Solute and macromolecule diffusion in cellular aqueous compartments

Alan S. Verkman

Diffusion of solutes and macromolecules in aqueous cellular compartments is required for numerous cellular processes including metabolism, second messenger signaling and protein–protein interactions. The view of the cell interior has evolved from that of a viscous gel to that of a watery but crowded compartment. Recent measurements of fluorescent probe diffusion using photobleaching, correlation microscopy and time-resolved anisotropy methods, have indicated unexpectedly high mobilities of small solutes and macromolecules. This review evaluates experimental evidence defining the rates and barriers for molecular diffusion in cells. Possible implications of regulated molecular diffusion as a rate-limiting step in cell metabolism, and with respect to the delivery of therapeutic agents, are discussed.

Alan S. Verkman
1246 Health Sciences East Tower, Cardiovascular Research Institute, University of California–San Francisco, San Francisco, CA 94143-0521, USA.
e-mail: verkman@itsa.ucsf.edu

The cytoplasm and the aqueous compartment of intracellular organelles such as mitochondria are crowded with solutes, soluble macromolecules, skeletal proteins and membranes. The consequences of this crowding remain controversial. Popular pictorial models of the aqueous environment within cells (such as those based on measured solute concentrations drawn by David Goodsell [1]) suggest that crowding might seriously hinder solute diffusion – a major determinant of metabolism, transport phenomena, protein processing and second messenger signaling. One possible consequence of molecular crowding and hindered diffusion is the need to compartmentalize metabolic pathways to overcome diffusive barriers. For example, it has been proposed that the mitochondrial matrix is so crowded that metabolism occurs by the 'channeling' of metabolites from one enzyme to an adjacent enzyme without the need of free aqueous-phase diffusion [2–4]. A second predicted consequence of molecular crowding is that the physical chemistry of cellular interactions, such as protein–protein interactions and enzyme reactions, is drastically altered [5]. Such alterations have been proposed to involve changes in solute activity coefficients, which result mechanistically from the effects of occluded volume and restricted diffusion.

The diffusion of small and macromolecular solutes in cellular aqueous compartments is determined by solute properties, and by the composition, organization and geometry of the cellular compartment. The first step in characterizing the determinants of molecular diffusion in cells is the accurate experimental measurement of the diffusion of biologically important molecules in various cellular compartments. For example, the diffusion of small solutes is relevant for metabolite uptake and second messenger signaling, the diffusion of enzymes is important for metabolism, and the diffusion of DNA is important in antisense and gene therapy. This review summarizes the experimental approaches for quantifying molecular diffusion in cells, and evaluates recent data for molecular diffusion in cytoplasm and in the aqueous compartments of organelles. The substantially slower diffusion of membrane-associated molecules is not discussed here, nor is active transport involving skeletal-associated molecular motors.

Experimental approaches to measure diffusion in cells

The continuous, high-resolution tracking of the motion of many individual solute molecules in three dimensions is the gold standard in describing diffusive phenomena. However, at present, this is not practical for diffusion measurements in aqueous cellular compartments. Probably the most useful method for quantitative measurement of the translational diffusion of fluorophores and fluorescently labeled macromolecules is fluorescence recovery after photobleaching. In this method, fluorescently labeled molecules are introduced into cells by microinjection or incubation, or by targeted expression of green fluorescent protein (GFP) chimeras. In spot photobleaching, fluorophores in a defined volume of a fluorescent sample are irreversibly bleached by a brief intense light pulse. Using an attenuated probe beam, the diffusion of unbleached fluorophores into the bleached volume is measured as a quantitative index of fluorophore translational diffusion (Fig. 1a, left). A variety of optical configurations, detection strategies and analysis methods have been used to quantify diffusive phenomena in photobleaching measurements (reviewed in Ref. [6]). Acquisition of a series of images after photobleaching is particularly useful for qualitative description of fluorophore diffusion in cells. For example, the presence of fluorophore-excluding regions and the continuity of organellar compartments can be observed.

There are several cautions in the interpretation of photobleaching data. For unrestricted diffusion of a single fluorescent species in a homogeneous unhindered environment, fluorescence recovers to the initial (pre-bleach) level and the recovery curve contains a single component whose shape depends on the geometry of the bleached region (Fig. 1a, right, 'simple' diffusion). However, fluorescence recovery in cell systems is often incomplete ('restricted' diffusion) or multi-component ('complex' diffusion). Incomplete

