Fast multidimensional NMR spectroscopy of proteins in solution

Development of new methods and application to the study of structure and kinetics

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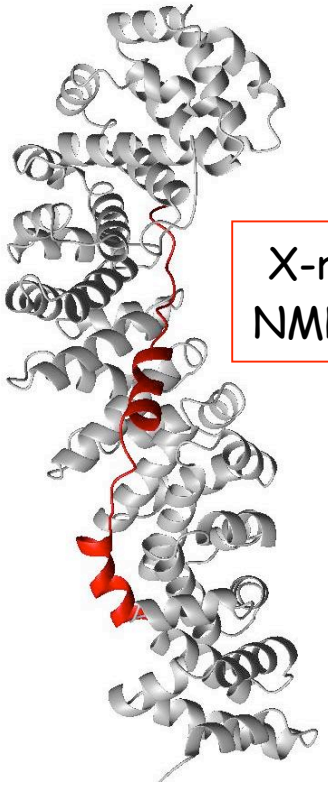
Major challenges in structural biology

Structures



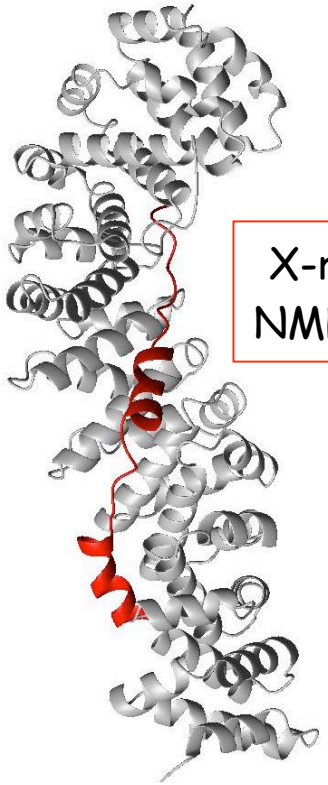
β-catenin in complex with the intrinsically unfolded protein tcf4

X-ray diffraction
NMR (20% of PDB)

Static

Major challenges in structural biology

Structures



β-catenin in complex with the intrinsically unfolded protein tcf4

X-ray diffraction
NMR (20% of PDB)

Static

Dynamics

Spectroscopies
IR
UV/Vis
NMR
...
Scattering

Diffraction

Transient Interactions

Folding

Unfolded proteins
Coupled binding/folding

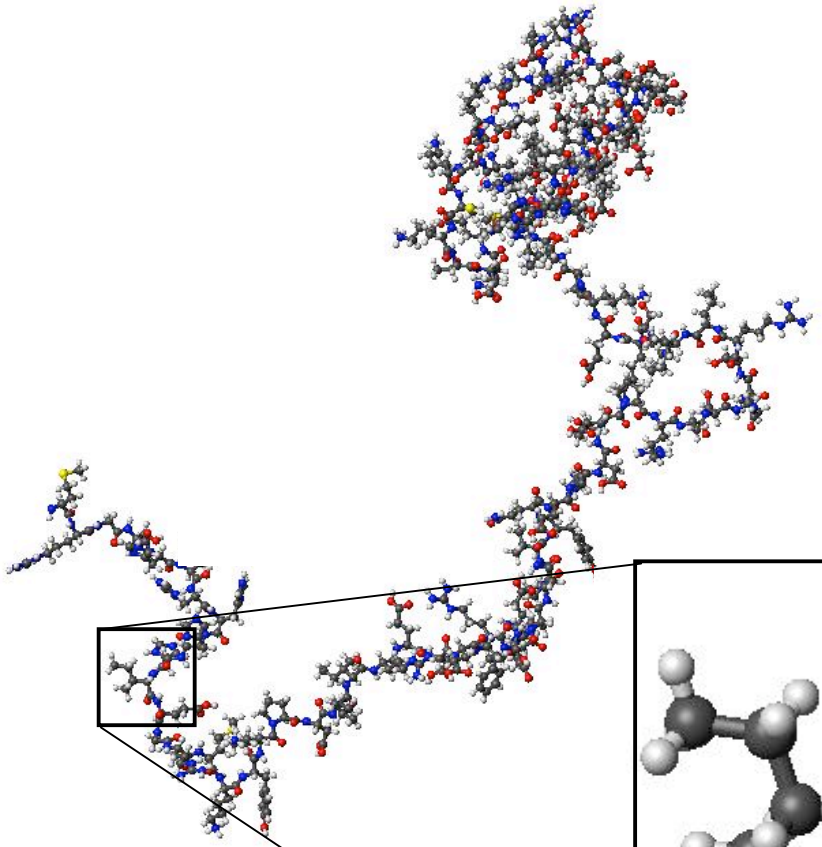
Catalysis

Misfolding and fibrils

...

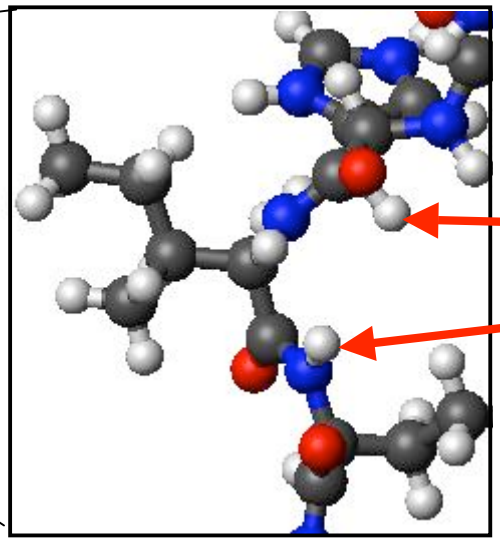
Ensembles of structures changing with time

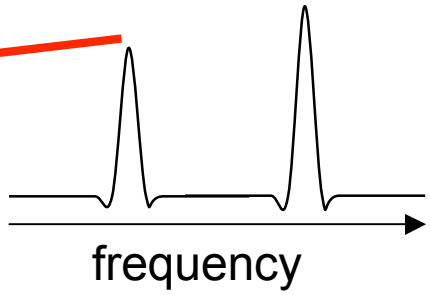
Nuclear spins report on the environment at hundreds of sites within the molecule



Each individual NMR-active nucleus (^{1}H, ^{2}H, ^{13}C, ^{15}N,…) gives local information

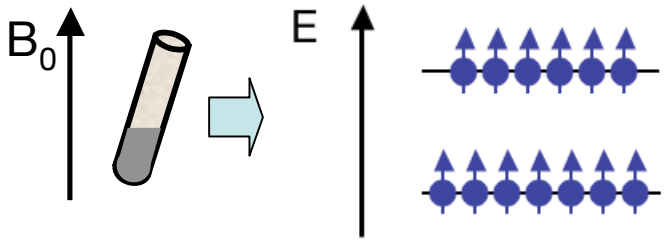
- Chemical environment
- Structure
- Dynamics





Requirements to observe individual atomic sites

1. Sensitivity



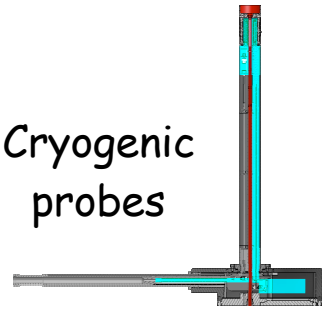
Only 1 out of ~10000 molecules contributes to NMR signal

