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The STINT-NMR Method for Studying In-cell Protein-Protein Interactions

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Abstract

This unit describes critical components and considerations required to study protein-protein structural interactions inside a living cell by using NMR spectroscopy (STINT-NMR). STINT-NMR entails sequentially expressing two (or more) proteins within a single bacterial cell in a time-controlled manner and monitoring their 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 the level of single amino acid residues. The advantages and limitations of STINT-NMR are discussed, along with the differences between studying macromolecular interactions in vitro and in vivo (in-cell). Also described are considerations in the design of STINT-NMR experiments, focusing on selecting appropriate overexpression plasmid vectors, sample requirements and instrumentation, and the analysis of STINT-NMR data, with specific examples drawn from published works. Applications of STINT-NMR, including an in-cell methodology to post-translationally modify interactor proteins and an in-cell NMR assay for screening small molecule interactor libraries (SMILI-NMR) are presented.

Keywords

in-cell biochemistry; in-cell NMR spectroscopy; protein-protein interactions; drug screening; proteomics

Introduction

The ultimate goal of structural and biochemical research is to understand how macromolecular interactions give rise to and regulate biological activity within living cells. The challenge is formidable due to the complexity that arises not only from the number of proteins (genes) expressed by the organism, but also from the combinatorial interactions between them (Rain et al., 2001; Sali et al., 2003). Despite ongoing efforts to decipher the complex nature of protein interactions, new methods for structurally characterizing protein complexes are needed to fully understand molecular networks (Gerstein, 2000). Until now, mostly in vitro techniques have been used to study macromolecular interactions that govern biological processes under conditions remote from those existing in the cell. However, recently developed in-cell NMR technology (Serber and Dotsch, 2001) affords atomic resolution information on proteins in their native environment. This unit describes a method for mapping the structural interactions that underlie the formation of protein-protein complexes. This method is called STINT-NMR for Structural Interactions using NMR spectroscopy (Burz et al., 2006b).

In its simplest form, STINT-NMR can identify the interacting surface of a target protein when a single interactor protein (ligand) binds to it. This is accomplished by sequentially expressing two proteins within a single bacterial cell in a time-controlled manner and monitoring their interaction using in-cell NMR spectroscopy. The resulting NMR data

provide a complete titration of the interaction and define structural details of the interacting surfaces at atomic resolution. The target protein, whose NMR structure must be known, is first overexpressed on uniformly labeled [U - ^{15}N] medium to yield a high-resolution, ^{15}N -isotope-edited, heteronuclear single quantum coherence (^{15}N -HSQC) spectrum of the target protein inside the bacterial cells. The growth medium is changed, and the unlabeled interactor is overexpressed. As the concentration of the interactor(s) increases, the HSQC spectrum of the target changes to reflect the different chemical environment of the residues that have been affected by the binding interaction.

STINT-NMR is extremely sensitive to low-affinity interactions ($K_d > 1 \mu\text{M}$), which may be of significant biological interest in the so-called “just-in-time assembly” of transient protein complexes. The effective protein concentration of overexpressed proteins inside the cell varies from 0.01 to 1 mM, and STINT-NMR can identify protein-protein interactions that span a range of binding affinities from micromolar to millimolar. Low-affinity interactions that result in chemical shift changes and/or signal broadening upon complex formation are most easily interpreted by in-cell NMR. However, large conformational changes upon complex formation, seen in some high-affinity interactions, make interpreting STINT-NMR results in terms of interaction surfaces more difficult without subsequent in vitro studies.

STINT-NMR technology was developed to study protein-protein interactions in bacterial cells. Tight control over the strength of protein overexpression allows regulation of the concentrations and stoichiometries of the interacting components. STINT-NMR can be used to study the interaction between components of macromolecular complexes containing three or more proteins, any one of which may be labeled as the target. The method can also be used to identify structural changes in protein interaction surfaces due to the presence of post-translational modifications (PTMs) by introducing PTM activities on inducible plasmids, a process sometimes called in-cell biochemistry. Finally, STINT-NMR can be combined with genetic and molecular selection in a technique called SMILI-NMR, for Screening of small Molecule Interactor Library by using In-cell NMR (Xie et al., 2009), to rapidly screen small molecule libraries for small druglike molecules capable of either enhancing or weakening the stability of a biomolecular complex. In this way, STINT-NMR serves as a direct assay for protein-drug interactions, facilitating high-throughput screening.

This unit summarizes the current state of STINT-NMR, emphasizing the critical components and considerations required to conduct an in-cell experiment. The first section spells out the advantages and limitations of STINT-NMR and discusses the differences between studying macromolecular interactions in vitro and in vivo (in-cell). The next section deals with experimental design, in particular, selection, regulation and compatibility of overexpression plasmid vectors, sample requirements and instrumentation. Typical data and data analysis is then presented with specific examples drawn from published works. The final section describes applications of STINT-NMR that have been developed in the authors' laboratory and a future prospectus for this rapidly evolving technique.

Advantages and Limitations

STINT-NMR is limited primarily by the level of interacting protein expression that can be achieved, hence the method cannot be used with proteins that bind very weakly or whose self-assembly linkage to binding requires very high concentrations of monomeric species to generate sufficiently high concentrations of competent binding species. Another limiting consideration is the integrity of the interacting proteins. For example, over-expressed proteins may degrade into components that bind nonspecifically to each other, thereby presenting multiple and/or incorrect interaction surfaces. For this reason, the target should

be stable over the course of the experiment. Should sample stability become questionable, SDS-PAGE and immunoblots can be used to assess the extent of degradation.