 0968-0004/02/$ – see front matter PII: S0968-0004(01)02003-5

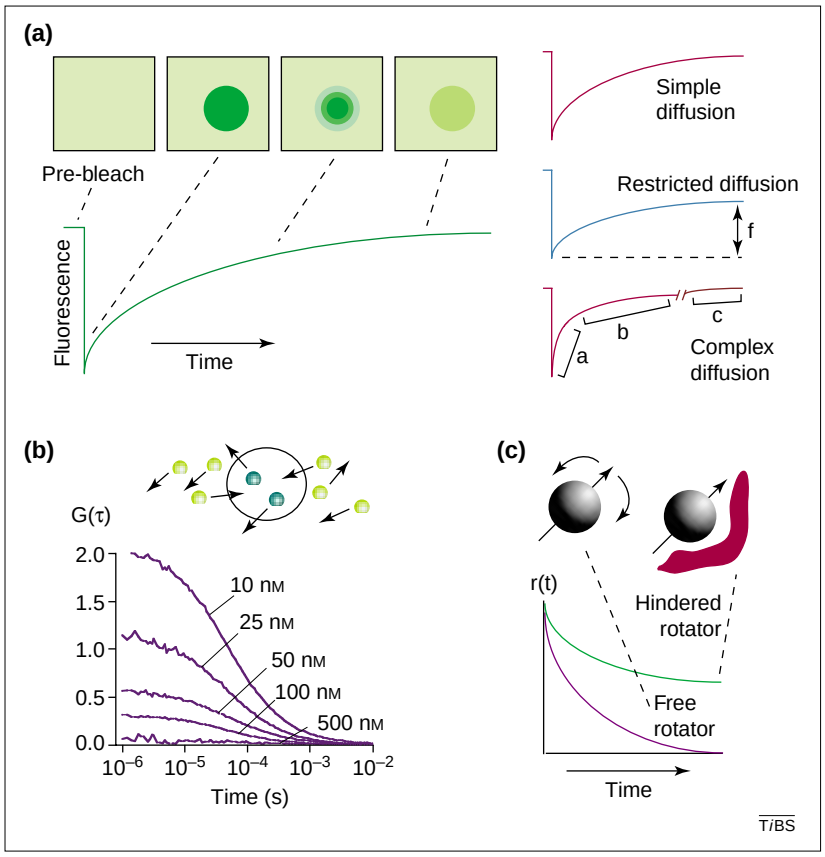

Fig. 1. Experimental approaches to measure molecular diffusion in cells. (a) Fluorescence recovery after photobleaching. (Left) Spot photobleaching showing fluorescence recovery into a circular bleached region by inward diffusion of unbleached fluorophores. (Right) Fluorescence recovery curves for simple, restricted and complex diffusion: 'f' is the fractional recovery of the photobleached signal, and 'a', 'b' and 'c' are recovery processes over different time scales. See text for explanations. (b) Fluorescence correlation microscopy. (Top) The diffusive movements of fluorophores into and out of a circular illuminated region. The fluctuations in measured fluorescence provide information about rates of diffusion. (Bottom) Example of diffusion of BODIPY-Fl (10–500 nM) in aqueous solution. G(τ) is the autocorrelation function. (c) Time-resolved fluorescence anisotropy. (Top) A rotating fluorophore undergoing free (left) and hindered (right) rotation. (Bottom) Time-resolved anisotropy, r(t), for free and hindered fluorophore rotation.

recovery is generally taken to indicate that a fraction of fluorescent molecules are immobile because they are either bound to slowly moving cellular components or become sequestered in non-contiguous compartments. However, some phenomena, for example, laser-induced photodamage and probe-beam photobleaching, can produce incomplete recovery. Moreover, because the extent of fluorescence recovery is measured at a finite time after photobleaching, very slow processes (process 'c' in Fig. 1a, right) might be overlooked and, thus, the presence of restricted diffusion can be misinterpreted [7]. The determination of solute diffusion coefficient(s) from fluorescence recovery curve shape is discussed elsewhere [8], and can become challenging when multiple diffusing species are present, or when diffusion is anomalous or geometrically restricted. As initially described for diffusion of small solutes in the cytoplasm [9], the quantitative comparison of recovery curve shape measured in cells to that in standards (fluorophore in thin layer of saline) is very useful in the experimental determination of diffusion coefficients.

There is another level of complexity that has been under-appreciated by cell biologists. It is generally assumed that laser-induced photobleaching is irreversible, so that any increase in fluorescence results from a diffusion of unbleached fluorophores into the bleached zone. However, there are situations in which the fluorescence of a whole cell can be bleached and then recover spontaneously without diffusion – a phenomenon called 'reversible' photobleaching. All fluorescent molecules, including GFP, can undergo reversible photobleaching. The best-understood process leading to reversible photobleaching is triplet-state recovery, during which excited-state fluorophores are sequestered into a triplet-state that decays slowly (generally milliseconds or less) to the ground state [10–12]. In addition, there are flicker and other poorly understood phenomena that lead to reversible photobleaching with recovery times of milliseconds to many seconds [13,14]. Neglecting reversible photobleaching has led to serious misinterpretations of data; for example, the incorrect assumption that fluorescence recovery indicates rapid fluorophore diffusion. Useful experimental approaches to identify a component of reversible photobleaching are measurements of fluorescence recovery either using different spot sizes (e.g. different lenses) or conducted subsequent to chemical fixation (e.g. paraformaldehyde). Irreversible (diffusion-dependent) photobleaching is strongly spot-size-dependent whereas reversible photobleaching is not. Fluorescence recovery from irreversible photobleaching is abolished by fluorophore fixation whereas recovery from reversible photobleaching is generally not affected.

There are several complementary methods to measure solute and macromolecule diffusion in cells. Fluorescence correlation microscopy relies on the analysis of fluctuations in the number of fluorescent particles in a very small volume defined by a focused laser spot (Fig. 1b, top) [15–17]. Increasing diffusion results in more rapid fluctuations and, consequently, in a smaller probability that a particle found in the beam initially will also be found in the beam at a later time. This probability is quantified by the autocorrelation function G(τ). As shown for a single diffusing fluorophore in Fig. 1b (bottom), the G(0) amplitude is related to fluorophore concentration, and the shape of G(τ) is related to the fluorophore diffusion coefficient. Although there has been recent interest in applying correlation methods to study molecular diffusion in cells, it remains unclear whether these methods will provide clear-cut quantitative information in the complex cellular environment. Correlation methods require very low fluorophore concentrations and are easily confounded by reversible photophysical processes, cell autofluorescence, and complexities in beam and cell geometry. Photobleaching and correlation microscopy methods provide information about fluorophore translational mobility. Determination of fluorophore rotation mobility is best