Solutions

High B_0-fields

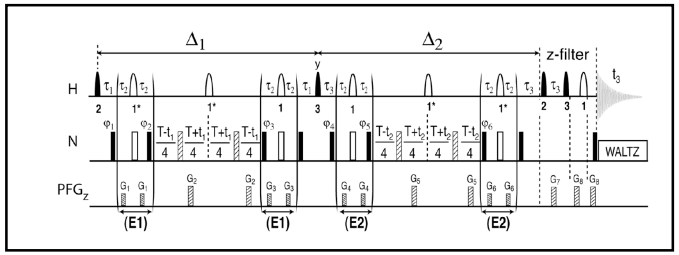
400 → 900MHz
factor ≈ 3.0

Cryogenic probes



factor ≈ 2-4

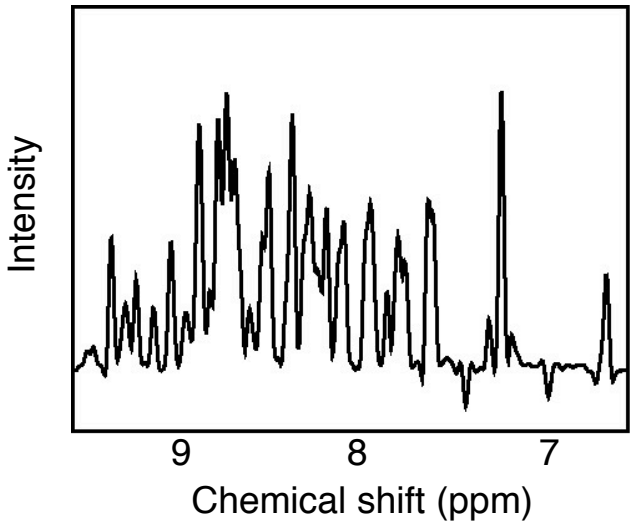
Optimized pulse sequences

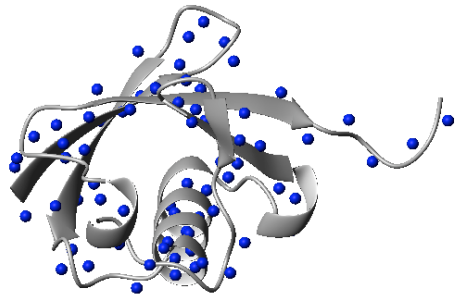


Sensitivity has advanced a lot in recent years making possible a **significant reduction of experimental durations**

Requirements to observe individual atomic sites

2. Resolution





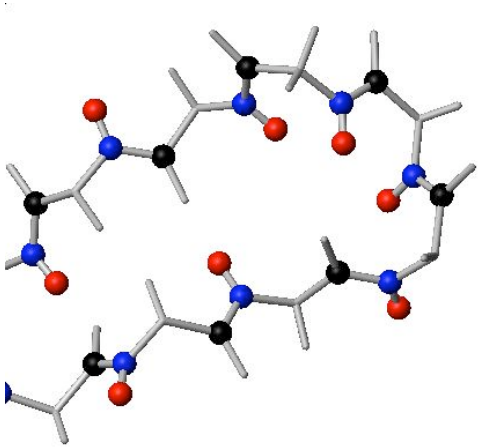
76 amino acids

Observation of signals of individual nuclei is hindered by signal overlap

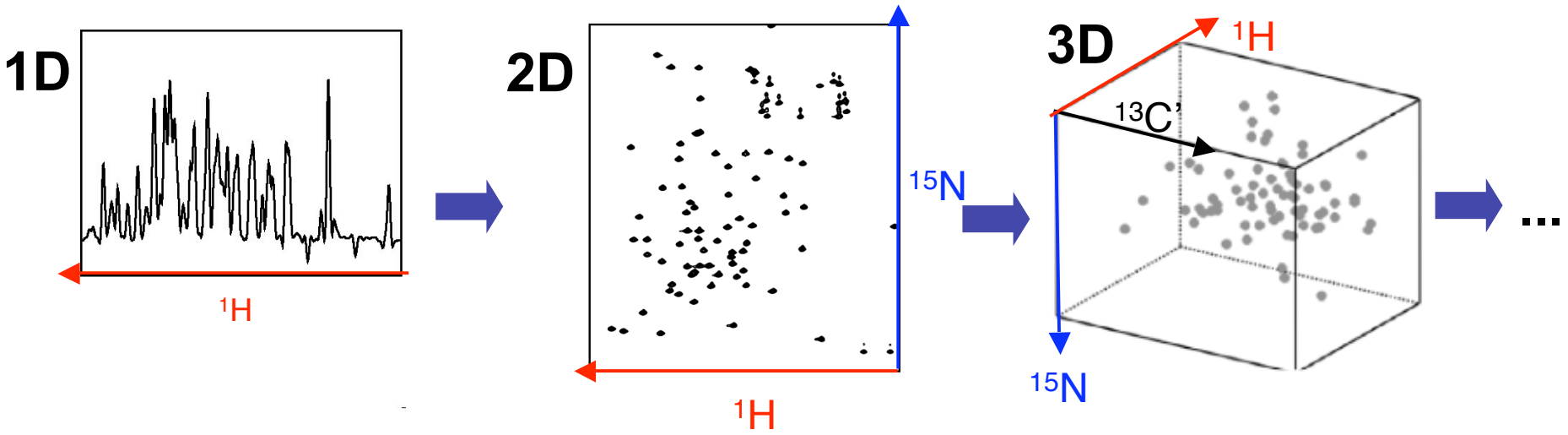
Solution

Multidimensional NMR

Multidimensional NMR



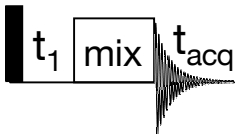
... correlates the frequencies of several interacting nuclear spins and spreads the signals over a multidimensional space

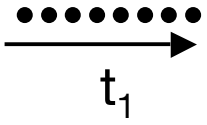


Time requirements of nD NMR

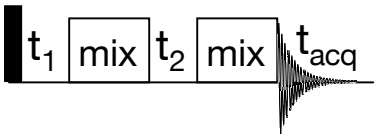
1D: seconds

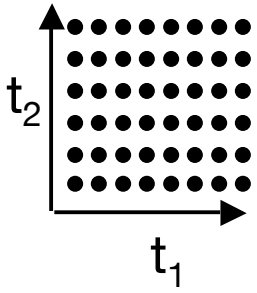
2D: minutes



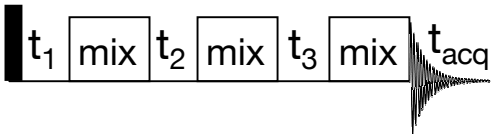


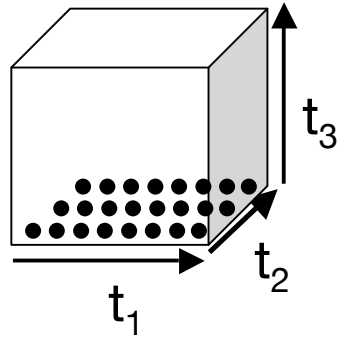
3D: hours



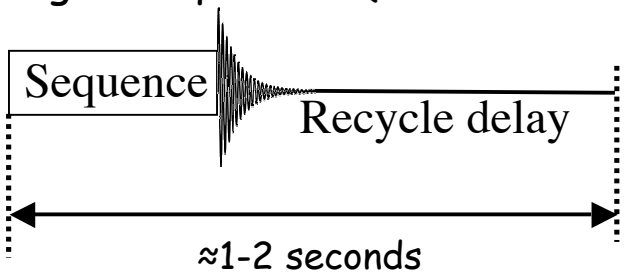


4D: weeks





Each point corresponds to
one signal acquisition (≈1-2 seconds)



In many cases, experimental durations are dictated by the sampling requirement, not by the sensitivity.

