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## Point of View

# The biochemistry of RNA metabolism studied in situ

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**Abbreviations:** FRAP, fluorescence recovery after photobleaching; FRET, Förster (or fluorescence) resonance energy transfer; FLIM, fluorescence lifetime imaging microscopy

**Key words:** in situ biochemistry, in vitro FRAP, FRAP, FRET, FLIM, SRm160, UAP56

In vitro assays have contributed important insights into the mechanisms of RNA metabolism in cells. A growing collection of microscopy techniques is allowing the measurement of macromolecular binding and complex formation in the context of a real cell. We will first discuss two of the more established techniques. Fluorescence resonance energy transfer (FRET) identifies binding partners, pairs of molecules residing in the same macromolecular complexes. The complimentary technique of fluorescence recovery after photobleaching (FRAP) measures the rates of binding and unbinding of those molecules in their complexes. A newer technique—in vitro FRAP—assesses the regulation of binding and complex formation by co-factors in the nucleus.

*Molecular biology is essentially the practice of biochemistry without a license.*—Erwin Chargaff

The study of biology took a most productive turn in the 19<sup>th</sup> century with the development of biochemistry, which reduced the incredible complexity and diversity of life to a few unifying principles of chemistry. Though the experiments of biochemistry have uncovered an enormous number of facts about molecules, reactions and pathways—it is the principles that have made biochemistry a logically consistent body of thought. It is the principles that have made it a powerful science.

These principles, indeed most phenomena of life itself, are reducible to the processes of binding and catalysis. There is also a non-catalytic, spontaneous chemistry in living organisms, but binding and catalysis are most central to our understanding of the uniqueness of life. All cell structures arise from binding interactions. All the macromolecular complexes responsible for creating and using RNA, including those responsible for transcription, RNA processing, RNA translocation and mRNA translation, are assembled by binding and disassembled by unbinding, and in their catalysis they bind substrates and unbind products. This catalysis is regulated by the

binding and unbinding of regulatory factors, some of which catalytically modify the complex.

In this article I will concentrate on the binding of macromolecules into complexes and structures, contrasting traditional in vitro approaches to an emerging set of techniques that can identify binding interactions and characterize the physical biochemistry of those interactions by microscopy. Of these many techniques I will only briefly examine the use of fluorescence resonance energy transfer (FRET) to identify the composition of macromolecular complexes in real cells, and the use of photobleaching techniques—including fluorescence recovery after photobleaching (FRAP)—to determine the binding constants governing the assembly and disassembly of those complexes. Only a few of the many applications of these techniques to RNA biology have been selected for discussion. More emphasis will be placed on “in vitro FRAP,” an extension of the FRAP method, which permits the identification of small molecules that regulate macromolecular complex assembly and the characterization of the mechanisms by which they do so.

These powerful, though often difficult, techniques allow the biochemical characterization of molecular interaction in cells, rather than in tubes. They overcome the major limitation on biochemistry in the past, and will help us refute the damning critique of Erwin Chargaff that, “...biochemistry is helpless before life, having to kill the organism before investigating it. Biochemistry is, in fact, much more successful in practicing the second part of its composite name than in following the prefix.”

## The Power of Biochemistry: Mechanism and Manipulation

In vitro biochemistry offers great power for the characterization of molecular interactions and reactions, because systems can be simplified to their essential components. Sometimes systems can even be reconstituted from isolated components, leaving no doubt that the system is self-contained. Even when this is not possible, individual components can be depleted and added back, with the consequences noted. This power of in vitro biochemistry comes with the significant and paradoxical costs of oversimplification and overcomplication. In vitro systems ignore components present in cells, but lost by the isolation protocols used to reconstitute simplified systems. On the other hand, in vitro systems may identify and characterize molecular interactions that might not function in cells. For example, two proteins may interact when they encounter, but may normally be in different cellular compartments. At least, until homogenization.

