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Mapping structural interactions using in-cell NMR spectroscopy (STINT-NMR)

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Abstract

We describe a high-throughput in-cell nuclear magnetic resonance (NMR)-based method for mapping the structural changes that accompany protein-protein interactions (STINT-NMR). The method entails sequentially expressing two (or more) proteins within a single bacterial cell in a time-controlled manner and monitoring the protein interactions using in-cell NMR spectroscopy. The resulting spectra provide a complete titration of the interaction and define structural details of the interacting surfaces at atomic resolution.

Proteins do not act in isolation; different levels contributing to complexity in biological systems arise not only from the number of proteins expressed in an organism, but also from the combinatorial interactions among them[1,2]. Despite the ongoing effort to decipher the complex nature of protein interactions, new methods entailing the structural characterization of protein complexes are needed to fully understand molecular networks[3].

Recently developed in-cell NMR technology[4] allows us to obtain atomic resolution information of proteins in a high-throughput manner. Though powerful, this technology is limited to characterizing one protein at a time. Simultaneous overexpression of more than one protein inside the cell leads to exceedingly complex NMR spectra. We propose to use sequential protein expression in combination with measuring changes of isotopic composition on induction to expand in-cell NMR technology to study protein-protein interactions. We used sequential expression to study time-dependent control of gene activity[5] and for *in vivo* studies of intein-based native protein ligation[6]. Sequential expression allows us to overexpress two or more proteins in a time-controlled fashion and to label only one protein with NMR-active nuclei, leaving the interactor protein(s) cryptic, thus reducing the NMR spectral complexity.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

We used this method to study ubiquitin interactions with two ligands. Ubiquitin is a highly-conserved 76-amino-acid protein found in all eukaryotic cells[7]. Post-translational modification of proteins by ubiquitin confers a spectrum of functions that depend on the number of ubiquitins bound. We are interested in understanding the network of interactions between ubiquitin and proteins containing the ubiquitin interacting motif[8] (UIM), and how these interactions regulate receptor recycling. We used two ubiquitin ligands containing the UIM: a 28-amino-acid peptide from ataxin 3 protein (AUIM) and a full-length protein, the signal transducing adaptor molecule STAM2 (ref. 9). AUIM peptide interacts with ubiquitin *in vitro* with low affinity (~230 μM)[10], but STAM2 binds ubiquitin with a higher affinity[11] as it has two ubiquitin interacting surfaces: one located in the UIM part of the molecule and another in the Vps27/Hrs/STAM (VHS) domain. These two systems simulate a broad range of protein-protein interactions of different affinities and various molecular weights.

We used two plasmids with two different, tightly controlled, inducible promoters for individual expression of two interacting proteins. We overexpressed the target protein, ubiquitin, in the uniformly labeled [U-^{15}N] medium (**Fig. 1a**). The NMR experiment, ^{1}H{^{15}N}HSQC (heteronuclear single quantum coherence), yielded high-resolution backbone spectra of the target protein inside the bacterial cells. We replaced the medium with unlabeled medium and overexpressed an interactor protein, STAM2 (**Fig. 1a**) or AUIM (data not shown); this yielded interactor proteins invisible to the isotope-edited NMR experiments. The HSQC spectrum of the target protein changes as a result of ligand-target interactions. The changes in the chemical shifts of the target protein define the interface between the protein and its ligand, and provide atomic resolution information on the protein-protein interaction.

For this experiment, we cotransformed the two plasmids into *E. coli* strain Rosetta(DE3) (Novagen). We induced [U-^{15}N] ubiquitin expression for 3 h by adding L-arabinose. Higher in-cell protein concentrations can be attained by extending the induction time. As we used an *E. coli* strain capable of metabolizing L-arabinose, we added a second aliquot of L-arabinose after 2 h of induction to insure robust protein induction. We centrifuged the cells and resuspended them in M9 medium containing antibiotics and casamino acids. We then induced STAM2 or AUIM expression for 3 h by adding isopropyl-β-D-thiogalactopyranoside (IPTG). We collected samples for in-cell NMR (25–100 ml) after the first 3-h induction and at various times after the second induction (**Fig. 1b**).