Limitations of nD NMR related to long experimental durations

* Sample stability and long acquisition times ?

* High throughput capabilities for structural genomics projects ?

* High-dimensional data sets as required for proteins with strong overlap (unfolded proteins) require unrealistically long times

* Spectral changes during a reaction occurring on a seconds time scale cannot be followed with atomic resolution

Outline

Part 1: Methods for fast multidimensional NMR

Concepts

Sensitive fast-pulsing experiments: SOFAST HMQC, BEST experiments

Part 2: Applications

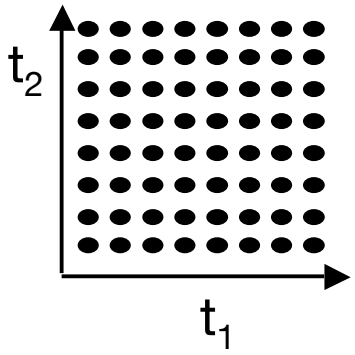
Application to the study of protein folding and unfolding

HET-SOFAST NMR: a fast tool for characterizing protein structure

Accelerating nD NMR

Concepts

1. Reduce the number of mandatory scans



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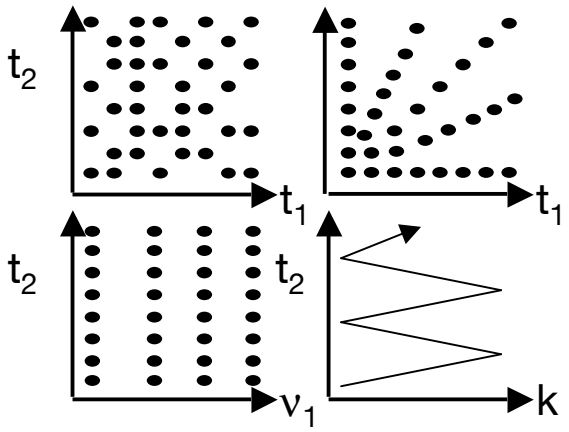

Alternative ways of data sampling:

Non-linear data sampling of time domain
Hadamard NMR spectroscopy (frequency domain)
"Single scan" NMR (spatial/orientational encoding)
...

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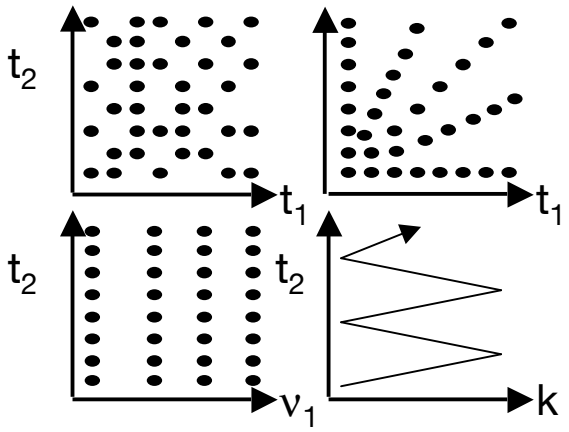

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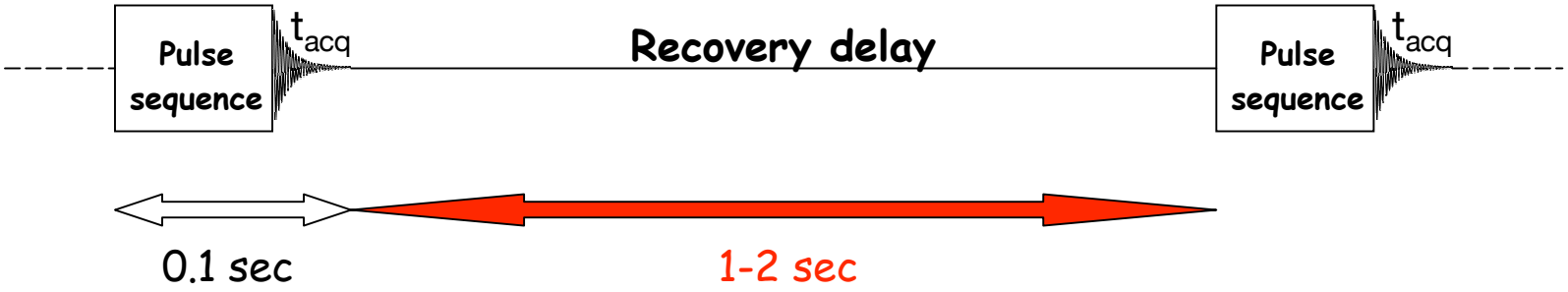

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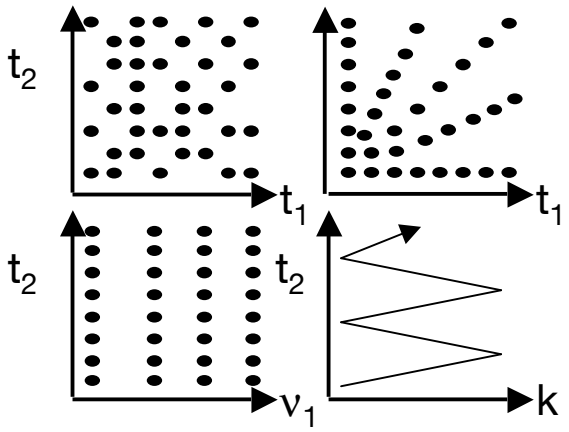