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Newer methods of in vitro biochemical analysis can yield too much information, cluttering and obscuring our understanding of mechanism. For example, co-immunoprecipitation combined with protein mass spectrometry can identify hundreds of potential binding partners. Not all binding interactions are equal in prevalence or importance in living cells; not all exist in cells. The inclusion of low-significance interactions on a protein mass spectrometry list can confound, rather than clarify, our thinking about biological function. SRm160 is an important mRNA splicing factor,<sup>1</sup> later found in mRNA export complexes. A proteomic study of its binding partners, identified 110 co-immunoprecipitating proteins.<sup>2</sup> Theoretically, by including only a single copy of SRm160 and of each partner in complexes we could assemble 110 different dimers, 12,100 different trimers, more than 1.3 million tetramers, and so on. It is important to supplement these powerful techniques of in vitro biochemistry with experimental approaches that detect the complexes that actually form in cells and at the locations where they exist.

## Determination of Molecular Interaction in Cells

FRET is also known as Förster resonance energy transfer in honor of Theodor Förster, who first recognized the principle and developed the theory.<sup>3</sup> After a fluorochrome in solution absorbs light, vibrational relaxation occurs rapidly. If there is a resonance of the energy transitions between the fluorochrome and a nearby, unexcited fluorochrome, there can be a transfer of energy and emission of light from the second fluorochrome. The emitted light from the second fluorochrome is at a higher wavelength than the fluoresced light from the first, and so is spectrally distinguishable. The application of this phenomenon to microscopy is presented in the cartoon of Figure 1.

In practice, FRET between two fluorophores occurs only when the distance between them is in the range of 1–10 nm.<sup>4</sup> At this distance we can be assured that both proteins are present within the same complex. In fact, because FRET efficiency decreases with the 6<sup>th</sup> power of the distance between two fluorophores, FRET might theoretically be used as a distance measurement. This would be difficult to achieve in practical cell biology. Nevertheless, the determination that two fluorochromes are present in the same 10 nm or smaller area, lets this technique evade the law of physics that would practically limit the spatial resolution of light microscopy to about 200 nm.<sup>5</sup>

In the case of SRm160, with its 110 potentially interacting proteins, we have recently used FRET to show that mRFP-SRm160 interacts with GFP-UAP56 at RNA splicing speckled domains.<sup>6</sup>

