both the location and the nature of lesions generated by laser ablation.

Not all strokes are the same. Blood vessels can be occluded (ischemia) or ruptured (hemorrhage) or they may be leaky (edema). A principal challenge in research is to create reproducible models of the different types of strokes. Here the authors have observed three different types of microvessel perturbations depending on how they apply the femtosecond laser pulses. The delivery of a train of pulses with an average energy per pulse of 0.8 μJ uniformly results in vessel rupture with hemorrhage (**Fig. 1**). In contrast, when they deliver pulses of lower intensity (0.1–0.5 μJ), there is extravasation of a fluorescein isothiocyanate–dextran tracer in the absence of erythrocyte loss approximately 80% of the time. A third type of injury is made by delivery of a single train of laser pulses capable of inducing extravasation, followed by the delivery of additional laser pulses of increased energy until blood flow stops. Using this method they are able to elicit occlusion 60% of the time. In addition, blood flow is monitored by line scans along single vessels. This approach provides mapping of blood flow dynamics in microvascular networks.

The main promise of this new technique is that individual components of the neurovascular unit can potentially be targeted. What happens to neuronal function when the microvessel is lesioned? What happens to microvessel dynamics when astrocytes are knocked out? The imprecision of older cerebral vascular injury models precluded the possibility of such discrete 'dissection' of the neurovascular unit. Of course, the use of femtosecond laser pulses to induce injury is not without limitations. The insult is nonphysiological and the mechanism of injury is not well understood or characterized at the cellular level. For example, the occlusive lesion generated by this technique contains fibrin but is not sensitive to tissue plasminogen activator. Thus, its nature is not apparent. Nonetheless, the precision of the injury coupled with the resolution of the two-photon laser-scanning microscopy enables unprecedented control in the perturbation and monitoring of the neurovascular unit *in vivo*.

Another advantage of this technique is afforded by the ability to map blood flow relatively deep in the brain. The cerebral microvascular is inherently three-dimensional and demonstrates high connectivity. It has been difficult to predict the quantity and spatial relationship of microvascular lesions that will result in ischemia. The ability to monitor flow dynamics as described in the article by Nishimura *et al.* will allow investigators to quantitatively assess the effects of specific patterns of vascular occlusion on blood flow and ischemia. The susceptibility of different microvascular beds to ischemia may differ by location. Indeed, the authors suggest that occlusion of deep microvessels results in a substantial reduction in flow in 'downstream' vessels, whereas occlusion of superficial vessels results in only a minor compromise of 'downstream' flow. The methodology described in this article will allow for systematic evaluation to determine how the anatomical locale and depth of a microvascular bed influence its response to occlusive insults. Such analysis could identify areas that are at high risk for ischemia. These issues and others, previously refractory to investigation, can now be addressed using the versatile stroke model described by Kleinfeld and coworkers.

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NMR mapping of protein interactions in living cells

Philipp Selenko & Gerhard Wagner

Intracellular protein-protein interactions form the basis of most biological processes. Structural aspects of these reactions can now be analyzed in living prokaryotic cells and in atomic detail by nuclear magnetic resonance spectroscopy.

Almost all biology has evolved within the boundaries of cellular structures, and every intracellular space represents a highly crowded, viscous solute that harbors an intricate network of biological activities simultaneously exerted by a vast number of macromolecules. Yet biochemical and structural investigations of biomolecules are typically confined to artificial, dilute, isolated *in vitro* experimental setups.

Departing from these limitations, in this issue of *Nature Methods*, Burz *et al.*[1] report a new application of in-cell NMR spectroscopy, 'STINT-NMR', which provides a means of analyzing the structural changes that accompany protein-protein interactions *in vivo* and at atomic resolution.

To allow the study of biomolecules in their natural environment (that is, in cells), recent attempts have aimed to develop new *in vivo* techniques for structural biology and cellular imaging[2]. X-ray crystallography and high-resolution electron microscopy are intrinsically excluded from *in vivo* approaches because of their requirement for pure samples and crystalline or vitrified specimen. Nuclear magnetic resonance (NMR) spectroscopy, as the only other method for the structural investigation of biomolecules at the atomic level, allows for the direct observation of 'NMR-active' atomic nuclei in any NMR-distinguishable environment and can thus be used to investigate labeled proteins structurally *in vivo* and inside cells[3].