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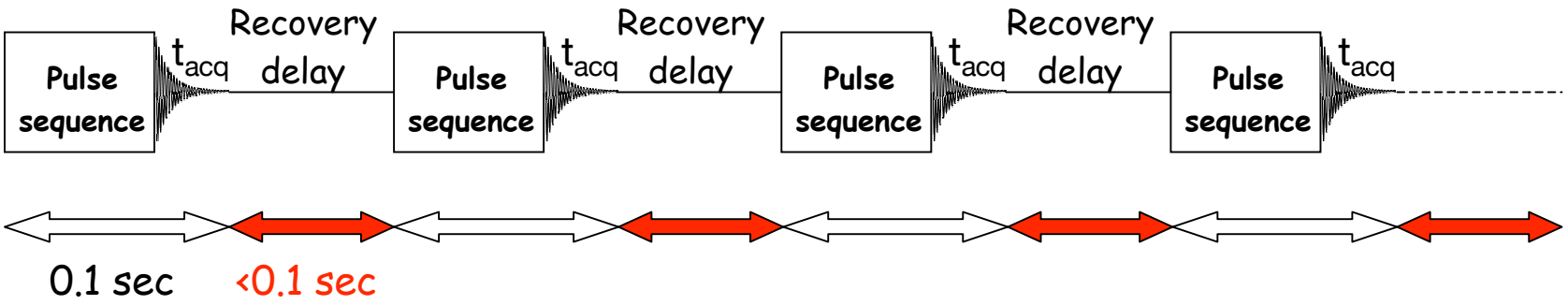
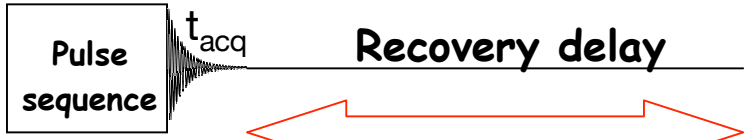
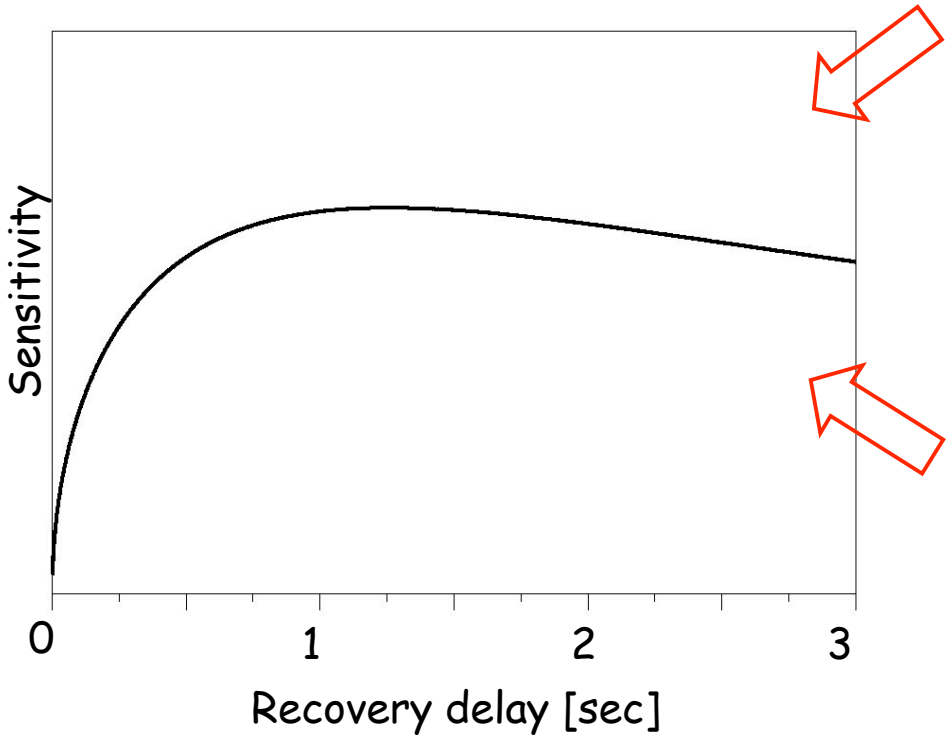
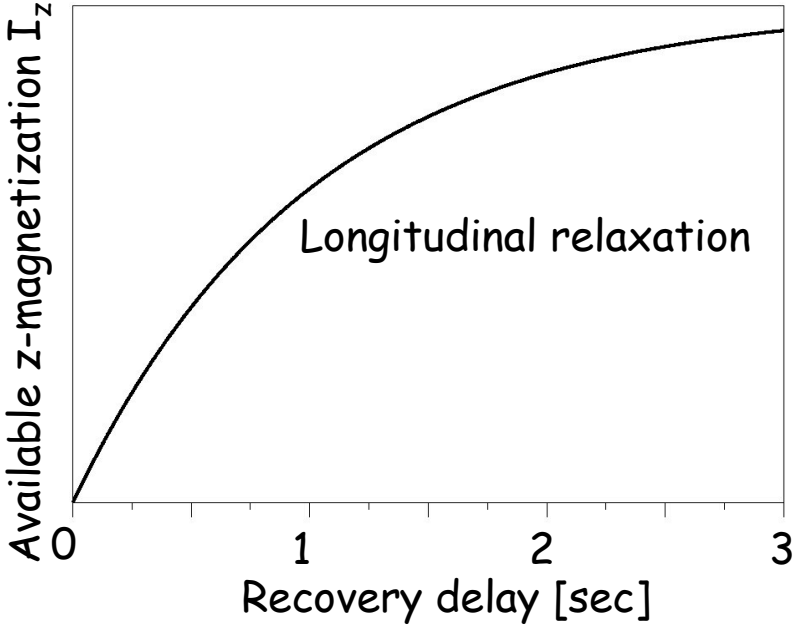
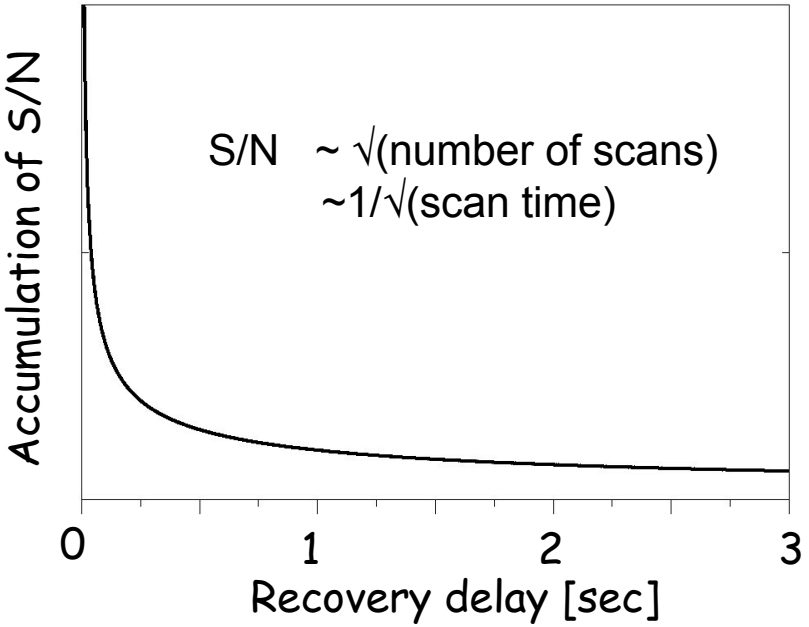


Considerations about sensitivity and interscan delay









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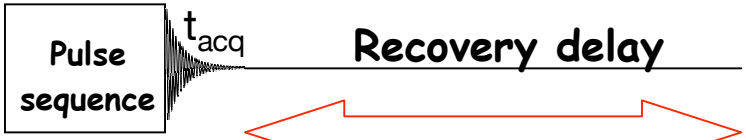