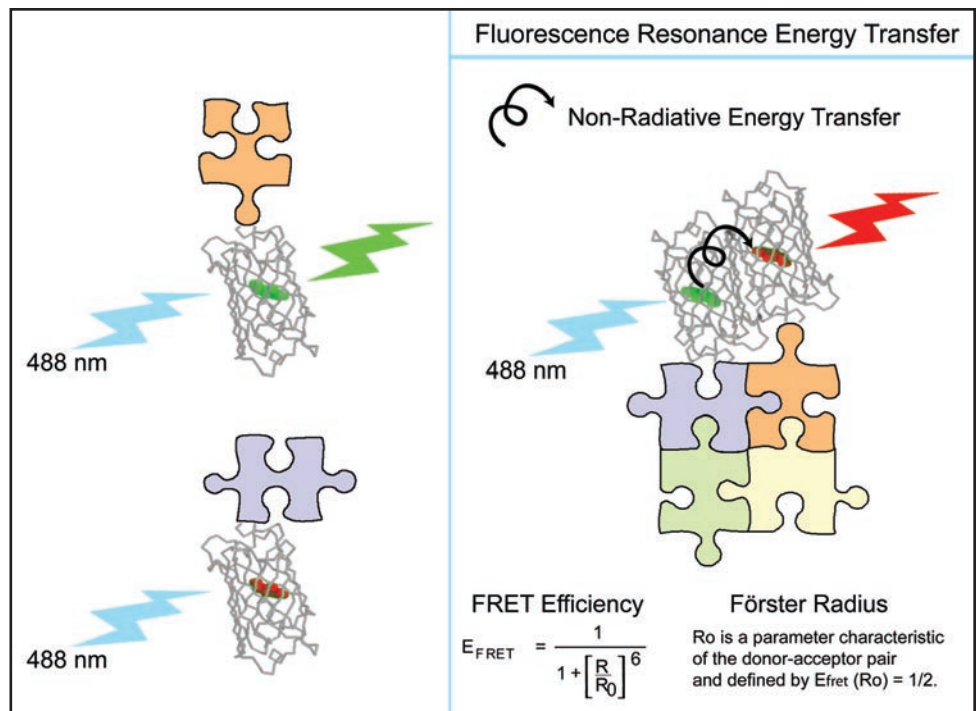


Figure 1. FRET measures macromolecular interactions. In this drawing of a typical FRET experiment, the puzzle pieces represent proteins fused to GFP and mRFP that can bind together in complexes. When they are not incorporated into the complex, only GFP is excited by light at 488 nm and only GFP emits fluoresced light. When both proteins are present in the same complex and their fluorescent domains are close together, GFP is excited at 488 nm but transfers some of the energy of excitation by a non-radiative process to the nearby mRFP. The mRFP, now excited by the transfer, can fluoresce light with an energy distribution of its own higher wavelength emission spectrum. The equation at the bottom shows that FRET efficiency falls off with the 6<sup>th</sup> power of the distance between the fluorescent domains, so that both must be 1–10 nm apart for the transfer to be detected. Other common FRET pairs include CFP-YFP.

UAP56 is an ATP-dependent RNA helicase of the DExD/H box family with, in various species, a role in RNA splicing and in mRNA export to the cytoplasm. In our studies, a point mutation that blocked ATP binding to UAP56, and had a dominant negative effect on mRNA export, also abolished FRET with SRm160 at RNA splicing speckled domains.<sup>6</sup>

This was not the first application of FRET to the study of RNA metabolism. One interesting, earlier use of FRET probed the role that Cajal Bodies in the nucleus play in the assembly of the U4/U6-U5 tri-snRNP complex.<sup>7</sup> The results were consistent with a mechanism in which some U4 snRNP and U6 snRNP complex assembly occurs in the nucleoplasm before these complexes move from the nucleoplasm into Cajal Bodies for the next step, U4/U6 snRNP assembly. Further FRET studies established a physical interaction between the Cajal Body protein coilin and the snRNP protein SMN and this interaction was localized to Cajal Bodies.<sup>8</sup>

FRET can be performed in live cells or after fixation. A common use of FRET is with fluorescent fusion proteins, but FRET between fluorescently tagged nucleic acids and proteins is also done. For fixed cells, fluorescently tagged antibodies can also be used. For example, molecular beacon-based FRET between snRNA and anti-fibrillarin antibodies (FITC) confirmed the interaction between fibrillarin and snRNA, identifying subnuclear sites of interaction in nuclei of *Giardia lamblia*.<sup>9</sup>

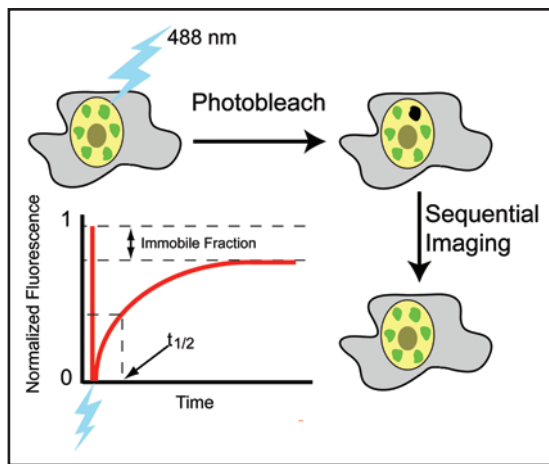


Figure 2. FRAP measures binding kinetics. This typical FRAP scheme measures the binding of a fluorescent mRNA splicing factor in complexes at RNA splicing speckled domains. The live cell is imaged to measure the pre-bleach distribution of the fluorescent protein. One speckle, or alternatively a region of the nucleus containing that speckle, is photobleached with high intensity laser light. A sequence of images is collected at time intervals after the bleach. In order for the fluorescence to recover in the bleached speckled domain, photobleached molecules must unbind from their complexes and still fluorescent molecules in unbleached speckled domains must also unbind. The two pools of splicing protein, fluorescent and dark, equilibrate by diffusion. Fluorescent molecules, diffusing into the bleach zone must then bind. For most proteins, this experiment is measuring binding or unbinding rates.<sup>13</sup> The fluorescence measurements after photobleaching are corrected for the fractional loss of fluorescence in the nucleus caused by the bleach. Data is often reported as a halftime ( $t_{1/2}$ ) of recovery. For many nuclear proteins it is observed that there are at least two populations: a population of photobleached molecules that are replaced, and an “immobile fraction” of molecules that are tightly bound, over the course of the experiment.