In-cell NMR yields lower quality spectra than that acquired using highly purified samples. This shortfall can be compensated for by increasing the sample size, the time of induction, or the number of scans per experiment. Another way to overcome poor spectra is to grow the culture in D₂O. Although the exchange of surface-exposed protons is generally rapid, protected protons in protein interiors may be refractory to such exchanges, leading to the disappearance of protected residues in HSQC spectra. An important factor in limiting the acquisition time of in-cell NMR experiments is cell lysis (Cruzeiro-Silva et al., 2006). Stabilizing the cells using cell protectants, such as glycerol or sucrose (Cruzeiro-Silva et al., 2006), as well as alginate encapsulation (Li et al., 2008) may extend the in-cell NMR acquisition time. Alternatively, the time required to acquire data can be reduced by using a cryoprobe. Finally, increased sensitivity may be attained by using transverse optimized spectroscopy (TROSY-HSQC) for high-molecular-weight complexes that have slow tumbling times.

STINT-NMR requires minimal sample preparation other than washing the cells. This method can also be used to study interacting proteins whose structures are unknown, as only one of the interacting species is typically labeled. The method may be applied to proteins that are soluble in vivo but are difficult to purify; however, in these instances, the applicability of the method must be assessed on a case-by-case basis. The interaction of the target protein and the interactor can be monitored by STINT-NMR as long as they remain soluble in the cell and have concentrations that are sufficient for in-cell NMR detection (generally ≥ 0.1 mg/ml of labeled protein). Therefore, this method is best suited for proteins that are well behaved and can be expressed to very high concentrations.

There are advantages to using in-cell systems instead of crude lysates for STINT-NMR. First, the proteolytic machinery is tightly regulated inside cells and this regulation is lost in lysates, which can result in rapid proteolysis of the sample (Groll et al., 2005). Second, overexpressing proteins in the cell results in higher local concentrations of interacting partners than in lysates, thereby increasing the chances of observing weaker interactions. Third, the ability to control PTMs in an environment that normally lacks the ability to provide such modifications, i.e., bacterial cells, affords an opportunity to examine the effects of PTMs on protein structure without competing reactions, in effect, turning the bacteria into “cellular test tubes.” Fourth, the cellular environment in which the interactions occur is more physiologically relevant than that obtained when working in dilute solutions and may confer biologically relevant structural conformations that cannot be duplicated in vitro.

To further dissect the differences between in-cell and in vitro studies of protein-protein interactions, the interactions between overexpressed proteins and other intracellular molecules can be divided into two parts: specific interactions with affinities lower than 10 μ M and nonspecific interactions with affinities much greater than 10 μ M. Intracellular proteins participating in specific interactions have to be present in stoichiometric ratios with the target protein(s) to be detected with in-cell NMR, so that the bulk of the labeled population is bound. Typically, during in-cell NMR, target proteins are overexpressed to concentrations 10- to 100-fold greater than their physiological concentrations, resulting in a large population of free protein and no changes in the NMR spectrum. ??Thus, specific interactions between overexpressed target protein(s) and intracellular physiological partners might be overlooked. Using STINT-NMR, these partners can be re-introduced later at concentrations comparable to that of the labeled proteins, thereby giving rise to detectable specific interactions inside the cell.??

Nonspecific interactions between target-labeled proteins and the rest of the cellular molecules are omnipresent and establish the proper physiological environment for the labeled proteins that uniquely distinguishes in-cell NMR from in vitro techniques. This is best evidenced by small differences in the NMR spectra and solution structures of protein measured in cells and in vitro (Sakakibara et al., 2009). Since the concentrations of molecules inside eukaryotic and prokaryotic cells are very similar to each other, we do not expect that the contribution of nonspecific interactions will be dramatically different in these two cases. One may expect that in-cell NMR spectra of cytosolic proteins in these two cases will be also very similar (Selenko et al., 2006; Inomata et al., 2009). At the same time, proteins that are known to interact with intracellular compartments are best studied in eukaryotic cells. Since the concentration of the overexpressed proteins is still only 1% of the total protein concentration, it can be assumed that protein overexpression does not strongly disturb normal cellular physiology. Indeed, recent experiments showed that protein overexpression does not change intracellular viscosity, a critical parameter for in-cell NMR (Slade et al., 2009). To confirm that cell physiology is not affected, growth rates of cells with and without protein overexpression can be compared.

Experimental Design

Overview

A detailed protocol for performing and analyzing STINT-NMR experiments using a labeled target and single interactor protein is available in Burz et al. (2006a) and a method for expressing two interactors and kinase activity can be found in Burz and Shekhtman (2008). STINT-NMR can be performed in different ways to provide a broad range of interactor-to-target protein concentration ratios for a complete structural titration. The order of protein expression is generally not critical and any or all proteins can be labeled using different strategies. The resulting NMR data provide a complete titration of the interaction and identify the amino acids that comprise the interaction surface of the target protein.

The most comprehensive experiment involves creating a matrix of samples (Fig. 17.11.1) wherein the target protein is overexpressed for 1, 2.5, and 4 hr, and the interactor protein is overexpressed for 1, 2.5, and 4 hr at each target concentration (Fig. 17.11.2). It is generally useful to first acquire a single sample for NMR analysis before generating the full matrix of samples. It is also important to acquire the in-cell NMR spectrum of the target protein before the interactor is overexpressed (Fig. 17.11.1). Occasionally, only a target-interactor complex will yield a high-quality NMR spectrum (Xie et al., 2008). In this case, STINT-NMR is limited to studying interactions of this complex with other interactors.

The culture volumes suggested ??in the protocol?? assume that there is little increase in the OD₆₀₀ during the course of induction (we typically observe increases of 10% to 30% in the OD₆₀₀ following the second induction). A minimal increase in cell density during induction helps maintain the in-cell concentrations of interacting proteins whose induction phase has ended, at a high level by minimizing redistribution during cell division (Fig. 17.11.2). This helps ensure that sufficient cell culture is available to provide adequate NMR samples for the complete experiment.

It is important to note that in-cell titrations lack the precision of binding isotherms obtained in vitro because of the variable levels of protein expression that are inherent when using living cells, and thus are largely qualitative. In each case, a unique structural endpoint is attained as evidenced by no further changes in the chemical shifts or differential broadening of specific peaks in the NMR spectra as the concentration of interactor protein increases. It is therefore unnecessary to quantify total cellular protein concentrations except to generate binding curves for determining binding affinities.