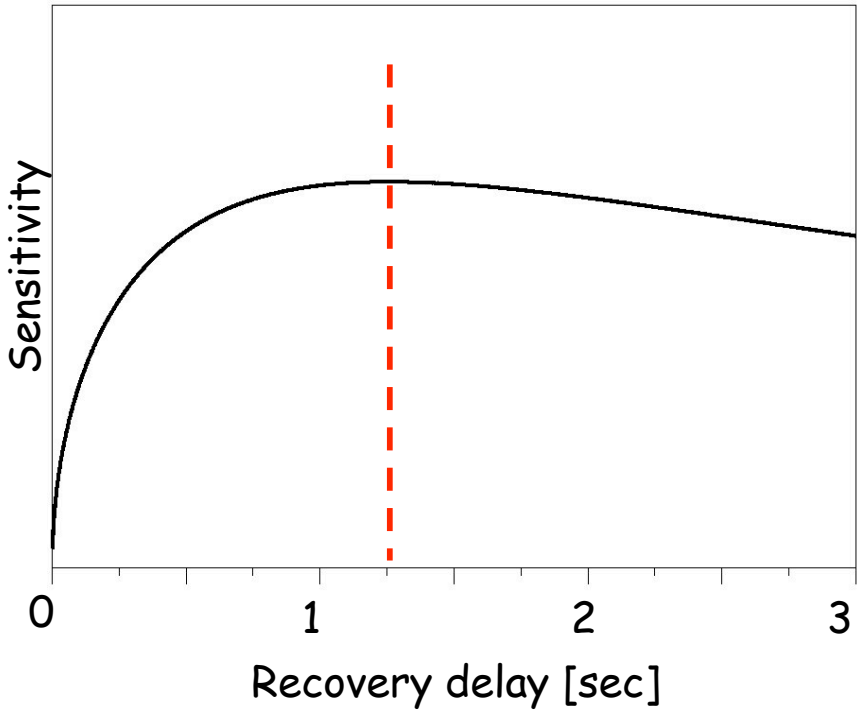

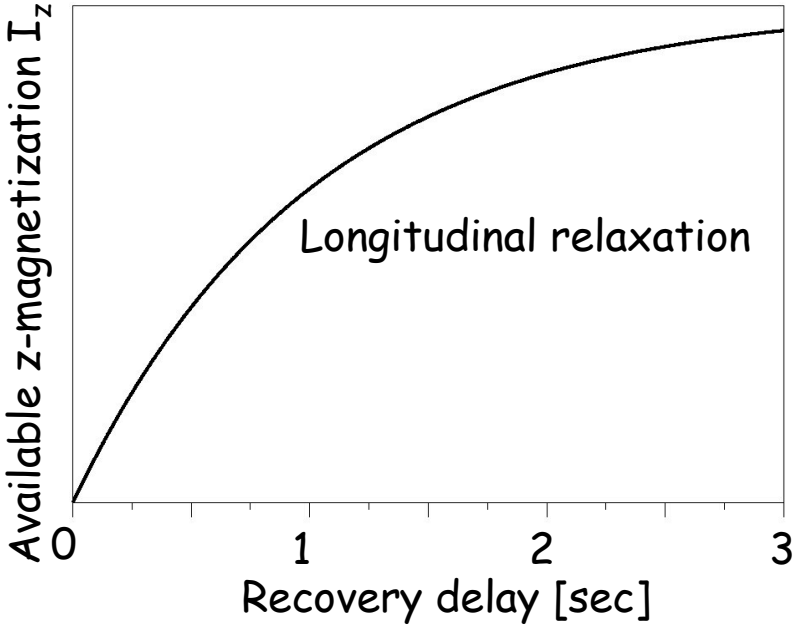

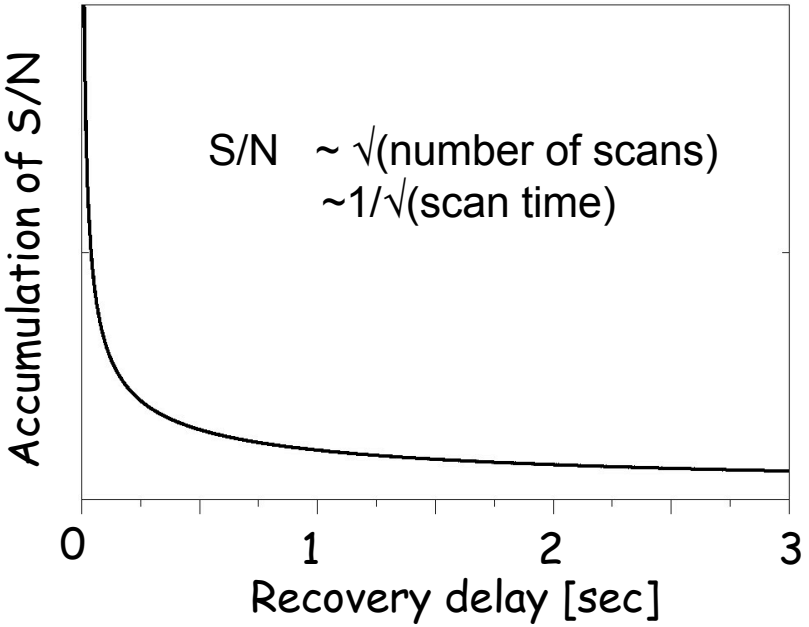

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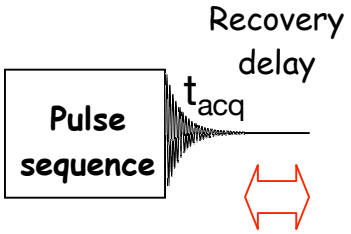