## FRET/FLIM

There are many microscopic protocols for measuring FRET,<sup>10</sup> all with substantial practical difficulties. Techniques for measuring FRET rely on fluorescence intensity measurements, but fluorescence intensity can be altered by other phenomena, and its measurement in FRET experiments is complicated by spectral overlap. More fundamentally, FRET can be difficult to establish as it depends on the distance between, orientation, and environment of the two molecules. As a result, a negative result cannot be interpreted as a lack of interaction and this, more than the technical issues, can make FRET a frustrating technique.

These difficulties in performing FRET studies have encouraged the search for alternative methods of FRET measurement. One promising approach detects FRET by the alteration of fluorescence excitation lifetime of the fluorochrome using fluorescence lifetime imaging microscopy (FLIM). Originally a spectroscopic technique, fluorescence lifetime measurements have been adapted to many microscopy systems. Fluorescence lifetime is very sensitive to changes in the microenvironment around a fluorochrome. For a GFP fusion protein, a very effective way to change that environment and to decrease fluorescence lifetime is to move a big FRETing fusion protein, for example one with mRFP, into the neighborhood. A big advantage of FLIM for detecting FRET is that it is a time measurement and not an intensity measurement.

Recently, FRET has been combined with FLIM to localize sites in cells where mRNA splicing complexes form.<sup>11</sup> The interacting proteins were the SR proteins SC35 and ASF/SF2, HCC1/CAPER, a 70K U1snRNP associated protein bound near the 5' splice site of the pre-mRNA, and the U2 snRNP-auxiliary factors U2AF35 and U2AF65 bound at the 3' splice site. Protein-protein interactions, first identified by *in vitro* assays as significant in splice site definition, were found to actually occur in cells at sites within the nucleoplasm and at RNA splicing speckled domains. In addition, interactions between HCC1/CAPER and both U2AF35 and U2AF65 were identified that had not been previously reported, consistent with a role for HCC1 in 3' splice site selection. FLIM may provide a more sensitive and higher contrast measure of FRET—fluorescence lifetime decreases with FRET—and FLIM can provide more information about the heterogeneity of complexes in a single voxel.<sup>12</sup>

## Binding Rate Constants Determined in Cells

While FRET can identify the molecules binding in complexes, the physical chemistry of binding, including the measurement of kinetic parameters, of equilibrium constants, and of the sizes of bound versus diffusing pools is readily studied in live cells by the complimentary technique of FRAP (Fig. 2), and its many derivatives.

In FRAP, a fluorescent molecule is introduced into a cell. A fluorescent spot or region of interest in the cell is photobleached. The rate of fluorescence recovery is measured. If there is recovery, then the photobleached or “dark” molecules in the bleach zone are replaced by homologous fluorescent molecules from other regions of the cell. When the fluorescent molecule is bound in a relatively immobile complex, the mechanism for this replacement must include unbinding of the “dark” molecules in the bleach zone, the unbinding of still fluorescent molecules outside of the bleach zone, the exchange of these two pools of molecules by diffusion, and the binding of fluorescent molecules to complexes in the bleach zone.<sup>13</sup> Thus the rate of recovery depends on binding and unbinding constants, and on diffusion coefficients.<sup>13</sup> If, as is usually the case in living cells, binding or unbinding are rate limiting then these binding constants, but not the diffusion coefficient, can be calculated from FRAP results.<sup>14,15</sup>