Plasmids

Sequentially overexpressing proteins requires having plasmids with compatible origins of replication, antibiotic selections, and transcriptional promoters under the control of separate inducers. There are at least four compatible origins available for the purpose of co-expression: ColE1, RSF1030, CloDF13, and p15A; and four commonly used antibiotics: ampicillin (Amp), kanamycin (Kan), streptomycin (Stm), and chloramphenicol (Cam). In addition, we have tested four induction systems on commercially available and in-house made plasmids: the IPTG-inducible T7 promoter-*lac* operator ($P_{T7}/lacOp$); the L-arabinose-inducible *araBAD* operator, the tetracycline (or anhydrotetracycline) inducible *tet* operator, and the L-rhamnose-inducible *rhaB* operator.

Table 17.11.1 lists plasmids that are available for STINT-NMR. For example, one combination of four commercially available and in-house built plasmids that would work together to perform sequential protein overexpression is: pASK3+ from IBA (Amp^R, tetracycline, ColE1 origin); pCDF from Novagen (Stm^R, IPTG, CloDF13 origin); and in-house made pDB1 (Kan^R, L-arabinose, RSF1030 origin); and pRHA (Cam^R, L-rhamnose, p15A origin). The authors have used these plasmids to produce sufficient labeled protein for in-cell NMR experiments.

Plasmid origins of replication regulate copy number, which in turn affects plasmid stability. A high-copy-number plasmid confers greater stability to the plasmid when random partitioning occurs at cell division, but generally decreases the growth rate, the latter a property that actually improves the quality of STINT-NMR experiments. As a rule, the larger the copy number, the higher the concentration of overexpressed protein. Plasmid origins also confer compatibility, i.e., the ability to replicate in the presence of other plasmids in the same bacterial cell. Two plasmids containing the same or similar origins generally cannot co-exist in the same cell; the plasmids will attempt to maintain a constant copy number, but the population distribution of the plasmids will vary from cell to cell, with some cells rejecting one of the plasmids altogether. Therefore, each protein or set of proteins expressed must be induced from compatible plasmids.

Bacterial plasmids contain a constitutively active gene that codes for proteins that confer antibiotic resistance; transformed cells are selected by their ability to grow in the presence of antibiotics. In addition, the presence of antibiotics in the growth medium applies selective pressure for the cell to maintain the plasmid. Cells containing two or more compatible plasmids, each of which confers a different antibiotic resistance, can be selected by growing in the presence of multiple antibiotics.

Promoter activity—A major feature of STINT-NMR is to provide a complete structural titration of the labeled target molecule by increasing the concentration of unlabeled interactor protein(s) in the cell. In this case, the relative concentrations of the interacting partner(s) need to be tightly controlled; this is especially critical if one wants to introduce post-translational modifications to change the properties of the interacting partners. Transcription of each protein or set of proteins must be individually induced; this requires that the plasmids contain distinctly regulated promoters. The in-cell concentration of overexpressed protein is primarily a function of the ??plasmid?? copy number, strength of the promoter, and the concentration of the inducer.

Expression vectors regulate transcription in bacteria by derepression. For each plasmid, a repressor gene is constitutively transcribed and the resulting protein binds to operator sites. For example, the *lacI* gene product produces Lac repressor, which binds to sites on the *lac* operator (*lacOp*), repressing transcription. Upon addition of lactose or the nonmetabolized lactose analog, IPTG, to the cells, the inducer binds to the DNA-bound repressor causing it

to dissociate from the DNA. The resulting derepression allows RNA polymerase to bind to the promoter and transcribe the gene.

In the plasmid systems the authors have used, the *lac* and *tet* promoter/operators exhibit the greatest levels of overexpression, followed by the medium-strength *araBAD* operator and the comparatively weak rhamnose operator. The *lac* and *tet* operators are induced by IPTG and tetracycline (or anhydrotetracycline), respectively, and the *araBAD* and rhamnose operators are induced by L-arabinose and L-rhamnose. The *tet*, *rhaB*, and *araBAD* operators utilize endogenous *E. coli* RNA polymerase for transcription and elicit linear transcriptional responses over the wide range of inducer concentrations typically used for overexpression, thus providing the level of control necessary to perform these experiments. In these cases, overexpression can be terminated by simply washing the cells to remove the inducer. On the other hand, IPTG induction results in a high level of expression that is not subject to a great deal of control as a function of the concentration of the inducer. IPTG induction continues for up to 4 hr after the cells have been washed. This is a result of using T7 RNA polymerase for transcription from the T7 promoter (P_{T7}) present in the plasmids used for STINT-NMR (Table 17.11.1). T7 RNA polymerase is introduced into *E. coli* through the DE3 lysogen where it is under the control of *Lac* repressor ($P_{T7}/lacOp$). Adding IPTG to these cells induces overexpression of T7 RNA polymerase, and T7 RNA polymerase expression continues even when the intracellular concentration of IPTG is low.

Some plasmids contain promoters whose activity is dependent on cAMP-binding protein (CAP). This is because CAP-dependent transcription may be “leaky,” meaning that there is a low constitutive level of transcription; the *araBAD* promoter exhibits this property. To maintain tight control over promoter activity, it is critical to eliminate leakiness. Undesired overexpression can be suppressed by adding 0.2% glucose to growth culture and plates. Glucose reduces the concentration of cAMP in these cells, preventing CAP from activating transcription.

Cloning strategies—STINT-NMR can be extended to systems in which two or more interactor and/or post-translational modifying proteins are sequentially overexpressed. This can be accomplished by (1) overexpressing proteins from two or more compatible plasmids or from a single plasmid with two separately inducible sites, such as pRSFDuet, pCDFDuet, or pACYC (Novagen), using the same inducer (Burz and Shekhtman, 2008); (2) overexpressing a cistronic construct of two or more genes using a single inducer (unpublished data); or (3) sequentially overexpressing protein from three or more compatible plasmids using separate inducers to generate interacting proteins (Burz and Shekhtman, 2008).

