popularity with the biological community. In general, we will discuss three broad categories: protein interactions, protein quantitation, and protein dynamics.

Introduction

Case study: The yeast Cdc42 actin-based polarity mechanism

The application of modeling to fluorescence data is a powerful approach to generate additional information about complex systems. One of the most often modeled mechanisms is the yeast actin-based polarity system.1 Though long-studied, the recent combination of modeling and biophysical measurements continue to shed new light on its inner workings. While not true cell migration, cell polarity represents some of the major features of migration in simplified form, including Rho-GTPase (Cdc42) activation, vesicle mediated recycling, membrane microdomains, and subsequent polarity-directed membrane growth. Given the demonstrated importance of Cdc42, a recent series of papers have been devoted to its recycling/activation dynamics from both an imaging and modeling standpoint.2-8

We will begin with an intro to polarity establishment of Cdc42. In budding yeast, Cdc42 shifts from an isotropic cortical distribution to a polarized distribution as a first step toward formation of a bud or mating projection. The Cdc42 concentrates in a “polar cap” at the site of future polarized growth, where it acts through numerous effector pathways to orient cellular components, most notably the actin cytoskeleton, and exocytic and endocytic pathways.9 Once localized at the polar cap, individual molecules of Cdc42 are not held by a scaffold, but rather are continuously recycled to balance rapid membrane diffusion. Dual protein-recycling pathways exist in yeast, an actin-based mechanism involving transport of Cdc42 inside of secretory vesicles along actin cables, and a cytosolic based mechanism that involves the adaptor protein Bem1 and the yeast Guanine nucleotide dissociation inhibitor (GDI), Rd1. These have been recently reviewed.10

The actin cytoskeleton has long been known to be important for robust polarization and polarized growth of both yeast and Cdc42 itself. In 2003, Rong Li and colleagues demonstrated with a combination of imaging and modeling that in the absence of any spatial cues, (i.e., bud scars in yeast) that an actin-based, positive feedback loop was sufficient for robust polarization of the GTP-locked Cdc42Q61L 3. This loop involves stochastic accumulation of Cdc42, followed by local stimulation of actin cable generation, which allowed for directed transport of Cdc42 along actin cables (Fig. 1A). This initial model did not include membrane diffusion, which turns out to be quite rapid. In 2007, the model was expanded to include a balance of directed targeting of Cdc42 along actin cables with rapid membrane diffusion and internalization of Cdc42 via actin-based endocytosis4 (Fig. 1B). Again, this work centered on GTP-locked Cdc42. Two years later, Li and colleagues examined wild type Cdc42, which is able to cycle between GTP and GDP bound forms. This work found again, using essentially the same model framework, a balanced relationship of directed delivery of Cdc42, membrane diffusion, and internalization through endocytosis.5 Interestingly, parameters of recycling were not tuned for maximum strength of polarity, but rather for a ‘flat’ shape of Cdc42 distribution at the polar cap that is optimal for round bud formation.

This model was further developed in 2011 by Layton and colleagues. This work called into question the actin-based mechanism altogether by pointing out a key simplification in previous models: when Cdc42 is delivered upon exocytosis of vesicles, insertion of membrane area had not been considered. If the concentration of Cdc42 on exocytic vesicles is equal to the concentration already at the cap, then docking of exocytic vesicles will not lead to further concentration of Cdc42 at the polar cap (Fig. 1C). This can be temporarily remedied if Cdc42 is concentrated from the internal pool onto exocytic vesicles. However, with this scenario the internal pool of Cdc42 will eventually be depleted, and polarization will cease as new vesicles with low...
Cdc42 concentration are inserted that actually dilute the polar cap.

This led to an interesting conundrum. Cells require actin to grow. And while cells treated with LatA or LatB, which eventually leads to disruption of actin structures, can polarize as long as the yeast GDI, Rdi1, is present, Rdi1 deletion cells cannot polarize in the presence of LatA or LatB.6,11 This suggests strongly that an actin mechanism contributes positively to polarization. Nonetheless, the modeling result that strong accumulation of Cdc42 onto exocytic vesicles was required for initial polarization, yet the polarization could not last over time, led to the questioning of the positive role of actin altogether.