The first FRAP studies were designed to measure the lateral mobility of membrane proteins and test the fluid mosaic model of membrane structure.<sup>16,17</sup> The theory for analyzing these experiments accounted only for diffusion. The FRAP experimental design was later adapted to three dimensional molecule mobility and laser scanning confocal microscopy.<sup>18</sup> The broadening of FRAP theory to include binding of fluorescent molecules to intracellular structures then allowed the measurement of binding constants.<sup>13-15,19-22</sup>

The first uses of FRAP to study the dynamic function of nuclear proteins important in RNA metabolism were by the Misteli and Hendzell laboratories. The results showed, for example, that the photobleach recovery of the GFP-ASF/SF2 splicing factor was two orders of magnitude slower than that of just GFP.<sup>23,24</sup> Photobleach recovery can be retarded by making the recovering molecule large, perhaps, as part of a tightly bound complex. However, as the diffusion coefficient is inversely proportional only to the cube root of the mass,<sup>25</sup> such a complex would need to be unreasonably large in order to account for such a large difference in recovery time between GFP-ASF/SF2 and GFP. It is more likely that the recovery is slowed by binding. The bleached molecules must be released from their binding

sites at speckled domains, fluorescent molecules bound outside of the bleach zone must also unbind. The two pools must equilibrate while fluorescent molecules bind at sites vacated by bleached ones. The recovery kinetics of ASF/SF2, similar to those of most RNA splicing factors studied since, are consistent with binding and not diffusion being rate limiting. A lack of the envelope estimate suggests that a molecule like ASF/SF2, recovering two orders of magnitude slower than would be expected for a diffusion-limited recovery, may be diffusing only 1% of the time and the rest of the time is bound. More sophisticated tools for determining whether diffusion, unbinding, or binding are rate limiting have been presented, as have more sophisticated tools for measuring binding residence.<sup>13-15</sup> An additional factor that can slow photobleach recovery is the tortuosity of the nucleus, but this has not yet been adequately measured.

A powerful application of FRAP to the study of transcriptional control has used tandem arrays of model genes, all integrated into chromosomes. The integrated arrays provide a large target for photobleaching fluorescent transcription factors. Such studies have shown a surprisingly high rate of transcription factor turnover on promoters (reviewed in refs. 26 and 27). Many related alternative protocols to FRAP have been presented, using photobleaching or using laser activation of fluorochromes, and all with advantages for some experiments. We will concentrate below on only one extension of FRAP that allows for biochemical manipulation.

The assumptions on which a FRAP experiment are built frequently include the thought that tacking a large GFP on one end of a protein molecule will not affect its binding or function. Since almost all FRAP experiments are overexpression experiments, it must also be assumed that this overexpression has not overwhelmed the number of available binding sites. We have tried to address this potential problem by using stable cell lines where possible, in the hope that the level of overexpression is at least not toxic, and by comparing FRAP data from cells with different expression levels in the same experiment.<sup>28</sup> While these underlying assumptions cannot be completely correct, it is remarkable that FRAP experiments have generally yielded internally consistent results that are also consistent with *in vitro* data.

### In vitro FRAP

We developed a novel FRAP technique that assesses the role that small molecules play in the binding and exchange of macromolecules in nuclear complexes.<sup>28</sup> This technique—in *in vitro* FRAP—is presented schematically in Figure 3. A fluorescent molecule, for example a fluorescent-fusion protein, is introduced into a cell, where it binds in a complex at a structure in the nucleus. The structure, or a region of the nucleus, is photobleached and the time course of recovery is measured, providing information about the binding and unbinding of the fluorescent molecule in a live cell. Cells are then extracted in 50  $\mu\text{g}/\text{ml}$  digitonin to permeabilize the cell membrane without removing the nuclear envelope, a preparation protocol originally developed to characterize nucleocytoplasmic transport.<sup>29</sup> Molecules smaller than the diffusion limit for nuclear pores (40 to 60 kDa), for example  $\text{Mg}^{2+}\text{ATP}$ , can be depleted from nuclei or added back to the nucleoplasm while larger molecules, including fluorescent fusion-proteins, are constrained within the nuclear volume. In the case of ATP, nuclear levels decline to 1 nM or less in of digitonin-treated cells. The fluorescent molecule can then be