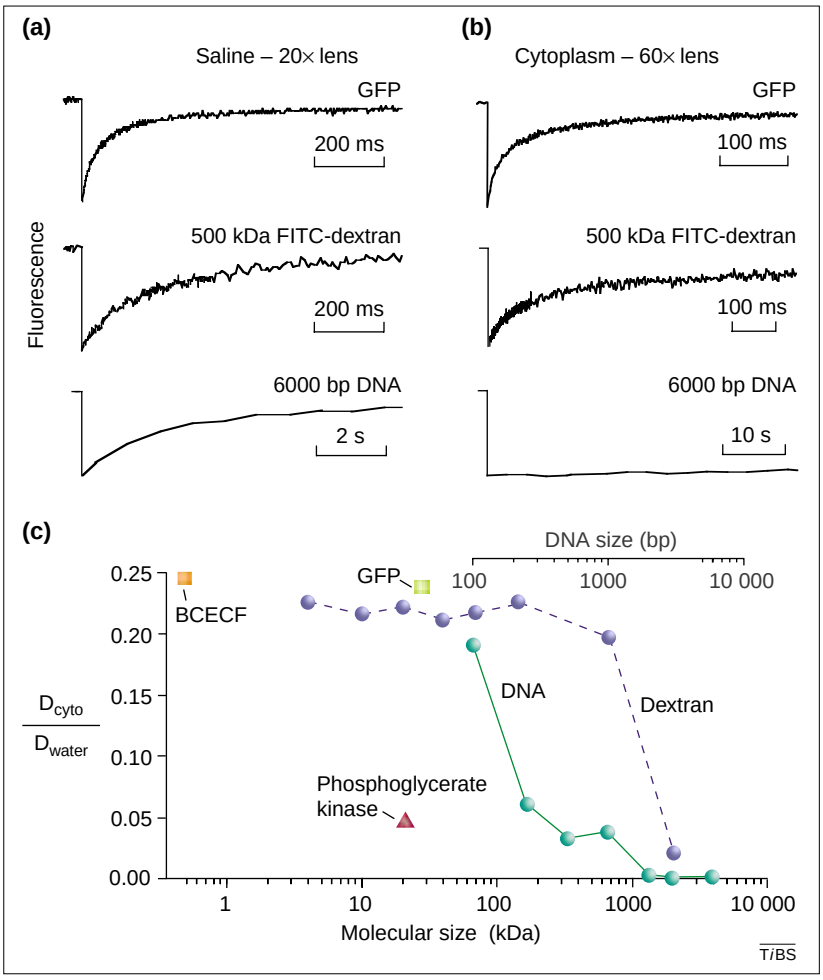

Fig. 2. Diffusion of solutes and macromolecules in cytoplasm. (a) Spot photobleaching (20× objective, short bleaching time) of dilute saline solutions of indicated compounds. (b) Spot photobleaching (60× objective, short bleaching time) of unconjugated green fluorescent protein in cytoplasm of transfected cells, or indicated fluorescein-labeled dextran and linear dsDNA fragment in microinjected cells. (c) Ratio of diffusion coefficient in cytoplasm versus saline (D_{cyto}/D_{water}) for indicated solute/macromolecules. Data taken, with permission, from Refs [9,12,26,31,36].

done by time-resolved anisotropy methods when rotation occurs over tens of nanoseconds or faster (Fig. 1c) [18]. Slower rotational mobilities can, in principle, be measured by polarization photobleaching or correlation methods, or by time-resolved phosphorescence anisotropy. Magnetic resonance methods have also been used to obtain indirect information about solute diffusion [19], although they are much less sensitive than fluorescence methods and do not permit cell-level spatial resolution.

Diffusion in cytoplasm

For transport of small solutes such as metabolites, second messengers and nucleotides, an important parameter describing cytoplasmic viscosity is the solute translational diffusion coefficient. We analyzed the determinants of the translational mobility of a small fluorescent probe, BCECF, in cytoplasm using spot photobleaching [9]. Diffusion of BCECF in cytoplasm was approximately four times slower than in water (D_{cyto}/D_{water} ~0.25). Three independently acting factors were identified that accounted quantitatively for the fourfold slowed diffusion: (1) slowed diffusion in fluid-phase cytoplasm, (2) probe binding to intracellular components, and (3) probe collisions with intracellular components (molecular crowding). Slowed diffusion in fluid-phase cytoplasm (factor 1, also called fluid-phase viscosity) is defined as the microviscosity sensed by a small solute in the absence of interactions with macromolecules and organelles. As measured by time-resolved anisotropy [18], the picosecond rotational correlation times of BCECF and other small solutes were only 10–30% slower in cytoplasm than in water, indicating that the fluid-phase viscosity of cytoplasm is not much greater than that of water. Subsequent experiments using a probe in which steady-state fluorescence sensed local viscosity confirmed that cytoplasmic fluid-phase viscosity is low [20]. Probe binding (factor 2), as quantified by confocal microscopy in digitonin-permeabilized cells, and independently by time-resolved anisotropy (amplitude of slowly rotating component), only accounted for ~20% of the slowed diffusion. Probe collisions (factor 3) was assessed by comparative measurements of cytoplasmic BCECF diffusion in shrunken and swollen cells versus BCECF diffusion in saline solutions containing different concentrations of dextran to mimic molecular crowding. Probe collisions were determined to be the principal diffusive barrier that accounted for the fourfold slowed diffusion of BCECF in cytoplasm versus water. Similar slowing of BCECF diffusion was measured in membrane-adjacent cytoplasm using total-internal-reflection photobleaching [21].