In 2013, we revisited these mechanisms after one peculiar observation. If imaged with high resolution both spatially and temporally, the Cdc42 accumulation at the polar cap was not uniform or distributed in a Gaussian fashion around the center of the cap. Rather, it was punctate, with areas of high concentration that overlapped with formins, which are nucleators of actin cables, and exocytic markers, and areas of low concentration that overlapped with future areas of endocytosis (prior to endocytic internalization).12 This led to many questions – such as why areas of high and low accumulation of Cdc42 can persist with the rapid average membrane diffusion coefficient of Cdc42? Is Cdc42 directly deposited into regions of high concentration or does Cdc42 migrate and ‘stick’ there? We also examined, using fluorescence correlation spectroscopy (FCS) in live yeast, the issue of Cdc42 concentration on exocytic vesicles that was brought into question in Layton et. al. From a modeling standpoint, the relationship of this concentration to the concentration of Cdc42 at a polarized or non-polarized region of the membrane should be considered. This was explored using calibrated imaging. The goal of this review is to discuss the specific biophysical approaches that allowed these measurements, and how these approaches were applied specifically to the actin-dependent model of Cdc42 symmetry breaking in yeast.

While methods for assessing heterotypic protein interactions such as fluorescence cross-correlation spectroscopy (FCCS), and fluorescence resonance energy transfer (FRET) have been covered at length13-16 there has been far less discussion of methods to assess homotypic protein interactions or vesicle occupancy. The reasons for this are simple: heterotypic interactions are often more easily probed by standard genetic and biochemical methods. Nevertheless, an investigation of biological phenomena from a systems standpoint necessitates a more complete picture which demands inclusion of homotypic interactions. Besides dimerization, interactions that may be of interest include larger complex formation as well as non-specific interactions such as vesicle occupancy, that may have implications in biological models.7,12

Methods for determination of homotypic interactions can be divided into two categories: qualitative tests for interactions and quantitative measurements of interaction stoichiometry. Qualitative tests are often similar to those for heterotypic interactions (biochemical interaction, FRET, homo-FRET, or FCCS between multiple tagged copies). Quantitation of interaction stoichiometry represents a significantly greater challenge. For specific interactions, a typical strategy involves structural biological investigation via structural modeling and/or mutagenesis. These approaches are limited by the availability/applicability of structural biological methods for a complex of interest. An underutilized approach, and one applicable to live cell studies, is to use fluorescence correlation spectroscopy (FCS) to measure the molecular brightness (relative stoichiometry) of a diffusing complex.5,17

**Fluorescence correlation spectroscopy**

In FCS, the fluctuating intensity of a sample is recorded over time as fluorescent molecules diffuse in and out of the fixed focal...
In order to generate a correlation curve, the intensity trace is subjected to correlation analysis to measure self-similarity of the signal over time:

\[
G(t) = \frac{\langle \delta I(t) \delta I(t + \tau) \rangle}{\langle I(t) \rangle^2}
\]

(1)

Intuitively, a sample containing only monomeric fluorophores (Example 1, Figure 2A) will have smaller, more rapid fluctuations in intensity than a sample with more total fluorophores yet the same number of diffusing complexes (i.e., each is an oligomer) (Example 2, Figure 2A). \(G_0\), the normalized variance of the signal, is inversely proportional to the average number \(N\) of molecules present in the focal volume:

\[
N = \frac{\gamma}{G_0}
\]

(2)

Here \(\gamma\) is a focal volume shape factor independent of the focal volume size. Once \(N\) is known, the average brightness \(\bar{e}\) of the species is simple to calculate (Fig. 2A):

\[
\langle \bar{e} \rangle = \frac{I}{N}
\]

(3)

where \(I\) is the average intensity of the time trace, usually in photon counts. The average occupation number, \(<\nu>\), of molecules present in each diffusing complex can then be calculated by comparing against the measured brightness of a known monomer sample. The monomer sample is an important tool in quantitative parameter measurement, as both concentration and brightness measurements of the monomer can be used as indexes against which other samples can be measured. We will come back to this theme again later in this text.

\[
\langle \lambda \rangle = \frac{\langle \bar{e} \rangle}{\bar{e}_{\text{monomer}}} - 1
\]

(5)

Here \(\lambda\) is the vesicle occupation. In cases where there are multiple species with different diffusion rates, it is possible to fit the correlation curve to two components to determine the brightness of each population separately. This is simplified considerably in the case of a priori knowledge of a monomeric state and known monomeric brightness for one of the populations. It is our experience, however, that in the absence of significant differences in diffusion rates, only the average brightness and number of molecules is obtainable in vivo due to limitations in signal to noise and acquisition time. Such scenarios may become addressable in an in vitro context or with added information from separate experiments or differences in diffusion rate (Cdc42).