For example, we have used IPTG to induce interactor protein overexpression simultaneously from both pCDF.1b (Novagen) and pRSF.1b (D. Burz and A. Shekhtman, unpub. observ.). pCDF.1b contains a CloBF13 origin, confers streptomycin resistance and uses the same T7 promoter and *lac* operator for protein induction as pRSF.1b, which contains a RSF1030 origin and confers kanamycin resistance. We have also used a combination of pASK, pCDF (or pCDFDuet), and pDB1 to sequentially overexpress a target, one or two interactor proteins, and kinase activity (Burz and Shekhtman, 2008). In this case, the three plasmids have mutually exclusive origins, antibiotic resistances, and inducers.

Another major consideration when performing STINT-NMR is the need to overexpress some of the proteins found in complexes simultaneously. This can be of paramount importance for maintaining the solubility of proteins in the cell. For example, in the absence of proper binding partners, proteins are often localized to insoluble inclusion bodies inside the cell. The commercially available plasmids, pRSFDuet, pCDFDuet, and pACYC-Duet

(Novagen), each of which have two expression cassettes available for cloning, as well as compatible origins and antibiotic selections, can overexpress up to six proteins from six T7 promoters simultaneously using IPTG induction. Since the strength of induction is comparable, this combination of plasmids can potentially alleviate solubility problems that may be encountered.

Two proteins can also be simultaneously overexpressed from compatible plasmids using two different inducers (Burz and Shekhtman, 2008). This experimental variation was used to overexpress post-translationally modified interactor protein. Overexpression of the interactor protein was induced for 3 to 4 hr off pCDF.1b or pCDF-Duet, using IPTG, while simultaneously inducing overexpression of a tyrosine kinase (Fyn kinase) from pDB1 using L-arabinose for the last 1 to 2 hr (Burz and Shekhtman, 2008).

Cell types—Cell types are selected for minimum background and the lowest potential for intrinsic intracellular binding partners. Bacterial cells provide an ideal environment for the study of eukaryotic proteins in this regard, but lack the biochemical machinery to provide chemical modifications that occur in eukaryotic cells and that may be necessary for an accurate assessment of the specific interaction being investigated; this can be overcome to some extent by expressing the biochemical activity required for such modifications. In all of our experiments, we have successfully used *E. coli* strains BL21(DE3) and BL21(DE3) codon + (Stratagene). These cell lines lack Lon protease, thus minimizing proteolytic degradation of overexpressed proteins. BL21(DE3) codon + contains a plasmid with a p15A origin and Cam^r that provides additional tRNAs to enhance eukaryotic gene overexpression; this cell line is incompatible with pACYC, pACYCDuet, and pRHA plasmids (Table 17.11.1).

Labeling strategies—STINT-NMR can be performed in different ways to provide a broad range of interactor-to-target concentration ratios for a complete structural titration. Any protein can be expressed first and any or all proteins can be labeled using different strategies. Labeling is performed in minimal growth medium (M9) and overexpressed proteins are typically uniformly labeled using ¹⁵N-ammonium chloride [*U*-, ¹⁵N] as the sole source of nitrogen. To assess major differences between in-cell and in vitro target protein structures, both ¹⁵N-ammonium chloride and ¹³C-glucose uniform labeling [*U*-, ¹⁵N, ¹³C] are used. When labeling proteins transcribed off plasmids whose promoter activity is CAP-dependent, it is important to substitute glycerol for glucose as the sole carbon source. Glucose reduces the concentration of cAMP in these cells, preventing CAP from activating transcription.

Different isotope labeling schemes can be employed to increase the quality of the in-cell NMR spectra. Molecular size is a limiting factor in analyzing protein complexes by STINT-NMR since an increase in molecular weight leads to a loss of spectral resolution and sensitivity due to reduced tumbling times. Growing bacterial cells in deuterated media allows us to successfully perform ¹H{¹⁵N}-TROSY-type experiments on molecular complexes up to ~100 kDa (Burz et al., 2006a). To extend the technology to complexes up to 1 MDa, a methyl-specific labeling scheme can be employed (Gardner and Kay, 1998). In this case, only methyl groups of Ile, Leu, and Val residues will be available for chemical shift mapping. For example, adding alpha-ketobutyrate 4-¹³C,3,3-d₂ to deuterated minimal medium results in the production of proteins with (¹H-δ₁ ¹³C methyl)-isoleucine. This approach will necessarily lead to the loss of atomic resolution but may result in higher quality NMR spectra, thus providing alternative pathways to study protein complexes by STINT-NMR. Sequential expression allows us to label two interacting proteins with two different NMR-active isotopes, e.g., ¹⁵N and ¹³C. This approach allows us to observe the

same protein complex without leaving interacting partners cryptic, but rather by collecting two, ^{15}N - and ^{13}C -edited, NMR spectra.

Sample Requirements and Handling

Cell viability

Cell lysis is an important factor limiting the acquisition time of in-cell NMR experiments (Cruzeiro-Silva et al., 2006). Glycerol (to 10%) is added to samples as a cryoprotectant for prolonged storage of the cells at -80°C . Adding glycerol to the NMR buffer maintains the viability of *E. coli* cells at densities sufficient to obtain in-cell NMR spectra at room temperature for more than 4 hr. The procedure produces reliable STINT-NMR samples with minimal cell lysis during NMR experiments. Usually the NMR signal from the [U -, ^{15}N]-protein in the supernatant does not exceed the noise level (Burz et al., 2006b). We find samples handled this way to be particularly robust.