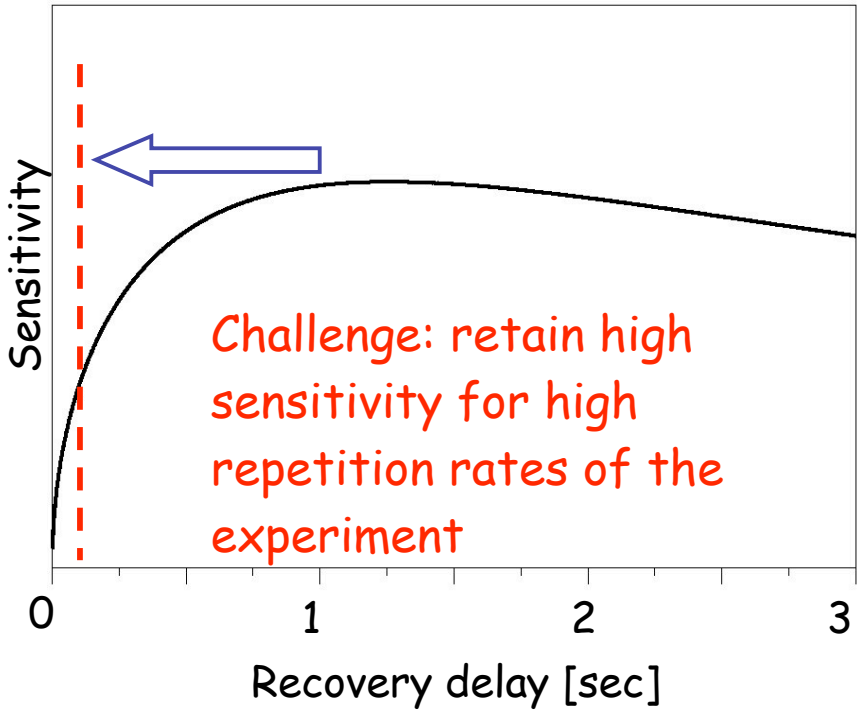

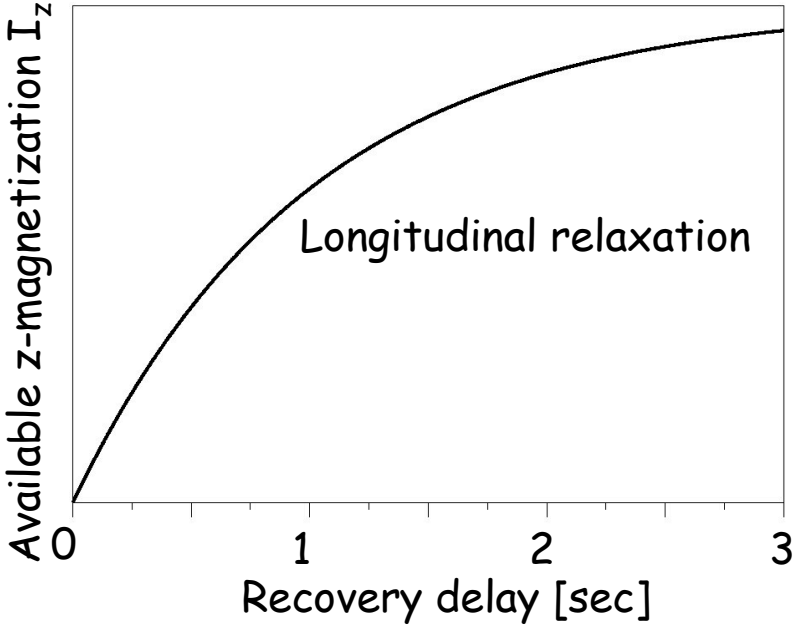

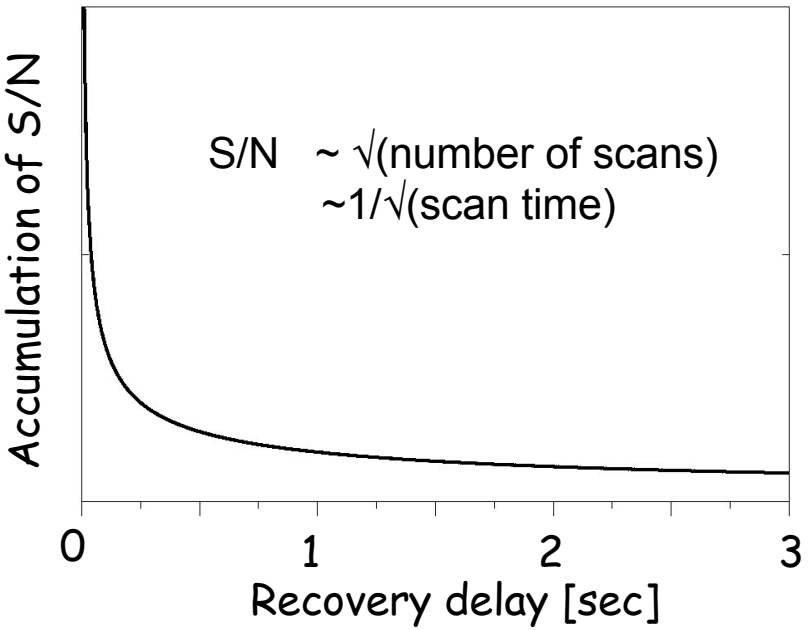

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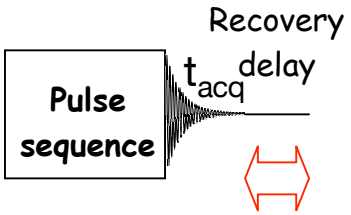
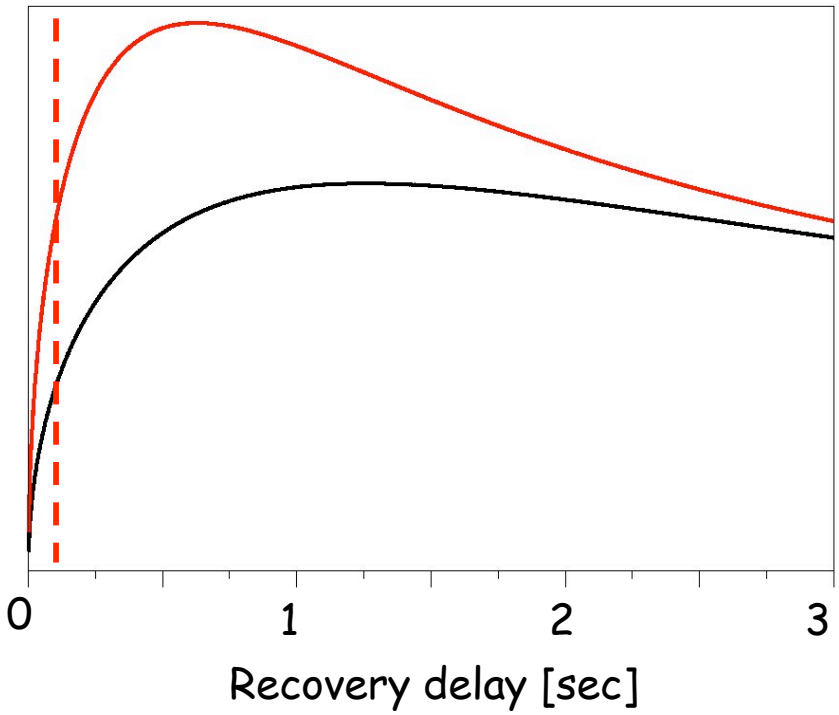
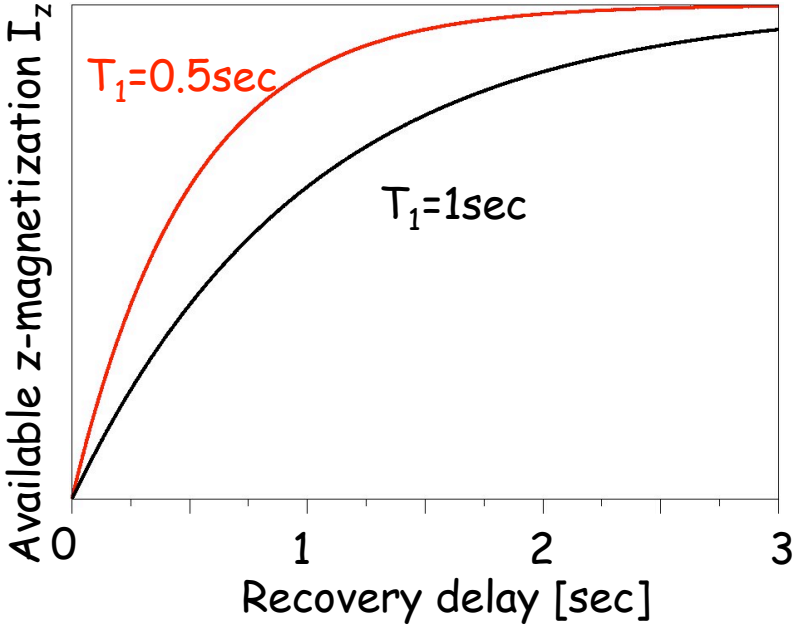
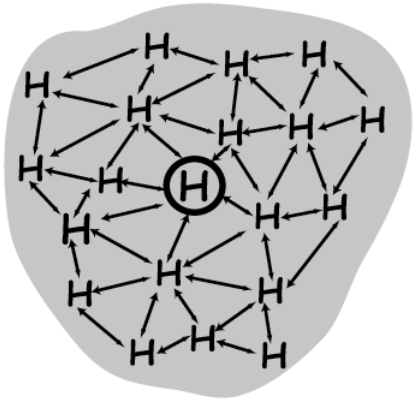






Accelerated longitudinal relaxation allows fast repetition of scans while retaining high sensitivity

Proton longitudinal relaxation in proteins



Most NMR experiments in liquids excite and detect proton spins.

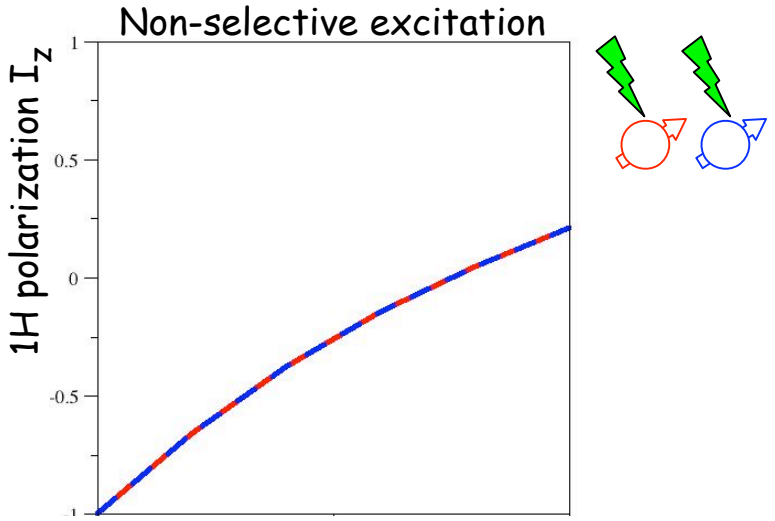
The ^{1}H longitudinal relaxation determines the sensitivity.