**Brightness analysis to determine concentration of Cdc42 on vesicles**

In the case of mobile Cdc42 in yeast, brightness analysis by FCS revealed an average brightness of GFP-Cdc42 that is higher than monomeric GFP. While this result is straightforward, its interpretation is not. With multiple mobile pools, and an average brightness in between that of a monomer and dimer GFP control, taken at face value it only implies that at least one of the pools (must be) is monomeric, and the second is some sort of oligomer. Fortunately, two diffusing pools were easily observed in the standard one-color FCS autocorrelation decay of Cdc42 –
Fluorescence lifetime imaging relies on the fact that the fluorescence lifetime (the inverse of the rate of fluorescence emission) reports directly on the fluorescent yield of the fluorophore: $\tau(x,y,z)/\kappa$. In other words, if the fluorescence lifetime of a GFP fusion protein in a complex is identical to the fluorescence lifetime of GFP expressed on its own in the cell cytoplasm, then the fusion construct and its localization can be interpreted as not perturbing the GFP fluorescence. This methodology can also be used to discover sources of heterogeneity. As an example, this method was used to discover and explore the heterogeneity in mCherry folding (and lack thereof for EGFP).\textsuperscript{12,21,23}

### Protein Quantitation: Quantitative Imaging

We define quantitative imaging as the determination of absolute protein levels from in vivo imaging data. This is an active and competitive area of research. The advantages of successful quantitative imaging are obvious. For example, it is one thing (and a simple thing) to measure a protein gradient and know that there is twice the protein at the front of the gradient as there is at the back. Knowing the concentration is e.g., 100 nM at the front and 50 nM at the back is significantly more complicated, and yet also much more useful for modeling studies. Quantitative imaging methods have also been proposed to determine stoichiometry of molecular complexes inside live cells.\textsuperscript{23-26}

#### Measuring protein concentrations with FCS

FCS is one method for quantitation that has been applied in vivo. With good data and mobile proteins, the absolute concentration of protein is simply calculated from the above formulas.\textsuperscript{15} For example, in the case of Cdc42, FCS was recently used to calculate the concentration of monomeric Cdc42 in the cytosol for different mutants of Cdc42.\textsuperscript{27} That work showed that increasing expression of GDI, which binds and shields the prenyl group of GTPases, thus allowing them to escape membranes, increased the concentration of quickly diffusing Cdc42, while the presence of Cdc42 in the slowly diffusing pool (attributed to vesicles) did not increase. The effect was lost in cells deleted for the lipid flipase Lem3, supporting (together with other data) the notion that extraction of Cdc42 by the GDI from the membrane is modulated by electrostatic interactions of Cdc42 with lipids.

#### Calibrating intensity measurements

The requirement of mobile complexes for FCS quickly restricts its usage in many important contexts. Nevertheless, such problems can at times be addressed by calibrating imaging results with FCS or other methods. Generally speaking, calibrated imaging involves the comparison of measured intensity values in the experimental sample with a measured reference brightness of some standard that can be related to the monomer FP. The reference brightness can be measured using one of several strategies: First, one can use FCS on soluble monomer fluorophores as described in the previous section to determine the brightness of monomeric GFP.\textsuperscript{23} FCS has the advantage that the brightness of the fluorescent protein can be measured directly in the cytoplasm.
of a living cell, without the need to use a separate cell type, or in vitro isolation of GFP. Following FCS, imaging can determine the relative fluorescence intensity of a given protein compared with the fluorescence intensity of the sample upon which the FCS was performed. A second category of methods involves imaging either single fluorophore molecules or a structure with a known number of fluorophores. In the case of fluorescent proteins, imaging single molecules is fraught with difficulty and certainly cannot be attempted in vivo except in cases where molecules are displayed near the coverslip and can be imaged with TIRF microscopy. Imaging complexes compared with a standard with known stoichiometry has been used more extensively\(^\text{23,24,28,29}\) assuming the consistency of the fluorophore’s fluorescence in the environment under which the complex is imaged (see above).