To ensure that the NMR signal comes from proteins located inside the cell, after each STINT-NMR experiment, the NMR sample is centrifuged and the supernatant is probed by collecting an additional ^{15}N -HSQC. *E. coli* cell viability is routinely tested by using a standard colony-plating test. In this test, the number of colonies grown on antibiotic selection plates inoculated with the in-cell sample before STINT-NMR is compared with the number of colonies grown using cells plated after the STINT-NMR experiment. Colonies are counted by using a molecular imager. The cells are considered to be viable if the numbers of colonies on the plates are within 10% of each other.

Instrumentation

A high-field NMR spectrometer, greater than 500 MHz, equipped with a cryoprobe is required. The high field provides the necessary resolution and sensitivity for in-cell studies. The cryoprobe further increases the sensitivity of the NMR spectrometer and allows for faster data acquisition, which is especially important given that the sample consists of living cells.

Data and Analysis

^{15}N -edited heteronuclear single quantum coherence spectra ($^1\text{H}\{^{15}\text{N}\}$ -HSQC) of the cells (Serber and Dotsch, 2001) are collected for each STINT-NMR sample at room temperature using a 500-MHz or greater NMR spectrometer equipped with a cryoprobe. Water suppression in the NMR spectrum is achieved using a Watergate pulse sequence with 3-9-19 selective inversion (Piotto et al., 1992). Generally, 32 scans (1 hr data collection time) are sufficient to obtain a good quality NMR spectrum with a signal to noise ratio of 5:1 on a sample made from 100 ml of cell culture. $^1\text{H}\{^{15}\text{N}\}$ -HSQC allows us to obtain high-resolution NMR spectra of the backbone amide proton and nitrogen nuclei of the target protein inside bacterial cells. When unlabeled interactor protein is overexpressed in these cells, the spectrum of the target changes due to target-ligand interactions. Unlike the case where interacting proteins are simultaneously overexpressed in labeling medium, in STINT-NMR the spectral complexity is minimized because only the target protein is labeled with NMR-active nuclei, leaving the interactor protein(s) cryptic.

Depending on the chemical exchange rate between the free and bound states of the target, affected NMR peaks, corresponding to backbone amides, can either shift, broaden their line shape, or disappear completely (Fig. 17.11.3). The ideal situation corresponds to a case where the residues that are perturbed upon binding are localized to the surface of the target. More frequently, changes in chemical shifts and differential broadening of some assigned peaks may be more widespread, reflecting rearrangements of secondary structural elements

or a global or allosteric change in the conformation of the target. To accurately assess the changes in the NMR spectrum of a target molecule when bound to an interactor molecule, it is imperative that the resonance assignments of the target protein are known beforehand and that the target is stable and well behaved in the absence of the interactor protein. Assigning target resonances is generally accomplished *in vitro* using purified protein.

Chemical shift changes indicate local perturbation of the structure, whereas peak broadening can also result from an increase in molecular size. In addition to these changes in the NMR spectrum, new peaks can appear if the protein was interacting with a large molecule, such as membranes or genomic DNA, prior to expressing the interactor, and the interactor competes away the large nonspecifically bound target. In these cases, we monitor changes in the NMR spectrum of the free target since only this species gives rise to visible peaks.

To identify the molecular interface we measure either the line broadening of the NMR peaks or the change in the chemical shifts of amide nitrogens and attached amide protons according to the equation:

$$\sqrt{(\delta_N^2/25 + \delta_H^2)}$$

where δ_H and δ_N represent changes in proton and nitrogen chemical shifts, respectively. Usually, residues exhibiting either a chemical shift change above 0.05 ppm or extreme broadening are considered to be affected by protein-protein interactions (Fig. 17.11.3 insets). Mapping these changes onto the three-dimensional structure of the labeled target protein reveals the protein-protein interaction surface (Zuiderweg, 2002; Fig. 17.11.4). In the case of ^{13}C methyl group labeling, we use a ^{13}C -edited heteronuclear zero quantum coherence ($^1\text{H}\{^{13}\text{C}\}$ -HZQC) experiment (Tugarinov et al., 2004), which provides superior sensitivity for large molecular complexes.

In-cell NMR spectroscopy allows estimation of binding affinities based on the magnitude of the chemical shift change, $\Delta\Omega$, and the rate constant between bound and free states, k_{off} . Chemical exchange can result in gradual changes in chemical shift values when $\Delta\Omega \ll k_{\text{off}}$ (fast exchange), line broadening when $\Delta\Omega \leq k_{\text{off}}$ (intermediate exchange), or the appearance of new peaks when $\Delta\Omega \gg k_{\text{off}}$ (slow exchange). Assuming that the binding reaction is diffusion limited and the average change of the chemical shift is ~ 0.01 ppm, the fast exchange regime will occur when the dissociation constant, K_d , is larger than 100 μM , and intermediate or slow exchange will occur when the dissociation constant is ≤ 10 μM . To provide a more accurate estimate of binding affinities, the concentrations of proteins inside the cell may be assessed by performing immunoblots on whole cell extracts. If available, purified target and interactor proteins can be used to corroborate binding affinities *in vitro*. We found that the effective concentration of overexpressed protein inside the cell typically varies from 0.01 to 1 mM, thus STINT-NMR can identify protein-protein interactions that span a range of binding affinities from micromolar to millimolar.

Applications

Understanding protein-protein interactions, from both experimental and bioinformatics perspectives, has been the subject of intense investigation over the last decade. The availability of genome-wide protein-protein interaction databases (Bader et al., 2003) from yeast-2-hybrid screens, synthetic lethal screens, and mass-spectroscopy of purified, tagged protein complexes (Krogan et al., 2006), among others, is critical for developing a comprehensive approach to understanding the proteome (Zhu et al., 2002). Despite the

ongoing effort to decipher the complex nature of protein interactions, they are still not entirely understood (Gerstein, 2000). Experimental techniques employed to characterize the interactome yield a considerable amount of information about protein-protein interactions (Jansen et al., 2002), but suffer from a significant number of false positives and negatives, complicating the interpretation of results. This ambiguity stems from the simple yes-no nature of the high-throughput experiments. Usually, further structural characterization of protein complexes is needed to fully understand molecular networks (Gerstein, 2000). Another major flaw of the existing experimental screens of protein-protein interactions is their heavy bias towards detecting high affinity interactions. Transient, context-dependent, low affinity interactions that predominate in some important signaling pathways can be missed. Identifying an interaction, however, is merely the first step towards understanding how the proteome gives rise to and regulates biological processes. What is needed, in addition, is detailed structural information that can be used to study and re-engineer the complex set of interactions occurring *in vivo*, thus facilitating new approaches to treating and eliminating the pathological states that arise when the normal network of interactions is disrupted.