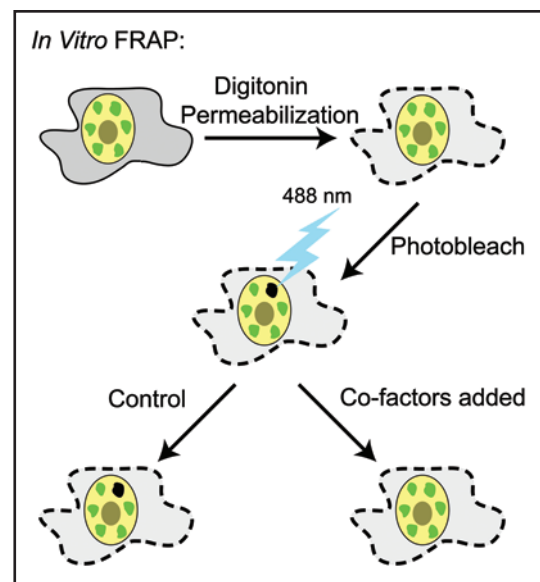


Figure 3. In *in vitro* FRAP identifies cofactors required for binding or unbinding. This extension of the FRAP assay measures the effect that cofactors have on the binding of fluorescent nuclear proteins. In the example shown, a fluorescent fusion protein is concentrated in the RNA splicing speckled domains of a live cell. The cell is extracted in digitonin which permeabilizes the cell membrane but leaves the nuclear envelope intact as a barrier to the diffusion of large molecules. Molecules smaller than 40 to 60 kDa are rapidly depleted from the nuclear interior by diffusion. The effect of this depletion on binding and unbinding can be measured by photobleaching before and after digitonin permeabilization. Small molecules can be added back through nuclear pores, and the kinetics of photobleach recovery again measured. If fluorescence recovery is restored, then the supplemented factor may be a co-factor necessary for the binding of the fluorescent protein into complexes at speckled domains. In the first application of this technique ATP was found to mediate the exchange of GFP-SRm160 in complexes at speckled domains.<sup>28</sup>

photobleached without and with the chosen small molecule and the recovery time courses can be compared. And then the cofactor can be added back and the photobleach recovery again measured.

The requirements of this technique are that the fluorescent molecule must be resident in the nucleus, must be too large to passively diffuse through nuclear pores, that the small cofactor molecule must be small enough to pass through pores, and that the binding site being photobleached must be relatively immobile over the time course of recovery. In control experiments, it is important to verify that the nuclear envelope remains impermeable to macromolecules after digitonin extraction. This can be measured by incubation with antibodies against nuclear proteins.<sup>29</sup> An extended *in vitro* FRAP protocol with partial solubilization of the nuclear envelope has been reported<sup>30</sup> and used to characterize the role of molecular chaperones in the nuclear mobility of steroid receptors.

We developed *in vitro* FRAP to address a paradox in the behavior of the mRNA splicing and export factor SRm160,<sup>28</sup> which, as we have discussed above, has 110 potential binding partners. We showed by FRET that some of these complexes in cells also contain UAP56.<sup>6</sup> Live cell FRAP studies revealed that there was a rapid exchange of GFP-SRm160 at RNA splicing speckled domains. However, when the cell was permeabilized the exchange stopped and the protein was stably bound for periods of up to an hour. It seemed possible that