**Measuring protein concentrations with calibrated imaging**

A common application of quantitative imaging for modeling applications is for the measurement of protein concentrations in regions much larger than the focal volume, which can’t be measured by FCS either because the concentration is too high or the particles are moving too slowly.

In this scenario, one must carefully account for the actual volume of the microscope focus. This can be done fairly easily by imaging fluorescent beads below the microscope resolution. From Figure 3 it is evident that 100 nm beads are sufficiently below the resolution to accomplish this task. The relationship between intensity and concentration is then given as follows:

\[
I(x, y, z) = NC(x, y, z)\frac{1}{V_{PSF}}e
\]  

(6)

where \(N\) is the number of molecules in the integrated focal volume, \(C\) is the concentration in molecules/unit volume, \(\varepsilon\) is the molecular brightness as defined above, and \(V_{PSF}\) is the focal volume. In another scenario, one may desire to find the concentration of protein on a cell membrane. In that case, the volume is replaced in the above equations by the cross-sectional area of the membrane when the focal volume is centered on it (radial area for lateral membranes and axial area for vertical membranes). It is crucial in these scenarios to account for the contribution of the cytoplasmic intensity to the measured membrane intensity. This can be accomplished through edge fitting for vertical membranes\(^\text{12}\) or simple background subtraction for lateral membranes with the caveat that both of these methods require a uniform cytoplasmic concentration (Fig. 2B). The FCS-based approach has been utilized to quantitatively determine the relationship of concentration of Cdc42 on exocytic vesicles to the concentration of Cdc42 both at the polar cap and the plasma membrane prior to polarization, which were used as direct input into a model of polarity establishment.\(^\text{12}\)

**Quantitative imaging of point sources**

Quantitative imaging of individual complexes, treated as point sources, can be used to establish a molecular brightness for use in calibrated imaging (see above) if the complex is of known stoichiometry, or, to determine the stoichiometry of a complex of interest. To understand quantitative imaging of individual complexes, it is first crucial to understand the concepts of image formation. We will limit our discussion to confocal microscopes for simplicity. Each imaged molecule emits light with a specified rate dependent on local excitation power. For a confocal microscope, that emitted light is spatially filtered. In this way, each molecule creates a Gaussian spot on the collected image with an intensity that is dependent on the distance above or below the focal detection region or point spread function (PSF) as follows:

\[
I_1(x, y, z) = \varepsilon \exp \left( -\frac{2[(x^2 + y^2) + (y - y')^2]}{w_0^2} + \frac{2(z - z')^2}{z_0^2} \right)
\]  

(7)

where the primed coordinates represent the position of the molecule, \(\varepsilon\) represents the molecular brightness of the fluorophore, \(w_0\) represents the beam waist in the \(xy\) plane and \(z_0\) represents the beam height. Note that the integrated volume of the focus is given by:

\[
V_{PSF} = w_0^2z_0(\pi/2)^{1/2} = 1.97w_0^2z_0
\]  

(8)

Once the brightness of a fluorophore is known, the calculation of the number of molecules in a point object is, in principle, quite simple:

\[
I(x, y, z) = nI_1(x, y, z)
\]  

(9)

where \(n\) is the number of fluorophores in the object. In other words, the intensity of the object will be the same as the single molecule but \(n\) fold brighter.

Of course, establishing that an object can be treated as a point source may not be trivial. The simplest tool for accomplishing this is to measure the size of the microscope resolution and compare the observed size of the imaged object to it. If the observed size of the object is identical to the measured size of the focal volume, one can confidently assume that the object is a point source. Figure 3B demonstrates that the observed amplitude drops fairly dramatically as the observed resolution decreases relative to the microscope resolution. A 10% increase in observed size correlates with a 20% decrease in amplitude. Nevertheless, this seemingly dramatic shift corresponds to a surprisingly large bead radius of \(~100\) nm for a 1.2NA (Numerical Aperture) objective. As Figure 3 shows, the size of objects which appear as point objects is significantly greater for lower NA objectives. Thus one strategy to maintain the assumption of a point object is simply to utilize a lower NA objective. We have omitted the effects of emission wavelength here for simplicity (here it is assumed to be \(~525\) nm) but one should note that resolution is inversely proportional to the wavelength of light.