Because of the strength of the T7 promoter, cell growth after IPTG induction was minimal; the cell density increased less than 0.2 OD_{600} over the 3-h incubation. Minimal growth prevents dilution of the labeled protein and ensures that the same concentration of the target protein and ligand are present in each cell. We then centrifuged the samples, washed them, and stored them at – 80 °C for subsequent NMR analysis (**Supplementary Methods** online). We found that freezing and thawing the cells produces minimal cell lysis, allowing the NMR samples to be stored for at least one month (**Supplementary Fig. 1** online).

The procedure we developed allows an increase in ligand concentration at a single, high concentration of target molecule. In some cases, the ratio of ligand to target may be too low for substantial binding to occur. To access different target-ligand concentration ratios, cells

can be sampled hourly during the first induction with L-arabinose and further processed as described above. The arabinose promoter is almost completely repressed once arabinose is depleted.

A gradual increase in the cellular concentration of AUIM resulted in small chemical shift changes of ubiquitin backbone amides (**Fig. 2a**). The changes are consistent with a low binding affinity of AUIM for ubiquitin[10], indicating that free and AUIM-bound ubiquitin are in fast exchange on the NMR timescale. An experiment in which AUIM was labeled and ubiquitin is unlabeled showed similar chemical shift changes of AUIM backbone amides (**Supplementary Fig. 2** online).

We mapped chemical shift changes obtained from this in-cell titration experiment onto the three-dimensional structure of ubiquitin (**Fig. 2**). The interaction surface between ubiquitin and AUIM includes ubiquitin residues Leu 8, Ile 44 and Ala 46, all known to interact with sequences containing the UIM motif[10,12]. This mapping result is also consistent with the titration of purified AUIM peptide against a solution of purified [^{15}N]ubiquitin (**Fig. 2b,e**). Since local concentrations of interacting proteins in cells are higher than in dilute solutions, the observed chemical shift changes were correspondingly larger. There are noticeable differences between the solution and in-cell NMR spectra of free ubiquitin with only 86% of the ubiquitin peaks observed in solution found within 0.1-p.p.m. distance of those observed in cells (**Supplementary Fig. 3** online). Peaks that underwent substantial (>40.1 p.p.m.) chemical shifts, came exclusively from the surface-exposed amides. The differences between the chemical shifts of in-cell and purified ubiquitin can be explained by the intracellular pH gradient and the influence of intracellular environment on ubiquitin conformation. This observation underscores the importance of performing titration experiments inside living cells where cellular environment may influence the conformations of interacting proteins. In addition, a negative control experiment, in which we titrated [^{15}N]ubiquitin in cells with a mutant AUIM (S16A) that does not bind to ubiquitin[13], demonstrated that small chemical shift changes result from a specific interaction and not merely from overexpression of the interacting molecules (**Supplementary Fig. 4** online).

Titrating [^{15}N]ubiquitin with STAM2 resulted in consistent broadening of selected ubiquitin peaks as the concentration of STAM2 in the cells increased (**Fig. 2c** and **Supplementary Fig. 5** online). Differential broadening of the NMR signals can be attributed to intermediate rates of exchange between free and STAM2-bound ubiquitin and is consistent with greater binding affinity of ubiquitin for STAM2 (ref. 11) than for AUIM. Mapping differentially broadened peaks onto the three-dimensional structure of ubiquitin revealed two distinct interfaces between STAM2 and ubiquitin (**Fig. 2f**). The first interface is formed by ubiquitin residues Lys 6, Leu 8, Ile 44 and Leu 70, and corresponds to the UIM binding surface. The second interface, unique to STAM2 and ubiquitin, is formed by Lys 11, Ile 13, Val 26, Lys 27 and Lys 33. We suggest that we detected this interface because of the interaction between ubiquitin and the VHS domain of STAM2. The increased affinity of STAM2 for ubiquitin can be explained by this additional interface.