In-cell biochemistry

Specific interactions between proteins are often modulated by post-translational modifications of protein structure, such as phosphorylation, glycosylation, and ubiquitination, and provide a mechanism for regulating cellular processes. PTMs can result in structural changes that manifest themselves through the interacting surface of the target protein. Such modifications can alter the strength or number of interactions in which these proteins engage and/or redirect them to subcellular compartments as required for proper functioning (Seet et al., 2006). For example, endocytosis of receptor tyrosine kinases (RTKs) requires tyrosine phosphorylation and monoubiquitination of the receptor and downstream components to sort endocytosed cargo for subsequent degradation (down regulation) or recycling to the cell surface (Clague and Urbe, 2001; Haglund et al., 2003; Marmor and Yarden, 2004). Ubiquitin binding mediates the processivity of a network of interactions required for proper functioning of the RTK sorting machinery. To examine the effect of PTMs on protein-protein interactions that occur along the RTK signaling pathway, we developed an in-cell biochemical methodology that, in combination with STINT-NMR, allows us to directly observe structural changes in the interaction surface of a target protein, ubiquitin, that result from phosphorylating tyrosine residues on interactor proteins (Burz and Shekhtman, 2008).

Since protein phosphorylation does not naturally occur in *E. coli*, we introduced the target protein, interactor proteins, and kinase activity on separately inducible plasmids. Tight temporal control over protein expression allowed us to post-translationally modify the interactor proteins and observe the structural details of the interaction surfaces in the absence and presence of PTMs within a cellular environment. To study the interaction between the target, ubiquitin, and post-translationally modified interactors, overexpression of the interactor protein was induced for 3 to 4 hr, with simultaneous induction of overexpression of a tyrosine kinase (Fyn) for the last 1 to 2 hr (Burz and Shekhtman, 2008). STINT-NMR revealed how ubiquitin interacts differentially with nonphosphorylated and phosphorylated components of the receptor tyrosine kinase (RTK) endocytic sorting machinery: the signal-transducing adaptor molecule (STAM2), hepatocyte growth factor regulated substrate (Hrs), and the STAM2-Hrs heterodimer (Burz and Shekhtman, 2008). The results are consistent with a weakening of the network of interactions when the interactor proteins are phosphorylated, as evidenced by a loss of interaction surface upon tyrosine phosphorylation of STAM2.

Further analysis of the ubiquitin-STAM2 interaction showed that the interaction surface is modulated by the phosphorylation state of two STAM2 tyrosines, Y371 and Y374, located in the conserved ITAM domain. To verify that the ubiquitin residues perturbed when STAM2 is phosphorylated are due to the post-translational modification, we mutated the ITAM tyrosine residues to phenylalanines (Y371/4F-STAM2). When this mutant was overexpressed and its interaction with ubiquitin examined using STINT-NMR, the resulting spectra and surface interaction maps for both the unphosphorylated and phosphorylated states were largely identical to those obtained using unphosphorylated wild-type STAM2 (Fig. 17.11.4). Small differences between the spectra are likely due to the fact that at least one additional Fyn-dependent tyrosine phosphorylation site (Y291) was still present in the mutant. Phosphorylation of select residues was confirmed by using immunoblots and mass spectroscopic analysis.

SMILI-NMR

Biomolecular complexes are attractive targets for controlling cellular processes in disease states by using small molecules as therapeutic agents and while high-throughput screening (HTS) of compound libraries has proven to be a viable approach to the drug discovery process, finding inhibitors or enhancers of biomolecular complex formation is difficult for HTS (Fernandes, 1998; Silverman et al., 1998). This is because in vitro assays often fail to consider physiologically relevant conformations of the complexes, and in vivo assays are hampered by the difficulty in distinguishing small molecules that directly affect the interactions of interest from those that exert their influence through other cellular components. In vitro NMR-based screening, utilized in drug discovery programs (Shuker et al., 1996; Peng et al., 2001; Hajduk and Burns, 2002; Pellecchia et al., 2002, 2008), has become a powerful method for identifying and analyzing low-molecular-weight compounds that bind specifically to biomolecular targets. To rapidly screen small molecule libraries for compounds capable of disrupting or enhancing specific interactions between two or more components of a biomolecular complex, we developed an in-cell NMR-based procedure called **SMILI-NMR**, for screening of small molecule interactor library using in-cell NMR (Xie et al., 2009).

SMILI-NMR employs STINT-NMR technology (Burz et al., 2006a,b; Burz and Shekhtman, 2009) to produce biomolecular complexes inside the cell in which one of the constituent proteins is uniformly [U-, ^{15}N] labeled with NMR-active nuclei. By monitoring the in-cell NMR spectrum of the labeled protein, we directly observe the formation of high-affinity ternary complexes and changes in the structure of that complex induced by the binding of small drug-like molecules that disrupt or enhance the stability of the complex. SMILI-NMR presents a unique approach to small molecule screening due to the in-cell nature of the assay combined with high resolution NMR as a readout. The formation of an interacting complex is critical to the success of this method, as it constitutes the basis spectrum from which any departure reveals the ability of the small molecule to potentially regulate the biological activity of the complex.