Another methodology commonly used to decide whether an object is a point source is size information from higher resolution methodologies and/or models. In this case, it is important to note that the object shape (and distribution of fluorescence within that shape) strongly influences the observed size.
Figure 3C shows the simulated relationship between the size of a Gaussian distributed cluster of objects and its observed size in a confocal microscope with different NA objectives. Gaussian distributed objects appear slightly larger than spherical ones due to the extended nature of the Gaussian distribution. Figure 3D shows the observed size for a hollow cylinder. Such objects appear dramatically larger than either Gaussian distributed or spherical objects.

If it is determined that the size of the complex of interest is neither below nor far above the microscope resolution, quantitative imaging becomes far more complex. If concentration is required, sub-resolution knowledge of the object structure is required. If only integrated molecular numbers are required, the problem becomes tractable through image integration:

$$I(x, y, z)dx dy dz = V_{PSF} e^{C(x, y, z)dx dy dz} = V_{PSF} e^{\lambda}$$  \hspace{1cm} (10)$$

where $\lambda$ is the total number of molecules in the object. In many cases the object will be sub-resolution in the axial direction but not the radial one. In such cases, integration is only required over the xy plane. If molecular density is required, one must have a sub-resolution model of the structure of interest to perform further analysis. From a practical standpoint, it is worthwhile to note that the peak intensity of a spot is determined with far more accuracy than the integrated intensity. This is mostly due to uncertainty in determining background levels and spot size. Of course, it is important to note that all of these methodologies are dramatically affected by competition with unlabeled endogenous proteins as well as overexpression. The examples we have referenced here are from yeast and bacterial studies where genomic integration of fluorescent protein tags is routine. Such methodologies are becoming more routine in tissue culture contexts with the advent of zfn, talen, and crispr/cas9 methodologies. Nevertheless, in some cases it is possible to achieve such results by careful selection of promoters and simultaneous knockdown of endogenous genes.

Measuring stoichiometry using stepwise photobleaching

A third methodology for determining brightness and/or stoichiometry is multi-step photobleaching. Here the strategy is to look for the stepwise bleaching of individual molecules as a complex is bleached. The limitations on this strategy are similar to those for imaging single molecules—TIRF accessible complexes have yielded the most convincing results. It is worthwhile to note that no stringent statistical criteria have been set for the quality of multistep photobleaching data. Particularly disturbing is the use of step-preserving filters (e.g., Chung-Kennedy, which can lead to the appearance of step-like transitions even in random data (Fig. 4). We would suggest that those utilizing this method take advantage of unbiased methodologies such as hidden-markov modeling, as has been used in other single molecule methodologies, to provide statistical criteria. At the very minimum, it is straightforward to generate exponential decays with Gaussian noise similar to that observed experimentally as we have done in Figure 4. This allows for unbiased assessment of the feasibility, reliability, and methodology for different transition finding methods.

Protein Dynamics

Protein dynamics can be quantified using a variety of methods. For mobile cytosolic proteins, or mobile membrane proteins, single point fluorescence correlation methods can very
Mathematical parameters can be solved with the combination of FRAP recovery rate and the steady-state amount of protein on the membrane relative to the cytosol, and has been applied to Cdc42.4,5

Role of membrane diffusion

This general model assumes an average membrane diffusion rate $D_f$. For Cdc42, this was determined using FRAP of Cdc42 in a situation where all recycling was eliminated, leaving only membrane diffusion as the mechanism for fluorescence recovery.4 The assumption of uniform membrane diffusion was revisited after the observation of discrete spots of high Cdc42 accumulation within the polar cap. We found high levels of Cdc42 overlap with regions of exocytic delivery, marked by either the actin cable nucleator Bni1, or the exocyst complex member Exo70. Regions of low Cdc42 accumulation centered on future regions of endocytosis (prior to the actual event of endocytosis). We knew from previous studies that if cells deleted for the GDI were treated with LatA to disrupt actin, the cells would eventually depolarize. With the goal of examining if depolarization would occur rapidly or slowly in regions of high accumulation of Cdc42, we applied the iFRAP measurement to the polar cap shortly after addition of LatA, prior to depolarization. Very surprisingly, given the average membrane diffusion rate of Cdc42, these hotspots of high accumulation of Cdc42 persisted for up to 100 s—far longer than expected given the apparent diffusion rate (Fig. 5B)—suggesting they were residing in ‘sticky’ regions.

Simulations were used to match the overall average decay rate of fluorescence loss following iFRAP with the relative protein distribution in each region, to extract the diffusion rates in the respective areas (Fig. 5B).