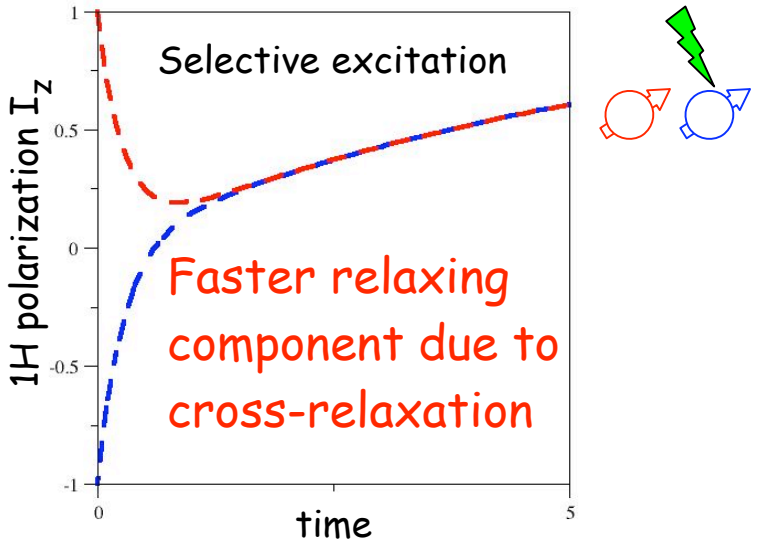
Longitudinal relaxation in a two spin system

$$-\frac{d}{dt}\begin{pmatrix} I_{1z}-I_{1z}^{0} \\ I_{2z}-I_{2z}^{0} \end{pmatrix} = \begin{pmatrix} \rho & \sigma \\ \sigma & \rho \end{pmatrix} \begin{pmatrix} I_{1z}-I_{1z}^{0} \\ I_{2z}-I_{2z}^{0} \end{pmatrix}$$

ρ Auto-relaxation rate constant

σ Cross-relaxation rate constant





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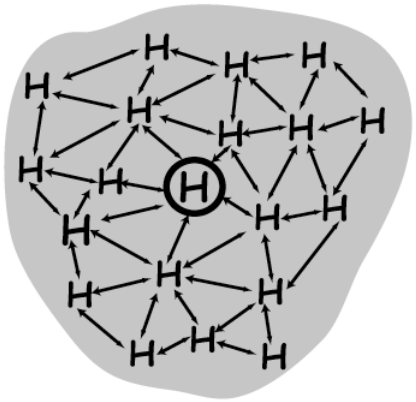


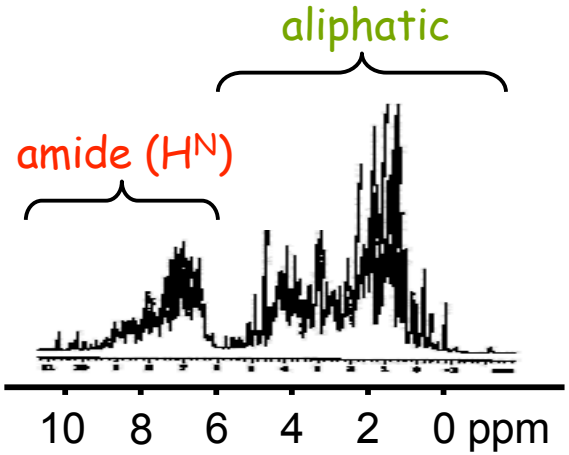
The relaxation in a multi-spin system can be considered as a sum of two-spin interactions.

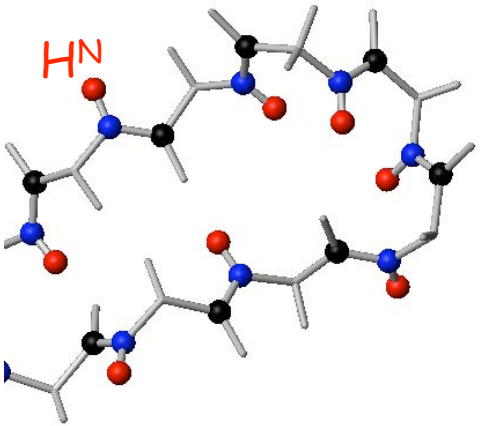
$$-\frac{d}{dt}\begin{pmatrix} I_{1z}-I_{1z}^{0} \\ I_{2z}-I_{2z}^{0} \\ \dots \\ I_{nz}-I_{nz}^{0} \end{pmatrix} = \begin{pmatrix} \Sigma_j\rho_{1j} & \sigma_{12} & \sigma_{13} & \dots & \sigma_{1n} \\ \sigma_{21} & \Sigma_j\rho_{2j} & \sigma_{23} & \dots & \sigma_{2n} \\ & & \dots & & \\ \sigma_{n1} & \sigma_{n2} & \sigma_{n3} & & \Sigma_j\rho_{nj} \end{pmatrix} \begin{pmatrix} I_{1z}-I_{1z}^{0} \\ I_{2z}-I_{2z}^{0} \\ \dots \\ I_{nz}-I_{nz}^{0} \end{pmatrix}$$