SMILI-NMR offers unique advantages for screening therapeutic agents against chosen disease-related protein targets. Changes in the interaction surface caused by a small molecule interfering with complex formation are used as a read-out of the assay. The in-cell nature of the experiment ensures that the small molecule is capable of penetrating the cell membrane and specifically engaging the target molecule(s). We can selectively screen for drug-like molecules that block the interaction surface that is critical for the normal functioning of the biocomplex. This greatly reduces the likelihood of compensatory mutations or suppressor gene products arising that may restore the function of the drug-compromised biocomplex.

SMILI-NMR requires a high magnetic field, preferably 700 MHz or higher, and a cryoprobe to perform in-cell NMR experiments. The cost of the assay is mitigated by the fact that SMILI-NMR obviates the need for multiple in vitro binding assays. The cell membrane provides a selectivity filter for druglike molecules capable of entering the cell, which increases the chance that a selected candidate will perform well in in vivo functional assays. In-cell protein overexpression results in higher local concentrations of interaction partners than in lysates thus increasing the likelihood of selecting weaker interactors for fragment-based screening (Hajduk and Burns, 2002; Schuffenhauer et al., 2005). SMILI-NMR can discriminate between binding to different surfaces on the target protein, further facilitating fragment based screening (Xie et al., 2009).

The atomic resolution nature of in-cell NMR increases the information content of SMILI-NMR, allowing deconvolution of mixtures of compounds used for screening. In general, the optimal number of mixture components will depend on the quality of the in-cell NMR spectrum of the biocomplex. By using a matrix method, $M \times N$ samples can be tested by examining $M+N$ samples in the first round of screening (Fig. 17.11.5). A further increase in the assay speed can be achieved by increasing the dimensions of the matrix, which would entail an increase in the number of mixture components. The utility of the method was demonstrated by screening a small library of 289 dipeptides against the FKBP-FRB protein complex involved in cell cycle arrest (Xie et al., 2009).

In the first round of screening, we identified a mixture of compounds that bind to the FKBP-FRB biocomplex with millimolar affinities. In the second round, we deconvoluted the mixture into contributions from individual compounds. As a final step, we found that at least one of the dipeptides, A-E, identified from the second round of screening, successfully inhibited the activity of the FKBP-FRB complex in promoting the growth of yeast cells (Heitman et al., 1991). These results proved that SMILI-NMR can be used to screen small libraries of carefully selected compounds, such as those used for fragment-based screening (Hajduk and Burns, 2002; Schuffenhauer et al., 2005).

The strongest appeal of SMILI-NMR is the biological relevance of the screening. Since observations are performed inside the cell, a library of compounds can be screened against biocomplexes under physiologically relevant conditions. Overall, SMILI-NMR promises to be a valuable addition to the repertoire of drug screening tools used in small molecule-protein complex interaction studies. The method bridges the gap between identifying small ligands capable of interfering with target biocomplexes and their functioning as inhibitors or enhancers of cellular processes.

Future directions/prospectus

The study of molecular interactions inside living cells has entailed utilizing complementary methods to resolve processes on different scales from micrometers to Angstroms. Light and fluorescence microscopy were successfully applied to study the localization and compartmentalization of macromolecules in vivo on the micrometer scale. Förster resonance energy transfer (FRET) extended our ability to identify protein-protein and protein-nucleic acid interactions on a scale of tens of nanometers and facilitated estimates of binding affinities and stoichiometries (Giepmans et al., 2006). The advent of STINT-NMR, which allows us to modify and examine protein-protein interaction surfaces at the level of single amino acid residues, has pushed the limits of resolution to the subnanometer scale. Though in its infancy, this technique has the potential to open a window for investigating life processes inside a living cell at a level of detail never seen before.

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Figure 17.11.1.

Flow chart to generate a matrix of samples for STINT-NMR analysis. Reprinted from Burz et al. (2006a).

Figure 17.11.2.

SDS-PAGE of ubiquitin and STAM2 sequential expression. The BL21(DE3) cells were analyzed immediately after overexpression; lane 1 is uninduced cells; lanes 2 to 5 are a timecourse of ubiquitin overexpression induced using L-arabinose for 0.5, 1, 2, and 3 hr, respectively; lanes 6 to 9 are sequential overexpression of STAM2 induced using IPTG for 0.5, 1, 2, and 3 hr, respectively. Note that the ubiquitin level remains essentially constant as STAM2 expression increases. Reprinted from Burz et al. (2006b). Abbreviations: ubq, ubiquitin.

Figure 17.11.3.

NMR spectra of the target protein, ubiquitin, complexed with interactor peptide, AUIM, and with interactor protein, STAM2. (A) Overlay of $^1\text{H}\{^{15}\text{N}\}$ HSQC spectra of *E. coli* cells after 3 hr of overexpressing [*U*-, ^{15}N] ubiquitin and 0 hr (black), 2 hr (red), and 3 hr (blue) of overexpressing AUIM. Individual peaks exhibiting large chemical shifts are labeled with corresponding assignments. The progression of colors in the $^1\text{H}\{^{15}\text{N}\}$ HSQC overlaid spectra was chosen for ease of viewing. Inset: Close-up of the chemical shift changes of Gly47 during titration. (B) $^1\text{H}\{^{15}\text{N}\}$ HSQC spectra of *E. coli* cells after 3 hr of overexpressing [*U*-, ^{15}N] ubiquitin and 3 hr of overexpressing STAM2. Resonance peaks exhibiting extreme broadening are indicated by crosses. Insets: One-dimensional traces of selected peaks exhibiting differential broadening after 3 hr of overexpressing [*U*-, ^{15}N] ubiquitin and 0 hr (black), 2 hr (red), and 3 hr (green) of overexpressing STAM2. Reprinted from Burz et al. (2006a). For the color version of this figure go to <http://www.currentprotocols.com/protocol/ps1711>.

Figure 17.11.4.