In theory, this form of model can be either expanded to include multiple mechanisms of protein dynamics, or dramatically simplified to determine internalization and export rates in situations where membrane diffusion is either absent or is sufficiently slow as to not contribute to the fluorescent recovery in FRAP.

STICS

FCS as described above is a single point method. In whole cells, for example migrating cells, it is often convenient to decipher motion or flow of bulk molecules. The case of coordinated directed motion has been addressed in detail, and this method and related methods are used commonly and with great results in the cell migration field.38-42 Here, we will focus on a less common application: the quantitation of confined and randomly directed motion. In the case where particles are sufficiently

### Figure 4. Chung-Kennedy filtering of simulated exponential decay data with Gaussian noise showing apparent bleaching transitions. The width of the Chung-Kennedy filter was 10 data points.  

nicely determine diffusion coefficients.35 However, single point correlation methods are limited in examination of bulk protein recycling, or determining on and off rates from static immobile structures. In this section, we focus on bleaching methods such as fluorescence recovery after photobleaching (FRAP) and inverse FRAP (iFRAP), and the image-correlation based STICS method.

Modeling of FRAP data

In Fluorescence recovery after photobleaching (FRAP) and inverse FRAP (iFRAP), an intense laser pulse is used to bleach fluorescent molecules in a particular region. In FRAP, the bleached region is monitored for recovery as fluorescent molecules from adjacent regions move into the bleached region, exchanging with the bleached molecules. In its simplest form, FRAP data can be used determine if a protein is mobile, what percentage of it is mobile, and can give rough estimates of how rapidly the protein is moving within the cell. In iFRAP, fluorescence is bleached in an adjacent region, and is monitored in an unbleached region. The decay of fluorescence is measured in the unbleached region as fluorescent molecules move out of this region and into the bleached region.

FRAP data formed the basis for the early experiments that discovered that Cdc42 is recycling dynamically at the polar cap, and helped decipher the mechanisms involved. For example, the rate of Cdc42 recycling was reduced upon disruption of actin with LatA, or disruption of the adaptor protein Bem1.36 These results formed the initial framework for future models. FRAP also played a crucial role in determination that the yeast GDI, Rdi1, played an active rather than passive role in polarizing Cdc42.5,11,27,37

Modeling of FRAP and iFRAP data are a straightforward way to extract rates of protein dynamics from live cell data. If a protein is localized both to the membrane and in internal pool, the amount and distribution, $(f)$, of protein on the membrane is mathematically described by the relationship of internalization rate $(m$ or $n$), export rate $(h)$, and the rate of membrane diffusion $(D_f)$ (see Figure 1B for a qualitative representation). For the models of Cdc42 recycling, two rates of internalization exist, $m$ for inside the delivery window, $n$ for outside, based on the higher accumulation of endocytic pits within the cap.

$$\frac{\partial f}{\partial t} = D_f \Delta f - m \chi f - n(1 - \chi)f + h \chi F_c$$  

(11)

Mathematical parameters can be solved with the combination of FRAP recovery rate and the steady-state amount of protein on the membrane relative to the cytosol, and has been applied to Cdc42.4,5

Rate ($m$ or $n$), export rate ($h$), and the rate of membrane diffusion ($D_f$) (see Figure 1B for a qualitative representation). For the models of Cdc42 recycling, two rates of internalization exist, $m$ for inside the delivery window, $n$ for outside, based on the higher accumulation of endocytic pits within the cap.

$$\frac{\partial f}{\partial t} = D_f \Delta f - m \chi f - n(1 - \chi)f + h \chi F_c$$  

(11)
bright, sufficiently slow, and of low enough density, particle tracking is the simplest method for such investigations. For spatial information, it is convenient to average tracks over segments of areas, which provides a limited resolution flow map. Also, once each track is generated, plots of mean square displacement (MSD) can be fit to determine if the particle motion is random, confined, or involves a transport component.

\[
\text{MSD}(\tau) = \langle \left| r(t + \tau) - r(t) \right|^2 \rangle = 4D\tau^\alpha
\]  \hspace{1cm} (12)

MSD is simply a way to visualize on average how far a particle travels on average over a time interval, \(\tau\). Because of the nature of diffusion, this quantity increases linearly for random motion \((\alpha = 1)\) with a slope equal to the diffusion coefficient, \(D\) (Fig. 5A). If the particles are actively transported, they will, on average, traverse further than simple diffusion would allow, and the MSD will increase in a quadratic fashion \((\alpha > 1)\). On the other hand, confinement leads to shorter distance traveled, in which case the MSD falls short of the linear prediction \((\alpha < 1)\). Of course, the motion of individual particles can deviate strongly from this average behavior for a short time. Therefore it is important to obtain large numbers of trajectories for robust analysis.