The paradigm of three distinct barriers to diffusion – fluid-phase viscosity, binding and crowding – is generally applicable to the analysis of solute diffusion in cellular compartments. An automobile analogy is useful to explain this concept – the time required for a car to travel from one point to another depends independently on its speed (fluid-phase viscosity), time spent at stop lights (binding) and route (molecular crowding).

Several laboratories have studied the diffusion of larger molecules. In early experiments by Luby-Phelps and colleagues [22,23], spot photobleaching was used to measure the translational diffusion of microinjected, fluorescently labeled dextrans and Ficolls. As dextrans and Ficolls are essentially non-interacting macromolecules, it was assumed that their slowing in cytoplasm is primarily caused by molecular crowding. As the molecular size of dextran or Ficoll was increased, diffusion in cytoplasm progressively decreased relative to that in water, suggesting a cytoplasmic 'sieving' mechanism that was proposed to involve the skeletal mesh. Qualitatively similar findings were observed for dextran diffusion in the cytoplasm of developing nerve processes [24] and skeletal muscle cells [25], although detailed comparisons are not possible because of differences in cell type and methods. Our laboratory conducted a series of

quantitative comparisons of the diffusion of size-fractionated FITC-dextrans and FITC-Ficolls introduced into cytoplasm by microinjection [26], and GFP introduced by transfection [12]. Examples of original spot-photobleaching recovery curves measured in saline and cytoplasm are provided in Fig. 2a,b. Figure 2c summarizes D_{cyto}/D_{water} values for different solutes and macromolecules. Diffusion of GFP was 3–5-fold slowed in cytoplasm versus saline, as was the diffusion of FITC-dextrans and FITC-Ficolls of molecular size ≤500 kDa (equivalent radius of gyration 20–30 nm). Similar slowing of GFP diffusion in cytoplasm was recently reported in photobleaching measurements in amoebae [27] and by fluorescence-correlation microscopy in mammalian cells [28]. The diffusion of very large molecules (e.g. 2000-kDa FITC-dextran) was remarkably impaired. Although the exact details of the D_{cyto}/D_{water} versus molecular size curve shape (Fig. 2c) probably depend on cell type and, to some extent, on analysis procedures (assumption of single component hindered diffusion versus complex diffusion), the main message from the work of several laboratories is that the diffusion of small macromolecules in cytoplasm is only mildly impaired whereas that for large macromolecules can be greatly impaired (see Ref. [29] for a recent review).

Although molecular crowding and sieving restrict the mobility of very large solutes, binding can severely restrict the mobility of smaller solutes. The diffusional mobility of DNA fragments in cytoplasm is thought to be an important determinant of the efficacy of DNA delivery in gene therapy and antisense oligonucleotide therapy [30]. Liposome-mediated gene transfer involves endocytic uptake, release from endosomes, dissociation of DNA from lipid, diffusion through cytoplasm, transport across nuclear pores and diffusion to nuclear target sites. We measured the translational diffusion of fluorescein-labeled double stranded (naked) DNA fragments from oligonucleotide size (21 base pairs) to plasmid size (6000 base pairs) after microinjection into cytoplasm [31]. As shown in Fig. 2b,c, the diffusion of DNAs of size >250 base pairs was remarkably reduced whereas diffusion of comparably sized FITC-dextrans was not impaired. This size-dependent diffusion of DNA might reflect the binding of DNA to cellular components and/or the non-spherical shape of the DNA molecules.

Similar to DNA, the diffusion of proteins in cytoplasm can be impaired by binding interactions, as shown by measurements of labeled protein diffusion in nerve processes [24], muscle cells [32], fibroblasts [33] and *Escherichia coli* [34]. Diffusion of several microinjected FITC-labeled glycolytic enzymes has been studied and found to be impaired compared with that of comparably sized FITC-dextrans. D_{cyto}/D_{water} data for phosphoglycerate kinase are shown in Fig. 2c. There is evidence that the diffusion of some glycolytic enzymes might by regulated by cell metabolic state. Metabolic depletion by 2-deoxyglucose in 3T3 cells resulted in an ~20% increase in the fraction of mobile aldolase [35]. In preliminary measurements, we found that D_{cyto}/D_{water} for pyruvate kinase was reduced from ~0.08 to 0.04 when cells were depleted of ATP, whereas D_{cyto}/D_{water} for phosphoglycerate kinase (~0.04) was not changed [36]. Altered enzyme binding to slowly diffusing cytoplasmic components might be responsible for the dependence of mobility of some enzymes on metabolic state. Altering enzyme diffusion in response to cell metabolic requirements could provide an elegant mechanism to regulate metabolism, although this speculation will require direct experimental verification.

Diffusion in organelles: mitochondrial matrix and endoplasmic reticulum

Solute diffusion in the aqueous lumen of intracellular organelles is involved in many processes, such as metabolism in mitochondria, and protein processing and recognition in the endoplasmic reticulum. The mitochondrion is spatially organized into a porous outer membrane, an intermembrane space, an inner membrane and an inner aqueous compartment known as the matrix. The inner membrane is the site of the electron transport pathway, whereas the matrix is the site of the tricarboxylic acid cycle and the fatty acid oxidation pathway. Although the sites, mechanisms and sequences of these and other mitochondrial reactions are, in general, well-established, much less is known about the spatial organization of metabolism. The matrix is a particularly interesting compartment because of its high density of enzymes and other proteins; these can constitute >60% of the matrix volume depending on metabolic state. Theoretical considerations have suggested that the diffusion of metabolite- and enzyme-sized solutes might be severely restricted in the mitochondrial matrix [1,2]. One report on solute diffusion in the matrix supported the notion that solute diffusion is severely restricted [37]; however, the high BCECF steady-state anisotropy in that study was subsequently shown to result from binding rather than from restricted diffusion [38].