High-resolution NMR measurements in living prokaryotic cells have gained popularity because they allow the collection of both qualitative structural[4] and quantitative dynamic[5] information on proteins in the context of intact living cells. All applications of in-cell NMR spectroscopy so far

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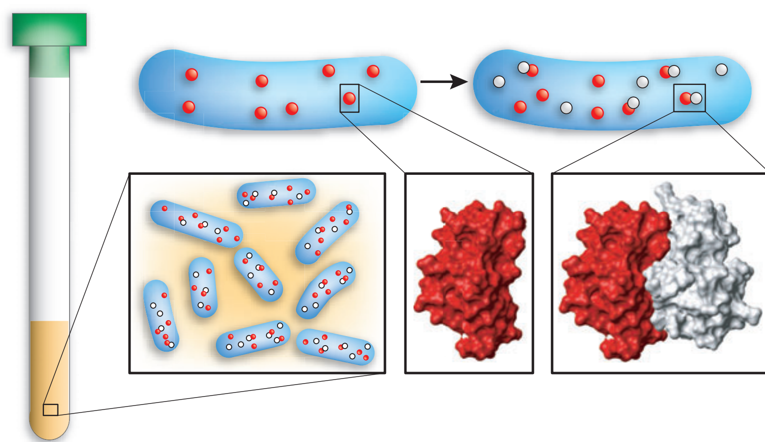

Figure 1 | In-cell NMR spectroscopy of protein-protein interactions. To analyze the structural details of a given protein-protein interaction, the two partners are sequentially expressed in *E. coli* and the suspension of live bacteria is used directly for NMR measurements.

have relied on the experimental rational originally proposed by Serber *et al.*[6]. Most isotopes of atomic nuclei in biomolecules are NMR-inactive and hence are 'invisible' to the 'spectroscopic eye'. Modern NMR methods use overexpression methods for substituting some of these nuclei with NMR-active isotopes. Multiple-resonance experiments then allow the selective detection of pairs of coupled nuclei, such as ^{1}H and ^{15}N, in a background that does not contain substantial amounts of such isotope pairs.

The expression process is generally referred to as 'isotope labeling' and involves recombinant protein production in growth media that provide isotope-substituted precursors. Because of the high protein expression typically achievable with prokaryotic protein production systems exploiting viral promoter and polymerases, labeled 'foreign' proteins typically accumulate rapidly after induction and generally outperform the protein synthesis rates of *Escherichia coli* gene products[3,7]. Additionally, when cells are grown in unlabeled medium first and are switched to labeled growth conditions only after the induction of recombinant protein synthesis, these macromolecules are essentially the only isotope-labeled species in this cellular context. Hence, without further purification, 'isotope-edited' correlation experiments of intact cells yield high-resolution conformational information on the intracellular recombinant proteins only[3].

Now Burz *et al*. have methodologically advanced that idea by using an additional round of cell growth in unlabeled medium, during which time the expression of a ligand-protein is induced from a second plasmid. The unlabeled interacting protein accumulates over time and binds to the labeled protein in the cytoplasm of these cells (**Fig. 1**). As changes in the chemical environment of labeled molecules, either by ligand binding or conformational rearrangements, result in differences in the 'chemical-shift' values of the residues involved, these can be directly measured and 'translated' into localized structural information on the labeled protein surface.

The complexity of the initial in-cell correlation spectrum remains unchanged because both the cellular environment and the induced protein ligand are unlabeled and therefore cannot be observed. The intracellular interaction of the labeled specimen with the unlabeled protein ligand can thus be analyzed in an indirect way or through an isotope-selective filter. Burz *et al.* use this method to study the *in vivo* binding of ubiquitin to the ubiquitin-interacting motif of the ataxin 3 protein and signal-transducing adaptor molecule 2. Although both of their systems represent model setups of low biological complexity, they clearly demonstrate the experimental feasibility of the approach.

What are the possible applications of STINT-NMR? Obviously, the method itself is appealing, because it allows for the structural interpretation of intermolecular, *in vivo* binding events in a cellular context. Mutational screens for ligands that are defective in their ability to interact *in vivo* can be done rapidly and with unprecedented detail in the experimental 'readout'. Qualitative *in vivo* binding affinities are inherently provided by the nature of the changes in resonance peak parameters in these NMR experiments and do not necessarily require additional measurements. Furthermore, STINT-NMR can provide a useful alternative to *in vitro* measurements when components are difficult to purify or are unstable in their pure form.

It is nevertheless important to keep in mind that although these measurements are done in a cellular, *in vivo* setting, they are not strictly 'native' in a biological sense. Both ubiquitin and its binding partners are nonprokaryotic, overexpressed proteins and their intracellular concentrations are above physiological relevance. Additionally, ubiquitin represents a highly stable, compact protein fold not likely to undergo proteolytic degradation or partial unfolding in a cellular environment. It remains to be seen to what extent STINT-NMR is applicable to other macromolecules.

In summary, in-cell NMR spectroscopy is emerging as a unique tool for providing atomic resolution structural information of biomolecules in living cells. It will be interesting to see how this technology can be extended to studies in eukaryotic cellular systems, in which a wide range of more complex biological activities could be analyzed *in vivo*. Some preliminary experiments in yeast, insect cells and, in particular, *Xenopus leavis* oocytes indicate that high-resolution, in-cell NMR measurements in eukaryotic cells are indeed technically feasible[8,9].

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