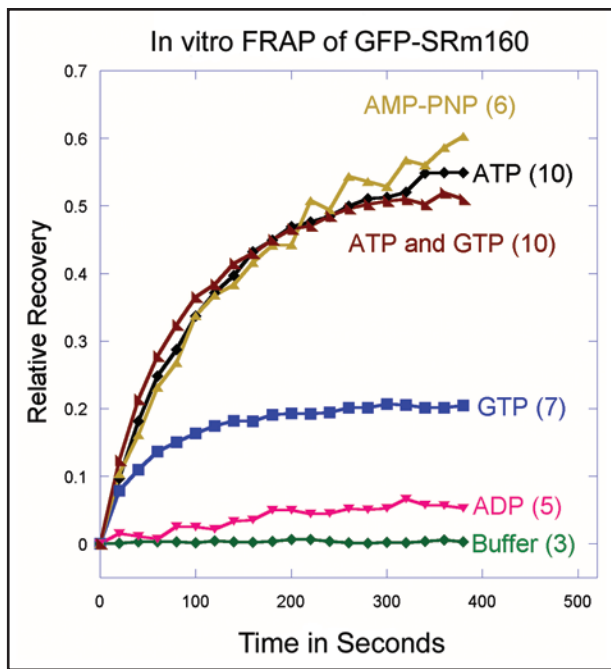


Figure 4. In vitro FRAP reveals the nucleotide specificity of GFP-SRm160 exchange in complexes at RNA splicing speckled domains. HeLa cells stably expressing GFP-SRm160 were permeabilized with digitonin. Before photobleaching, either buffer alone, 5 mM ADP, 5 mM ATP, 5 mM GTP, 5 mM AMP-PNP or a combination of ATP and GTP (5 mM each) were added. Each point represents the mean for the number of photobleached cells indicated to the right of the curve. This figure is adapted from Wagner et al. 2004 in the *Journal of Cell Biology*.<sup>28</sup>

a co-factor mediating exchange was lost when the nuclear envelope was extracted.

One possible cofactor was ATP. We first tried reducing ATP levels by treating cells with 10 mM sodium azide plus 6 mM 2-deoxyglucose in the absence of glucose for 15 to 30 minutes. This resulted in a small but significant slowing of photobleach recovery in treated cells. When we measured the resulting cellular ATP levels, they were reduced, but ATP concentrations were still higher than the expected  $K_m$  for ATP of a typical kinase.<sup>28</sup> There might still have been sufficient ATP persisting to maintain an ATP-dependent EGFP-SRm160 exchange.

Depletion of ATP from nuclei by digitonin permeabilization rapidly removed ATP from the nucleus and stopped the exchange of EGFP-SRm160 at speckled domains; there was no recovery of fluorescence after photobleach. The rate at which control proteins, including GFP-hnRNPA2, recovered after photobleaching was unchanged. Adding back 1–5 mM ATP/Mg<sup>2+</sup> restored the recovery of GFP-SRm160 after photobleaching. We concluded that the unbinding of SRm160 from complexes at speckled domains required ATP.<sup>28</sup>

One of the advantages of in vitro FRAP is that it studies complex assembly in real nuclei, but it offers a capacity for biochemical definition and manipulation that is more characteristic of an in vitro system. For example, the nucleotide specificity of SRm160 unbinding from complexes at speckled domains could be studied (Fig. 4). The big surprise from those experiments was that non-hydrolyzable analogues of ATP fully restored the exchange of SRm160. Thus, the