The ability to target GFP to the mitochondrial matrix provided a unique opportunity to test the hypothesis that solute diffusion is greatly slowed in the matrix. The diffusion of unconjugated GFP (targeted to matrix using cleavable COX8 leader sequence) was measured in the mitochondrial matrix of fibroblast, liver, skeletal muscle and epithelial cell lines [38]. GFP was expressed selectively in the mitochondrial matrix (Fig. 3a). Spot photobleaching of GFP with a 100× objective (0.8 μm spot diameter) gave a half-time for fluorescence recovery of ~20 msec with >90% of the GFP being mobile (Fig. 3b, left). Control studies showing slowed or abolished fluorescence recovery after paraformaldehyde fixation (Fig. 3b, right), or with increased spot size or bleach time, confirmed that the recovery represented authentic diffusion. The fluorescence recovery data were

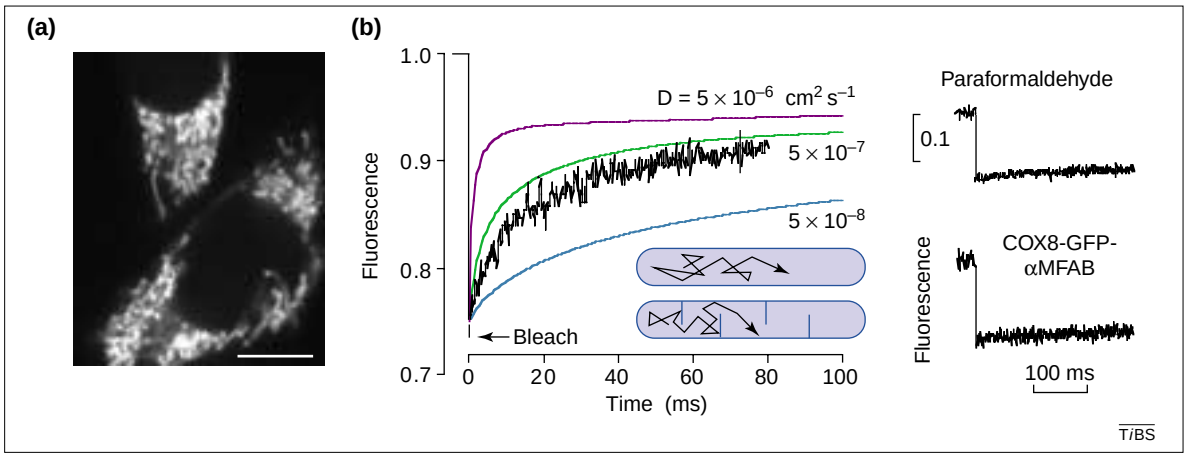

Fig. 3. Diffusion of green fluorescent protein (GFP) in the mitochondrial matrix. (a) Fluorescence micrograph of GFP in the mitochondrial matrix of transfected CHO cells. (b) (Left) Spot photobleaching of unconjugated GFP (100× objective) shown with model predictions for fluorescence recovery with diffusion coefficients (D). Inset: diffusion in the mitochondrial matrix without (top) and with (bottom) obstructions. (Right) Recovery data for the same system after paraformaldehyde fixation (top) and where the mitochondrial matrix was targeted with a GFP–matrix enzyme chimera (α-subunit of the mitochondrial β-fatty acid oxidation pathway). Scale bar = 5 μm. Adapted, with permission, from Ref. [38].

analyzed using a mathematical model of matrix diffusion. Mitochondria were modeled as long, continuously open cylinders with a specified orientational distribution (Fig. 3b, inset, top). Fluorescence recoveries were computed from analytical solutions to the diffusion equation. Predicted recovery curves for different diffusion coefficients are shown in Fig. 3b (left) along with experimental data. The fitted diffusion coefficient was $2–3 \times 10^{-7}$ cm^2 s^{-1}, only 3–4-fold less than that for GFP diffusion in water. The actual GFP diffusion coefficient is probably slightly higher because the mitochondria contain internal barriers called cristae (Fig. 3b, inset, bottom). A Monte-Carlo mathematical model was developed to evaluate the effects of cristae on apparent diffusion coefficients [39]; an interesting conclusion was that barriers had to be very large (occluding >90% of space) to slow diffusion significantly. Whereas unconjugated GFP diffused rapidly in the mitochondrial matrix, little fluorescence recovery was found for bleaching of GFP in fusion with subunits of the fatty acid α-oxidation multienzyme complex (Fig. 3b, right, bottom) and other enzymes (isocitrate dehydrogenase, malate dehydrogenase, succinyl CoA synthetase [36]) that are normally present in the matrix. The rapid and unrestricted diffusion of GFP in the mitochondrial matrix suggested that classical metabolite channeling might not be required. It was proposed that the clustering of matrix enzymes in membrane-associated complexes might serve to establish a relatively uncrowded aqueous space in which solutes can freely diffuse, thus reducing metabolite transit times. There is good kinetic and biochemical evidence for functionally important enzyme–enzyme interactions that are consistent with the notion of clustering [2].