Interaction surface maps of ubiquitin-ligand complexes. Interaction surface of ubiquitin mapped onto the three-dimensional structure of ubiquitin (PDB code 1D3Z). Individual residues exhibiting either a chemical shift change >0.05 ppm or differential broadening are indicated in red. All perturbed residues lie on the ubiquitin surface and, therefore, reflect changes in the interaction surface of the molecule rather than changes in tertiary or quaternary structure. (A) Y371/4F-STAM2-Ubq interaction; (B) phosphorylated Y371/4F-STAM2-Ubq interaction (YP-Y371/4F-STAM2). Ubiquitin ligands are indicated in each panel. This interaction map was used to prove that Y371/4F mutation leads to a loss of the dependence of STAM2-ubiquitin interaction on the phosphorylation state of STAM2. Reprinted from Burz and Shekhtman (2008). For the color version of this figure go to <http://www.currentprotocols.com/protocol/ps1711>.

	X-A	X-D	X-E	X-F	X-G	X-H	X-I	X-K	X-L	X-N	X-Q	X-R	X-S	X-T	X-V	X-W	X-Y
A-X	AA	AD	AE	AF	AG	AH	AI	AK	AL	AN	AQ	AR	AS	AT	AV	AW	AY
D-X	DA	DD	DE	DF	DG	DH	DI	DK	DL	DN	DQ	DR	DS	DT	DV	DW	DY
E-X	EA	ED	EE	EF	EG	EH	EI	EK	EL	EN	EQ	ER	ES	ET	EV	EW	EY
F-X	FA	FD	FE	FF	FG	FH	FI	FK	FL	FN	FQ	FR	FS	FT	FV	FW	FY
G-X	GA	GD	GE	GF	GG	GH	GI	GK	GL	GN	GQ	GR	GS	GT	GV	GW	GY
H-X	HA	HD	HE	HF	HG	HH	HI	HK	HL	HN	HQ	HR	HS	HT	HV	HW	HY
I-X	IA	ID	IE	IF	IG	IH	II	IK	IL	IN	IQ	IR	IS	IT	IV	IW	IY
K-X	KA	KD	KE	KF	KG	KH	KI	KK	KL	KN	KQ	KR	KS	KT	KV	KW	KY
L-X	LA	LD	LE	LF	LG	LH	LI	LK	LL	LN	LQ	LR	LS	LT	LV	LW	LY
N-X	NA	ND	NE	NF	NG	NH	NI	NK	NL	NN	NQ	NR	NS	NT	NV	NW	NY
Q-X	QA	QD	QE	QF	QG	QH	QI	QK	QL	QN	QQ	QR	QS	QT	QV	QW	QY
R-X	RA	RD	RE	RF	RG	RH	RI	RK	RL	RN	RQ	RR	RS	RT	RV	RW	RY
S-X	SA	SD	SE	SF	SG	SH	SI	SK	SL	SN	SQ	SR	SS	ST	SV	SW	SY
T-X	TA	TD	TE	TF	TG	TH	TI	TK	TL	TN	TQ	TR	TS	TT	TV	TW	TY
V-X	VA	VD	VE	VF	VG	VH	VI	VK	VL	VN	VQ	VR	VS	VT	VV	VW	VY
W-X	WA	WD	WE	WF	WG	WH	WI	WK	WL	WN	WQ	WR	WS	WT	WV	WW	WY
Y-X	YA	YD	YE	YF	YG	YH	YI	YK	YL	YN	YQ	YR	YS	YT	YV	YW	YY

Figure 17.11.5.

Matrix method of screening chemical libraries. A library containing 289 dipeptide compounds is screened by examining individual mixtures located in the first row and first column of a matrix plate. Mixtures that result in similar changes in the in-cell NMR spectrum, so called hits, located at the intersection of rows (red) and columns (blue), are used in the second round of screening to validate the hit. Reprinted with permission from Xie et al (2009). For the color version of this figure go to <http://www.currentprotocols.com/protocol/ps1711>.

Table 17.11.1

STINT-NMR Compatible Plasmids

Plasmid	Origin	Antibiotic selection ^a	Copy inducer	Operator ^b	Number	Source
pBAD-HisA	ColE1	Amp	L-arabinose	<i>araBAD</i>	~40	Invitrogen
pBAD202	ColE1	Kan	L-arabinose	<i>araBAD</i>	~40	Invitrogen
pRSF-1b	RSF1030	Kan	IPTG	<i>P_{Trj}/lacOp</i>	>100	Novagen
pRSFDuet	RSF1030	Kan	IPTG	<i>P_{Trj}/lacOp</i>	>100	Novagen
pDB1	RSF1030	Kan	L-arabinose	<i>araBAD</i>	>10	In-house ^c
pCDF-1b	CloDF13	Stm	IPTG	<i>P_{Trj}/lacOp</i>	20-40	Novagen
pCDFDuet	CloDF13	Stm	IPTG	<i>P_{Trj}/lacOp</i>	20-40	Novagen
pASK-3+	ColE1	Amp	Tet/ahTet	<i>tet</i>	~40	IBA
pACYC	p15A	Cam	IPTG	<i>P_{Trj}/lacOp</i>	10-12	Novagen
pACYCDuet	p15A	Cam	IPTG	<i>P_{Trj}/lacOp</i>	10-12	Novagen
pRHA	p15A	Cam	L-rhamnose	<i>rhaB</i>	10-12	In-house ^d

NOTE: Anhydrotetracycline binds to the *tet* promoter 35 times more strongly than tetracycline and it circumvents cell death due to lack of resistance to that antibiotic.

^a Amp (ampicillin), Kan (kanamycin), Stm (streptomycin), Cam (chloramphenicol)

^b *araBAD* (arabinose promoter), *P_{Trj}/lacOp* (T7 promoter, *lac* operon), *tet* (tetracycline promoter) IPTG (isothiolgalactopyranoside), Tet (tetracycline), ahTet (anhydrotetracycline)

^c Unpublished

^d Published in Burz and Shekhtman, 2008