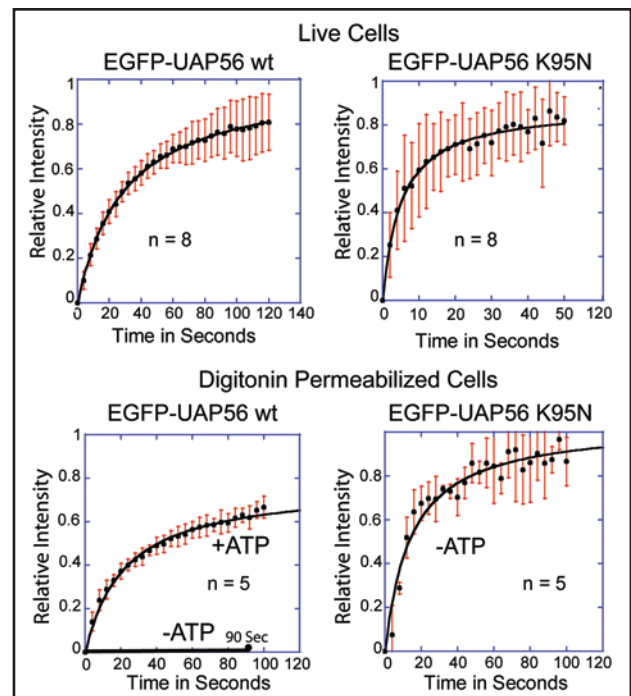


Figure 5. In vitro FRAP shows that the binding of GFP-UAP56, but not its K95N mutant, at RNA splicing speckled domains is ATP dependent. HeLa cells were expressing GFP-UAP56 wt or the GFP-UAP56 K95N point mutant that cannot bind ATP. After 24 hours, a nuclear region of interest was photobleached. The fluorescence recovery of EGFP-UAP56 or its K95N mutant at a speckled domain was recorded. In live cells, the fluorescence of both proteins recovered after photobleaching showing that UAP56 was exchanging on binding sites at speckled domains. After digitonin permeabilization, the FRAP recovery of EGFP-UAP56 stopped. Addition of 1 mM ATP restored FRAP recovery to EGFP-UAP56, showing that the FRAP mobility, that is the exchange at speckled domain binding sites, is ATP dependent. In contrast, after digitonin permeabilization GFP-UAP56 K95N recovered after photobleaching showing that its exchange at speckled domains was ATP independent. Shown are the calculated recovery curves with the number of cells noted on each graph. Means are plotted with error bars for standard deviations. This figure is adapted from Kota et al. 2008 in the *Journal of Cell Science*.<sup>6</sup>

factor responsible for mediating the ATP dependence of exchange was unlikely to be a protein kinase. As we could find no evidence that SRm160 itself had an ATP binding site, this result suggested ATP-dependent RNA helicases as candidates. Several DExD/H box helicases, including UAP56 are present in splicing and export complexes with SRm160, and there is evidence that some members of this family of helicases can remodel RNA-protein structures before ATP hydrolysis.

In vitro FRAP experiments showed that ATP was also required for the binding of UAP56 into complexes at RNA splicing speckled domains (Fig. 5). This ATP-dependence was lost when a point mutation blocking the binding of ATP was made.<sup>6</sup> This same mutation in UAP56 had a dominant negative effect on the export to the cytoplasm of spliced RNA reporter constructs.<sup>6</sup> However, expression of the dominant negative UAP56 did not influence the rate of GFP-SRm160 photobleach recovery in live cells or change the ATP-dependence of GFP-SRm160 recovery in the in vitro FRAP assay. This suggests that UAP56 is not the ATP-binding factor that mediates the ATP-dependent release of SRm160 from complexes at RNA splicing speckled domains.

The *in vitro* FRAP assay provides a tool for evaluating the effect that co-factor molecules may have on the binding of larger proteins into nuclear complexes, and for evaluating this in the context of an intact nucleus. Co-factors suitable for this assay need only be smaller than the diffusion limit of nuclear pores, so even small proteins and individual protein domains might be used. The assay can also be combined with pre-treatments of the live cell that include drugs, the knockdown of partner proteins, and expression of mutant forms of either the fluorescent protein or of its putative binding partners. The combination of the *in vitro* FRAP assay with high throughput microscopy systems may even allow this assay to be used as a tool for drug discovery, evaluating candidate compounds small enough for passive diffusion into the nucleus.

## Conclusion

We have witnessed an explosion of new microscopy technologies in recent years and are only beginning to exploit them. It is likely that the current progress in microscope development and application will accelerate. These technological advances have facilitated a growing repertoire of techniques that allow an *in situ* analysis of the biochemistry of living systems. The key advantages of these approaches are that the cellular context is preserved, providing spatial and architectural information, and that the molecular interactions studied are biologically relevant. The major disadvantages are that the techniques can be experimentally slow and difficult, and they require expensive instrumentation. For example, establishing a protein-protein interaction by co-immunoprecipitation is generally much faster, easier and cheaper than showing that this interaction is biologically relevant by FRET.

Advances in microscopy may change the very way in which we think about biology. As the resolution of physics improved with quantum mechanics, the universe was revealed to be more jiggly and stochastic, and less determinant than had been imagined. An improvement in the spatial and temporal resolution of microscopy may reveal similar properties underlying biology.

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