The ER is an extended tubular/plate structure that serves as the organelle for the initial processing and quality control of newly synthesized proteins. The ER lumen is densely filled with small solutes, such as calcium and glutathione, and proteins (100–200 mg protein ml^{-1}) including lipid synthases and molecular chaperones. GFP was targeted to the ER lumen by transient transfection with a cDNA encoding GFP with a C terminus KDEL retention sequence and an upstream prolactin secretory sequence [40]. Figure 4a shows a series of ER images before and after bleaching of a large spot denoted by the dashed white circle. Fluorescence recovered over time, such that the ER appeared to be identical before the bleach and after 30 sec, except for lower overall fluorescence because a substantial fraction of the total number of ER-associated GFP molecules were bleached (note bright unbleached cell denoted by arrow). Repeated laser illumination at the same very small spot resulted in complete bleaching of ER-associated GFP throughout the cell (Fig. 4b), indicating a continuous ER lumen. Interestingly, after 60 bleach pulses, the remaining fluorescence (<2% of original signal) had a Golgi-like pattern, suggesting imperfect KDEL retention. Quantitative spot photobleaching with a single brief bleach laser pulse (<0.1 msec) indicated that GFP was fully mobile (Fig. 4c). Direct comparison of GFP bleaching in the ER versus the cytoplasm indicated an approximately threefold slowing in the ER. However, as modeled mathematically [39], 25–50% of the apparent slowing in the ER is a consequence of its complex geometry – diffusion through interconnected tubes or plates is mildly slowed compared with diffusion in open space. The main message is that diffusion of unconjugated GFP is free and rapid in the ER lumen. ER diffusion of chimeras of GFP and nascent proteins could be useful in evaluating protein folding and interactions with molecular chaperones.

Perspective and directions

Photobleaching and related dynamic fluorescence measurements have provided quantitative information about the dynamics of biologically important solutes and macromolecules in cellular aqueous compartments. Rapid advances in fluorophore-targeting methods make possible the selective labeling of cellular components with a wide variety of chromophores. The description of diffusive phenomena in terms of three independent barriers provides a useful framework to understand how many types of solutes diffuse in specific cellular

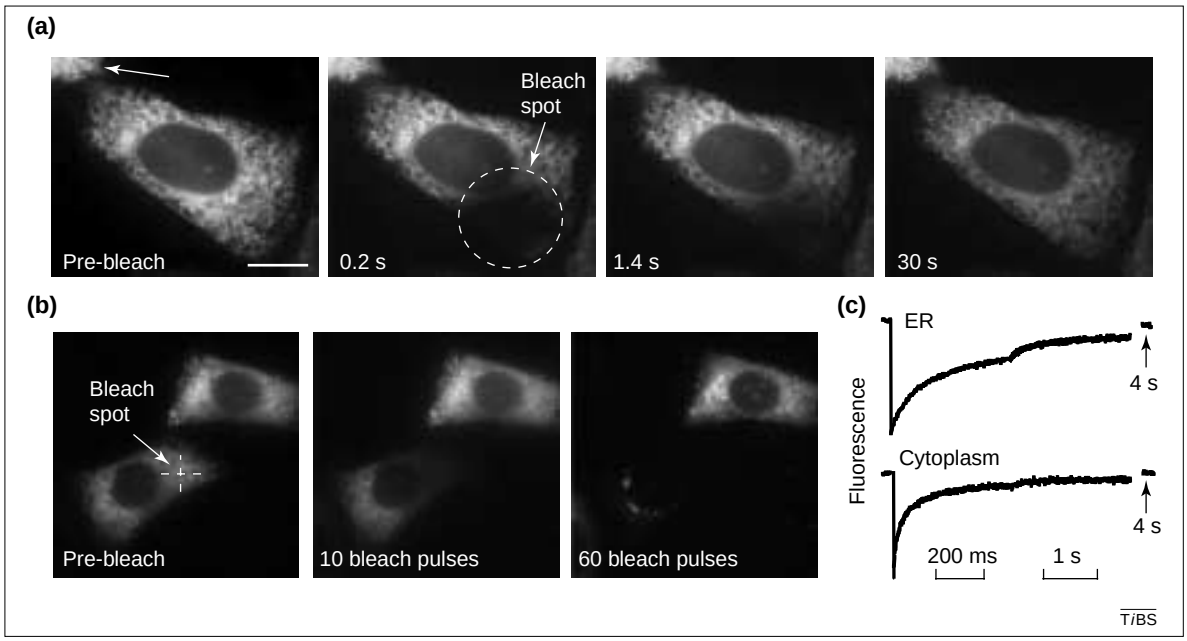

Fig. 4. Diffusion of unconjugated green fluorescent protein (GFP) in the lumen of endoplasmic reticulum (ER) in transfected CHO cells. (a) Serial micrographs showing ER fluorescence before (pre-bleach) and at indicated times after bleaching (bleach time 200 ms). Bleach spot is denoted by a dashed white circle. Arrow points to an adjacent unbleached cell. Scale bar = 5 μm. (b) Micrographs showing fluorescence depletion from the ER before and after indicated number of bleach pulses using a small (<1 μm) bleach spot. (c) Spot photobleaching (60× objective) of GFP in ER versus cytoplasm. Adapted, with permission, from Ref. [40].

compartments. Binding to fixed and mobile cellular components constitutes a major barrier for the diffusion of many types of molecules such as enzymes and DNA. Molecular crowding and geometric effects become rate-limiting for the diffusion of large (≥500 kDa) macromolecules and molecular complexes. However, these general paradigms have limited predictive value because of the complex and heterogeneous makeup of the intracellular milieu. For example, the reasons for the strong size-dependence of naked DNA diffusion in cytoplasm remain unknown.

The implications of restricted molecular diffusion for cell function remain a major unresolved issue. Limited data support the hypothesis that regulated enzyme mobility might provide a novel metabolic control mechanism. The impaired cytoplasmic mobility of plasmid-size DNA suggests that cytoplasmic diffusion might be a key rate-limiting barrier in non-viral gene delivery. Experimental attempts to alter molecular diffusion, for example using metabolic or skeletal disruption maneuvers, could be useful not only in understanding diffusive barriers, but also in improving the efficacy of drug and/or gene delivery. Finally, the understanding of solute mobility and mitochondrial metabolism might be important in disease pathogenesis. There are dozens of neuromuscular diseases caused by maternally inherited mutations in mitochondrial DNA encoding components involved in oxidative phosphorylation. The role of altered solute diffusion and macromolecule associations in disease pathogenesis mandates investigation.