In this work, we optimized the method for a bacterial system in which proteins can be easily and rapidly overexpressed with minimal proteolysis. We chose the ubiquitin system to

demonstrate the feasibility of the method; other interacting systems may not be as straightforward to optimize. The method is limited primarily by the level of interacting protein expression that can be achieved. There are several advantages to using in-cell NMR technology instead of analyzing crude lysates: the proteolytic machinery in cells is tightly regulated and this regulation is lost in lysates, which can result in rapid proteolysis of the sample[14] (**Supplementary Fig. 6** and 7 online); in-cell protein overexpression results in higher local concentrations of interacting partners than in lysates, thus increasing the likelihood of detecting weaker interactions; and the interaction occurs within a natural cellular environment, which may confer biologically relevant structural conformations that cannot be duplicated *in vitro*.

STINT-NMR can be used to study interacting proteins whose structure is unknown, as only one of the interacting species may be labeled. It can also be used with proteins that are hard to purify or are proteolytically labile (for example, STAM2), as there is no need to purify the proteins, and only modest concentrations may be needed to produce structural changes if the interaction is of high affinity. The method can be extended to include expression of three or more interacting molecules, and can potentially be applied to eukaryotic expression systems (for example, yeast) to permit post-translational modification of the protein structure (glycosylation, methylation) that does not occur in bacteria. Finally, the method allows the study of proteins that span a broad range of sizes; in this study we examined the interaction of ubiquitin (8.5 kDa) with AUIM (4 kDa) and with STAM2 (50 kDa). Extending STINT-NMR to the study of still higher molecular weight complexes can be accomplished by deuterating the target protein and using transverse optimized spectroscopy (TROSY-HSQC) and related methods[15] for detecting protein-protein interactions by in-cell NMR.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

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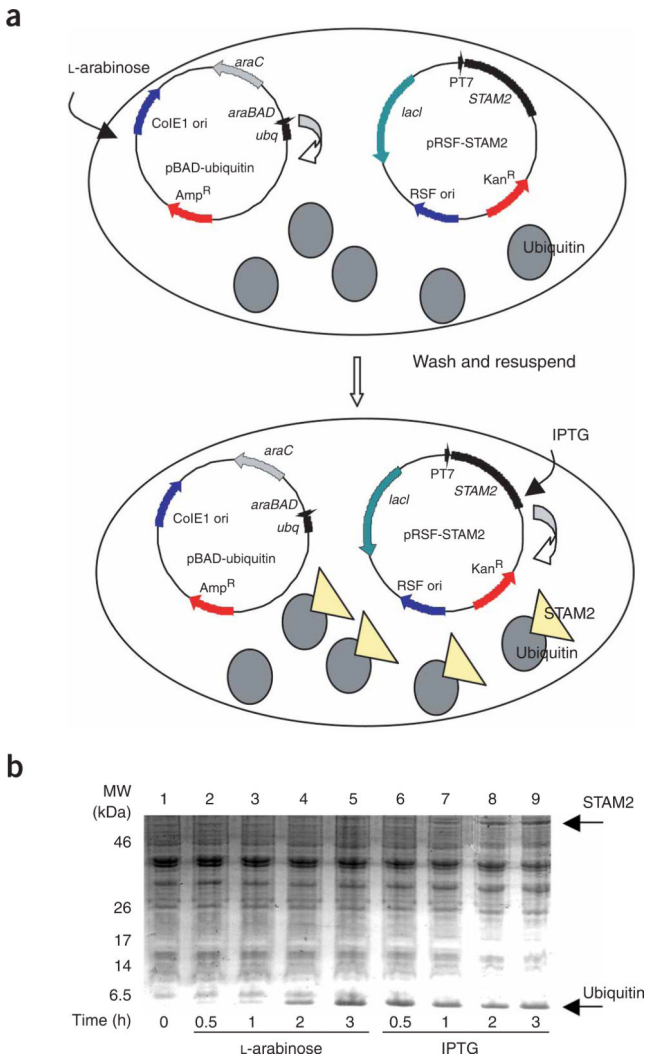

Figure 1.

