Biomolecular Science Based on In Situ Observation by Magnetic Resonance

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Protein structures and dynamics are closely related to physiological functions, but are strongly affected by temperature, pH, ionic strength, pressure, and molecular crowding. Within cells, biomolecules are densely packed, and the intracellular space is compartmentalized by cytoskeletal structures and organelles. Furthermore, this environment is non-equilibrium, constantly adapting to external stimuli. To accurately understand protein function, it is therefore essential to investigate biomolecules in their native contexts. We apply magnetic resonance techniques—particularly in-cell NMR—to observe the atomic-level structures and dynamics of proteins directly in living cells, where they perform their natural roles. Our group also develops methodological innovations to improve sensitivity and resolution, expanding the scope of in-cell NMR. By correlating structural and dynamic information with cellular physiology, we aim to reveal the molecular basis of complex biological systems and establish a foundation for biomolecular science based on in situ magnetic resonance observation.

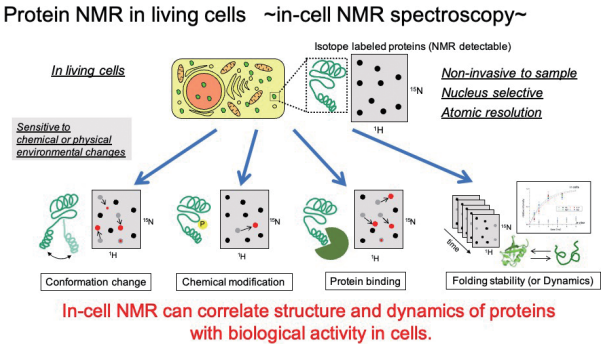

Figure 1. Overview of in-cell NMR spectroscopy.

Selected Publications

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- Y. Hikone, G. Hirai, M. Mishima, K. Inomata, T. Ikeya, S. Arai, M. Shirakawa, M. Sodeoka and Y. Ito., "A New Carbamidemiethy-Linked Lanthanoid Chelating Tag for PCS NMR Spectroscopy of Proteins in Living HeLa Cells," *J. Biomol. NMR* **66**, 99–110 (2016).
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1. In-Cell NMR Analysis of Keap1-Nrf2 Interactions Enabled by Controlling Intracellular Degradation

We investigated the interaction between Nuclear factor erythroid 2-related factor 2 (Nrf2) and its regulator Keap1 in living mammalian cells using in-cell NMR spectroscopy. Nrf2 is a transcription factor that protects cells from oxidative stress by inducing antioxidant response genes, and its stability is tightly controlled by Keap1-mediated ubiquitination and proteasomal degradation. Dysregulation of this system is implicated in cancer, neurodegeneration, and other diseases.

A major challenge we faced was the rapid intracellular degradation of delivered ^{15}N-labeled Nrf2-Neh2, which hindered direct NMR observation. To overcome this, we optimized cell preparation conditions by applying proteasome inhibitors such as MG132 and bortezomib. This successfully stabilized Nrf2-Neh2 after electroporation into HeLa and other cell lines. By contrast, mutating ubiquitination sites (Nrf2-Neh2 R7) or deleting Keap1 alone did not prevent degradation, showing that proteasomal pathways beyond Keap1-dependent ubiquitination contribute significantly.

Using degradation-controlled conditions, we observed clear in-cell NMR spectra of Nrf2-Neh2, which provided atomic-level evidence of its interaction with endogenous Keap1. We detected strong signal attenuation around the DLG and ETGE motifs, confirming their role in binding. By comparing HeLa cells with A549 lung carcinoma cells harboring the Keap1 G333C mutation, we revealed that this mutation weakens binding in dilute solution but displays selective effects inside cells, particularly near the ETGE motif. This demonstrates that the intracellular environment modulates the consequences of disease-associated mutations in ways not apparent from in vitro experiments.

We further evaluated the small-molecule inhibitor KI696, a nanomolar-affinity binder of Keap1. In dilute solution, KI696 efficiently disrupted the Nrf2-Keap1 complex, restoring Nrf2-Neh2 signals. In living mouse embryonic fibroblast cells reconstituted with Keap1, KI696 also increased Nrf2-Neh2 signal intensity, though to a lesser degree than in vitro. These results highlight how molecular crowding and intracellular kinetics reduce inhibitor potency, underscoring the importance of evaluating drug action directly in the cellular context.

Our study also revealed that the Nrf2-Keap1 system in cells exhibits rapid association–dissociation dynamics, producing extensive NMR signal broadening not predicted by concentration ratios alone. We interpret this as ubiquitination-dependent cycling of Nrf2, even under proteasome inhibition, reflecting the highly dynamic nature of the intracellular environment.

Taken together, we established a robust methodology to overcome proteasome-mediated degradation, enabling in-cell NMR spectroscopy of unstable proteins. Applying this platform to the Nrf2-Keap1 system, we uncovered unique insights into binding dynamics, mutation effects, and inhibitor activity in living cells. These findings not only advance mechanistic understanding of Nrf2 regulation but also demonstrate the broad potential of in-cell NMR as a tool for probing protein–protein interactions and evaluating therapeutic compounds under physiologically relevant conditions.

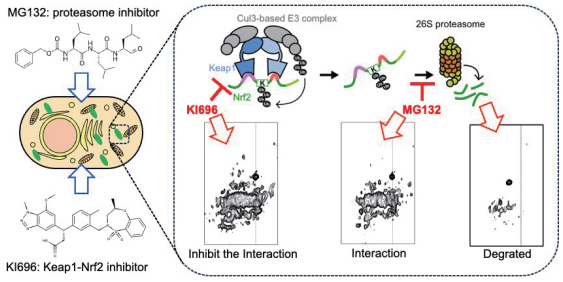

Figure 2. Schematic representation of Keap1-Nrf2 interactions in protein degradation-controlled mammalian cells.

2. In-Cell NMR Study of Protein Diffusion In Mammalian Cells

Protein diffusion in living cells is a crucial factor that determines localization, interactions, and functional regulation of biomolecules. Unlike dilute solution conditions, the intracellular environment is crowded and viscous, leading to significant restrictions on molecular mobility. To quantitatively characterize such behavior, we are developing an in-cell NMR platform that enables direct measurement of translational and rotational diffusion of proteins in mammalian cells.

As an initial step, we implemented fast, high-sensitivity NMR pulse sequences for intracellular diffusion measurements. Using ^{15}N-labeled ubiquitin mutants (L8A, I44A, V70A) introduced into HeLa cells, we successfully applied DOSY-SOFAST-HMQC experiments. Data acquisition was performed under optimized conditions, which enabled the detection of intracellular diffusion signals with sufficient sensitivity.

Diffusion analysis revealed a clear contrast between intracellular and dilute solution conditions. The translational diffusion coefficient was 2.18×10^{-10} m^2/s in buffer, but decreased to 4.96×10^{-11} m^2/s in HeLa cells—approximately a fourfold reduction. This result demonstrates that the dense intracellular milieu strongly impedes protein motion. Importantly, the ability to capture this quantitative difference confirms that our in-cell NMR setup is capable of monitoring protein dynamics under physiologically relevant conditions.

Looking forward, we plan to expand this model system to measure rotational diffusion in addition to translational motion. Rotational diffusion reflects molecular shape and local interactions and will complement the information gained from translational mobility. By integrating both, we aim to obtain a broader picture of protein dynamics across multiple timescales. In the coming year, we will refine our methodology and apply it to other protein systems, seeking to establish a versatile platform for in-cell structural biology.

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