References

1 Goodsell, D.S. (1991) Inside a living cell. *Trends Biochem. Sci.* 16, 203–206
2 Ovadi, J. and Srere, P.A. (2000) Macromolecular compartmentation and channeling. *Int. Rev. Cytol.* 192, 255–280
3 Rohwer, J.M. *et al.* (1998) Implications of macromolecular crowding for signal transduction and metabolite channeling. *Proc. Natl. Acad. Sci. U. S. A.* 95, 10547–10552
4 Welch, G.R. and Easterby, J.S. (1994) Metabolic channeling versus free diffusion: transition-time analysis. *Trends Biochem. Sci.* 19, 193–197
5 Minton, A.P. (2001) The influence of macromolecular crowding and macromolecular confinement on biochemical reactions in physiological media. *J. Biol. Chem.* 276, 10577–10580
6 Verkman, A.S. *et al.* (2001) Diffusion measurements by photobleaching recovery methods. In *Methods in Cellular Imaging* (Periasamy, A., ed.), pp. 112–127, Oxford University Press
7 Feder, T.J. *et al.* (1996) Constrained diffusion or immobile fraction on cell surfaces: a new interpretation. *Biophys. J.* 70, 2767–2773
8 Periasamy, N. and Verkman, A.S. (1998) Analysis of fluorophore diffusion by continuous distributions of diffusion coefficients: application to photobleaching measurements of simple and anomalous diffusion. *Biophys. J.* 75, 557–567
9 Kao, H.P. *et al.* (1993) Determinants of the translational diffusion of a small solute in cell cytoplasm. *J. Cell Biol.* 120, 175–184
10 Song, L. *et al.* (1996) Influence of the triplet excited state on the photobleaching kinetics of fluorescein in microscopy. *Biophys. J.* 70, 2959–2968
11 Periasamy, N. *et al.* (1996) Reversible photobleaching of fluorescein conjugates in air-saturated viscous solutions: molecular tryptophan as a triplet state quencher. *Photochem. Photobiol.* 63, 265–271
12 Swaminathan, R. *et al.* (1997) Photochemical properties of green fluorescent protein GFP-S65T in solution and transfected CHO cells: analysis of cytoplasmic viscosity by GFP translational and rotational diffusion. *Biophys. J.* 72, 1900–1907
13 Levin, M.H. *et al.* (2001) Diffusion in the endoplasmic reticulum of an aquaporin-2 mutant causing human nephrogenic diabetes insipidus. *J. Biol. Chem.* 276, 21331–21336
14 Haupts, U. *et al.* (1998) Dynamics of fluorescence fluctuations in green fluorescent protein observed by fluorescence correlation spectroscopy. *Proc. Natl. Acad. Sci. U. S. A.* 95, 13573–13578
15 Maiti, S. *et al.* (1997) Fluorescence correlation spectroscopy: diagnostics for sparse molecules. *Proc. Natl. Acad. Sci. U. S. A.* 94, 11753–11757
16 Widengren, J. and Rigler, R. (1998) Fluorescence correlation spectroscopy as a tool to investigate chemical reactions in solutions and on cell surfaces. *Cell. Mol. Biol.* 44, 857–879
17 Magde, D. *et al.* (1974) Fluorescence correlation spectroscopy. II. An experimental realization. *Biopolymers* 13, 29–61
18 Fushimi, K. and Verkman, A.S. (1991) Low viscosity in the aqueous domain of cell cytoplasm measured by picosecond polarization microscopy. *J. Cell Biol.* 112, 719–725
19 Mastro, A.M. and Keith, A.D. (1984) Diffusion in the aqueous compartment. *J. Cell Biol.* 99, 180s–187s
20 Luby-Phelps, K. *et al.* (1993) A novel fluorescence ratiometric method confirms the low solvent viscosity of the cytoplasm. *Biophys. J.* 65, 236–242