In many cases particles are too crowded or are moving too rapidly to allow for accurate tracking. In this case, spatio-temporal image correlation spectroscopy can be employed to decipher the nature of the molecular motion. This has been used to examine bulk protein dynamics in migratory\(^{38,39}\) and non-migratory cell types.\(^{44}\) Even in the absence of bulk flow, in the presence of non-directed diffusion, STICS is able to provide information about average diffusion coefficient and the nature of the motion, whether directed, random, or confined. This less common application of STICS relies on the depletion of the spatial correlation over time.\(^{43,45}\) STICS is simply the spatial correlation of each image of a movie with images taken at a time shift, \(\tau\), later:

\[
G(\rho, \tau) = \frac{\langle (I(t + \tau, r + \rho) - I(t, r))^2 \rangle_{tx}}{\langle I^2 \rangle_{tx}}
= G(\tau)\exp \left( -\frac{\rho^2}{w^2_0 + \text{MSD}(\tau)} \right)
\]  \hspace{1cm} (13)

Here \(\rho\) and \(\tau\) are spatial (radial) and temporal shifts. Here, as before, \(w_0\) represents the focal volume waist, or twice its spatial standard deviation. As we\(^{43}\) and others\(^{45,49}\) have shown, the above form can be extended to anomalous diffusion. If we plot the right hand side of the equation above for simulated diffusion, normalized to ignore the \(G(\tau)\) component, we see the utility of this analysis. The result is essentially a Gaussian peak with a starting width related to the microscope resolution and increasing with \(\tau\) value. The MSD can be easily calculated from this width as follows:

\[
\text{MSD}(\tau) = 2\sigma^2 - 0.5w^2_0 = 2(\sigma^2 - \sigma^2_0)
\]  \hspace{1cm} (14)

where \(\sigma\) is the observed standard deviation of the correlation function and the second equation demonstrates that the microscope resolution (denoted by \(\sigma_0\)) can be determined from the time zero point of the correlation plot. Given that we are describing the scenario in which motion is non-directional, it is

Figure 5. A. Spatio-temporal image correlation spectroscopy (STICS) can be used to determine the mean squared displacement (MSD) of molecules as is shown in this simulation. The MSD is linear for random diffusion (red) but shows slowing displacement with time shift for confined diffusion (purple) and accelerating displacement for transport (green). B. Simulations of iFRAP decays can distinguish between uniform and anomalous models of membrane diffusion.
appropriate to calculate the radial average of the correlation function which, when normalized, gives us a nice visual representation of the temporal spread of particles over time (Fig. 5A).

Once anomalous diffusion is properly assessed, it can be a powerful tool to model seemingly random diffusive processes. Such modeling can go far beyond what is available with FRAP analyses but can also be used in concert with FRAP analysis to gain a complete picture of micro and macroscopic protein transport phenomena.

**Conclusions**

The field of cell migration is one familiar with advanced imaging concepts to track collective particle dynamics. Less common is the use of fluorescence correlation spectroscopy and calibrated imaging methods to determine absolute concentration of mobile and fixed particles and protein structures. In this review, we have highlighted the use of these methods in establishing an accurate view of the mechanisms involved in actin-based yeast polarity.

The genetic simplicity of the yeast model system allows for quantitative measurements which have enabled sophisticated and powerful systems biology modeling. Such models have revealed nuanced mechanisms for both polarity establishment and maintenance. Many of these methods are directly applicable to migratory cell types and will become more so as genomic editing methods become available to allow measurements on endogenously expressed proteins. We anticipate that as use of these methods increases, quantitative determination of parameters will ensure that will allow for increasingly complex and accurate models of these systems. In many cases, new parameters of the system will support and/or confirm current models. It is also likely that in some cases, newly determined quantitative parameters will require models to be re-examined. Just as the yeast model system has provided genetic and biological insight into migratory mechanisms, it is our hope that quantitative imaging methods often applied in yeast will gain in popularity in more complex migratory systems.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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