Overview of STINT-NMR methodology. (**a**) In-cell NMR of interacting proteins. Target protein was over-expressed in uniformly labeled [U-^{15}N] medium and a sample containing no interactor protein was prepared for in-cell NMR (top). Cells were then washed and resuspended in label-free medium. Samples were taken as the interacting protein was overexpressed (bottom). Changes in the target protein structure are measured as the concentration of the interactor is increased. (**b**) SDS-PAGE of ubiquitin and STAM2 sequential expression. Rosetta(DE3) cells were induced with L-arabinose and then with IPTG for the indicated amounts of time to overexpress ubiquitin and STAM2, respectively. Lane 1, uninduced Rosetta(DE3) cells. Note that the ubiquitin level remains essentially constant as STAM2 overexpression increases.

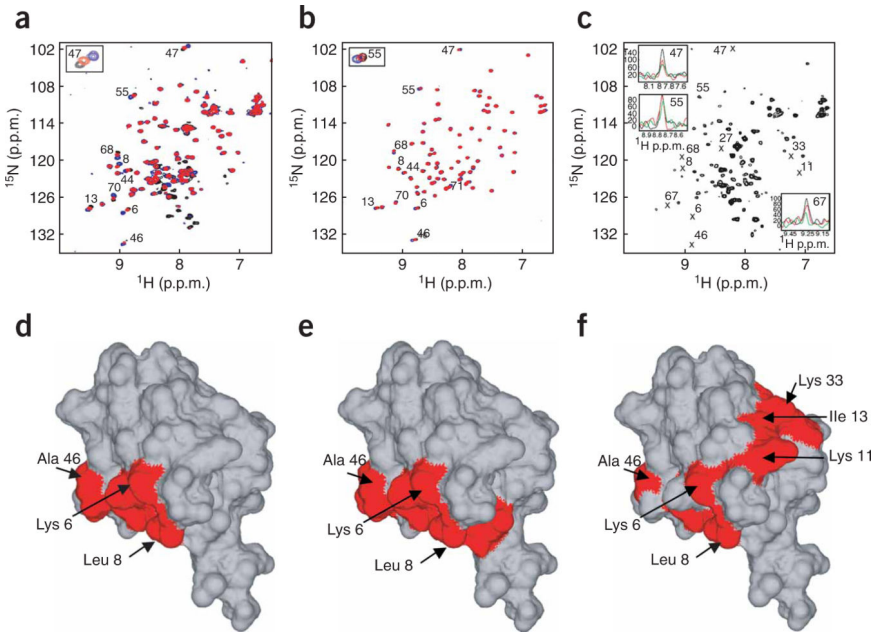

Figure 2.

NMR spectra and interaction maps of ubiquitin-ligand complexes. (**a**) Overlay of ^{1}H{^{15}N}HSQC-spectra of *E. coli* after 3-h overexpression of [^{15}N]ubiquitin and 0-h (black), 2-h (red) and 3-h (blue) overexpression of AUIM. Individual peaks exhibiting large chemical shifts are labeled with corresponding assignments. Progression of colors in the ^{1}H{^{15}N}HSQC spectrum was chosen for ease of viewing. (**b**) Overlay of ^{1}H{^{15}N}HSQC-spectra of free [^{15}N]ubiquitin (black), [^{15}N]ubiquitin-AUIM complex at a molar ratio of 1:1 (red) and at a molar ratio of 1:2 (blue). (**c**) ^{1}H{^{15}N}HSQC-spectrum of *E. coli* after 3-h overexpression of [^{15}N]ubiquitin and 3-h overexpression of STAM2. Resonance peaks exhibiting extreme broadening are indicated by crosses. Insets , one-dimensional traces of selected peaks exhibiting differential broadening after 3-h overexpression of [^{15}N]ubiquitin and 0-h (black), 2-h (red) and 3-h (green) overexpression of STAM2. (**d**) Interaction interface of ubiquitin-AUIM mapped on the three-dimensional structure of ubiquitin (PDB code 1D3Z) based on the chemical shift changes from the in-cell titration experiments. (**e**) Interaction interface of ubiquitin-AUIM complex based on the chemical shift changes from the *in vitro* titration experiment. Ubiquitin residues exhibiting either chemical shift change above 0.1 p.p.m. or extreme broadening are colored in red. (**f**) Interaction interface of ubiquitin-STAM2 based on the differentially broadened peaks from the in-cell titration experiment. The HSQC spectra were acquired as described in **Supplementary Methods**.