21 Swaminathan, R. *et al.* (1996) Cytoplasmic viscosity near the cell plasma membrane: translation of BCECF measured by total internal reflection-fluorescence photobleaching recovery. *Biophys. J.* 71, 1140–1151
22 Luby-Phelps, K. *et al.* (1986) Probing the structure of the cytoplasm. *J. Cell Biol.* 102, 2015–2022
23 Luby-Phelps, K. *et al.* (1987) Hindered diffusion of inert tracer particles in the cytoplasm of mouse 3T3 cells. *Proc. Natl. Acad. Sci. U. S. A.* 84, 4910–4913
24 Popov, S. and Poo, M.M. (1992) Diffusional transport of macromolecule in developing nerve processes. *J. Neurosci.* 12, 77–85
25 Arrio-Dupont, M. *et al.* (1996) Diffusion of fluorescence labeled macromolecules in cultured muscle cells. *Biophys. J.* 70, 2327–2332
26 Seksek, O. *et al.* (1997) Translational diffusion of macromolecule-size solutes in cytoplasm and nucleus. *J. Cell Biol.* 138, 131–142
27 Potma, E.O. *et al.* (2001) Reduced protein diffusion rate by cytoskeleton in vegetative and polarized dictyostelium cells. *Biophys. J.* 81, 2010–2019
28 Wachsmuth, M. *et al.* (2000) Anomalous diffusion of fluorescent probes inside living cell nuclei investigated by spatially-resolved fluorescence correlation spectroscopy. *J. Mol. Biol.* 298, 677–689
29 Luby-Phelps, K. (2000) Cytoarchitecture and physical properties of cytoplasm: volume, viscosity, diffusion, intracellular surface area. *Int. Rev. Cytol.* 192, 189–221
30 Bally, M.D. *et al.* (1999) Biological barriers to cellular delivery of lipid-based DNA carriers. *Adv. Drug Deliv. Rev.* 38, 291–315
31 Lukacs, G.L. *et al.* (2000) Size-dependent DNA mobility in cytoplasm and nucleus. *J. Biol. Chem.* 275, 1625–1629
32 Arrio-Dupont, M. *et al.* (2000) Translational diffusion of globular proteins in the cytoplasm of cultured muscle cells. *Biophys. J.* 78, 901–907
33 Pagliaro, L. and Taylor, D.L. (1988) Aldolase exists in both the fluid and solid phases of cytoplasm. *J. Cell Biol.* 107, 981–991
34 Elowitz, M.B. *et al.* (1999) Protein mobility in the cytoplasm of *Escherichia coli*. *J. Bacteriol.* 181, 197–203
35 Pagliaro, L. and Taylor, D.L. (1992) 2-Deoxyglucose and cytochalasin D modulate aldolase mobility in living 3T3 cells. *J. Cell Biol.* 118, 859–863
36 Haggie, P. *et al.* (2001) Diffusion of metabolic enzymes in mitochondria and the cytoplasm measured by fluorescence photobleaching. *Biophys. J.* 80, 280a–281a
37 Scalettar, B.A. *et al.* (1991) Dynamics, structure, and function are coupled in the mitochondrial matrix. *Proc. Natl. Acad. Sci. U. S. A.* 88, 8057–8061
38 Partikian, A. *et al.* (1998) Rapid diffusion of green fluorescent protein in the mitochondrial matrix. *J. Cell Biol.* 140, 821–829
39 Ölveczky, B.P. and Verkman, A.S. (1998) Monte-Carlo analysis of obstructed diffusion in 3 dimensions: application to molecular diffusion in organelles. *Biophys. J.* 74, 2722–2730
40 Dayel, M. *et al.* (1999) Diffusion of green fluorescent protein in the aqueous lumen of endoplasmic reticulum. *Biophys. J.* 76, 2843–2851

Nitric oxide and cytochrome oxidase: substrate, inhibitor or effector?

Chris E. Cooper

Endogenously produced nitric oxide (NO) controls oxygen consumption by inhibiting cytochrome *c* oxidase, the terminal electron acceptor of the mitochondrial electron transport chain. The oxygen-binding site of the enzyme is an iron/copper (haem a_3/Cu_B) binuclear centre. At high substrate (ferrocytochrome *c*) concentrations, NO binds reversibly to the reduced iron in competition with oxygen. At low substrate concentrations, NO binds to the oxidized copper. Inhibition at the haem iron site is relieved by dissociation of the NO from the reduced iron. Inhibition at the copper site is relieved by oxidation of the bound NO and subsequent dissociation of nitrite from the enzyme. Therefore, NO can be a substrate, inhibitor or effector of cytochrome oxidase, depending on cellular conditions.

Chris E. Cooper
Dept of Biological Sciences, Central Campus, University of Essex, Wivenhoe Park, Colchester, UK CO4 3SQ.
e-mail: ccooper@essex.ac.uk

Cytochrome oxidase is the terminal electron acceptor of mitochondrial, and many bacterial, electron transfer chains. Reduced cytochrome *c* (in all eukaryotes and some prokaryotes) or quinol (in some prokaryotes) is oxidized by the enzyme and, in the process, oxygen is reduced to water. The redox energy in this process is converted to a proton motive force, which subsequently drives ATP synthesis (Fig. 1). The enzyme contains three redox-active metal sites: Cu_A, haem *a* and a haem a_3/Cu_B binuclear centre. The copper–copper dimer (Cu_A) and haem iron (haem *a*) catalyse electron transfer from the substrate to the oxygen reduction site, where the binuclear haem–copper coupled centre (haem a_3/Cu_B) then catalyses the reduction of oxygen to water (Fig. 2). A zinc and magnesium ion play as yet undiscovered, probably structural, roles. The structure and function of cytochrome oxidase has recently been reviewed by Yoshikawa [1].

Nitric oxide (NO) is a small gaseous free radical that binds readily to haem iron [2]. NO interactions with cytochrome oxidase have been studied for many years. The first report of the spectral changes following NO binding to the enzyme were by Wainio in 1955 [3]. Since then, several studies have used NO as a probe of the structure and function of the haem a_3/Cu_B binuclear centre, both via optical and electron paramagnetic resonance (EPR) techniques [4,5]. Indeed, the hyperfine structure of the EPR spectrum of the NO complex of the reduced haem a_3 was the first evidence that histidine was the proximal ligand to the haem iron [6].

With the discovery in the late 1980s that NO was a eukaryotic intercellular messenger [7] – produced by the nitric oxide synthase (NOS) enzyme family and reacting with the haem protein guanylate cyclase to produce cyclic GMP – interest was rekindled in other possible targets of NO action. Early on, mitochondria were cited as possible secondary targets for NO; suggestions focused on a role for the immune system in producing excess NO to damage mitochondrial proteins in, for example, tumour cells [8]. Mitochondrial iron-sulfur enzymes (e.g. NADH dehydrogenase, succinate dehydrogenase, aconitase)

 0968-0004/02/$ – see front matter PII: S0968-0004(01)02035-7