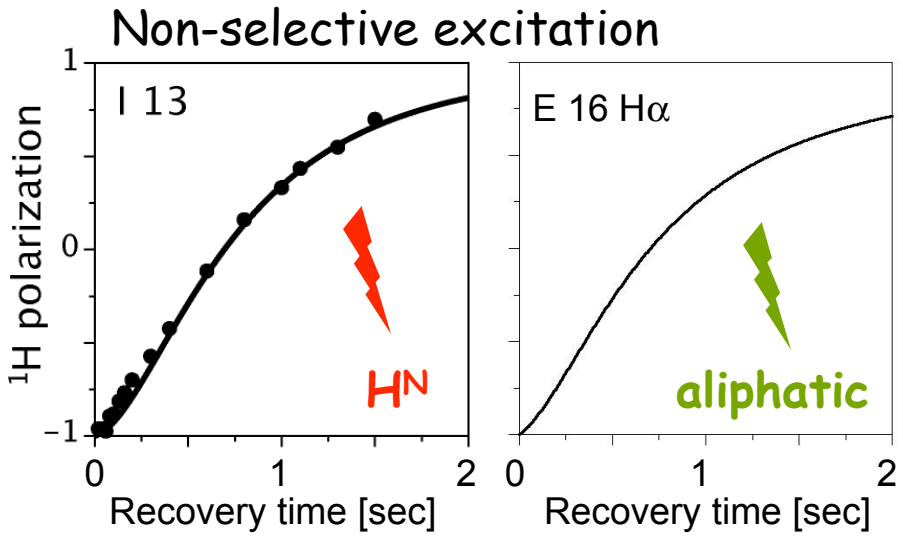
ρ Auto-relaxation rate constant

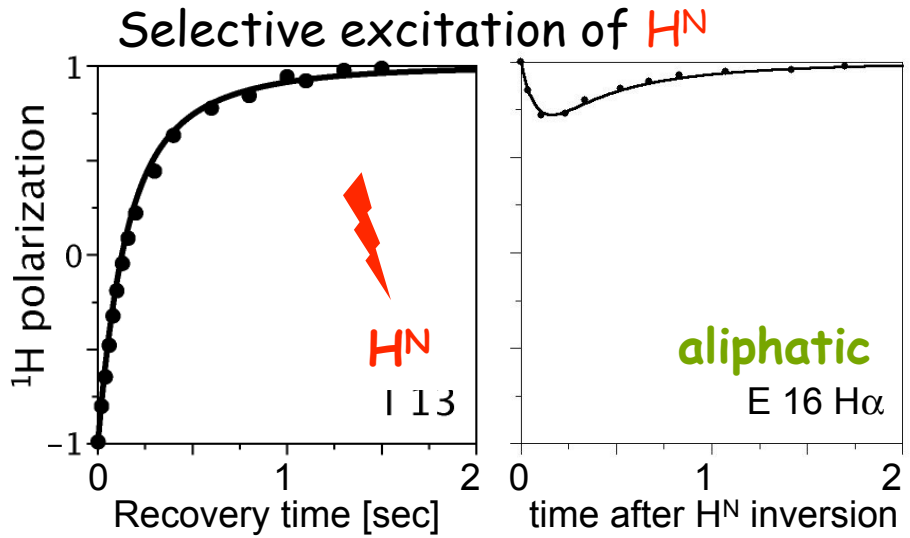
σ Cross-relaxation rate constant

Amide ^{1}H relaxation after non-selective/selective excitation









Outline

Part 1: Methods for fast multidimensional NMR

Concepts

Sensitive fast-pulsing experiments: SOFAST HMQC, BEST experiments

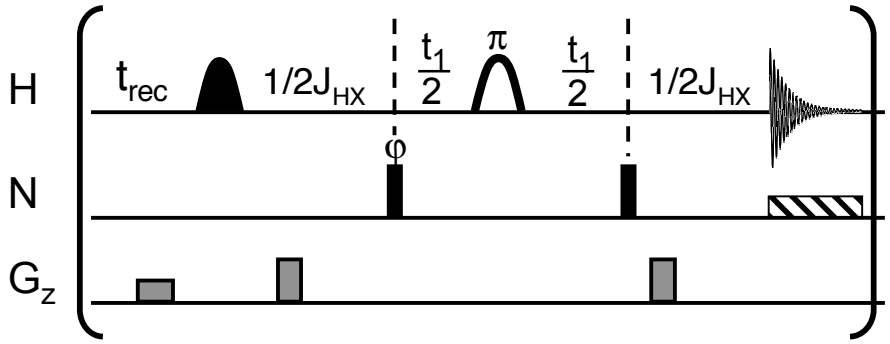
Part 2: Applications

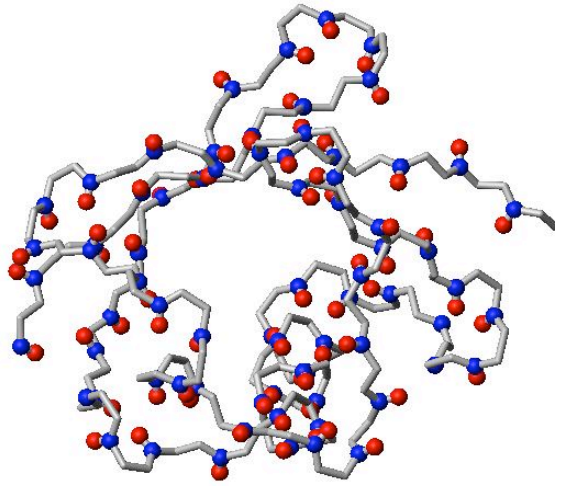
Application to the study of protein folding and unfolding

HET-SOFAST NMR: a fast tool for characterizing protein structure

2D ^{1}H-^{15}N correlation experiment: SOFAST-HMQC

Band-**S**elective **O**ptimized-
Flip-**A**ngle **S**hort-**T**ransient





Properties:

* Selective pulses
* Small number of rf pulses: reduces loss due to B_1 inhomogeneity
* Good water suppression in only one scan

* Schanda & Brutscher, *J. Am. Chem. Soc.* (2005) 127, 8014. Schanda, Kupce & Brutscher, *J. Biomol. NMR* (2005) 33, 199.

^{1}H-^{15}N correlation experiment: SOFAST-HMQC

Band-**S**elective **O**ptimized-
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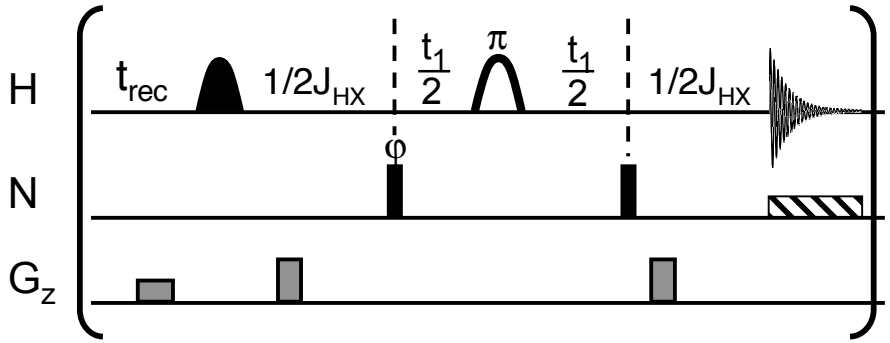

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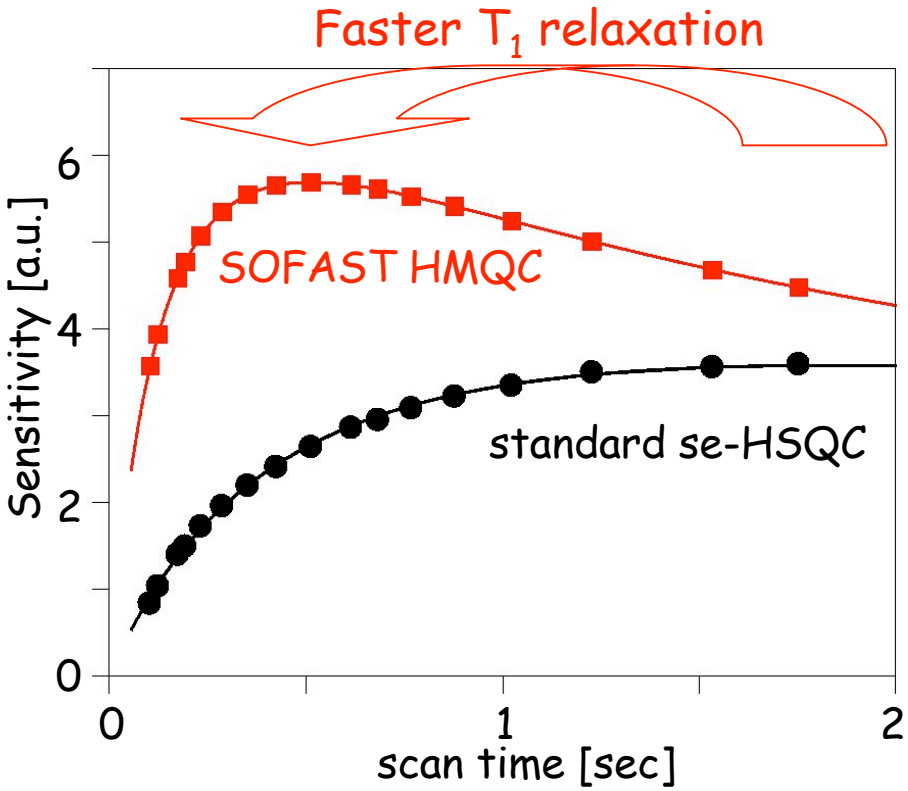
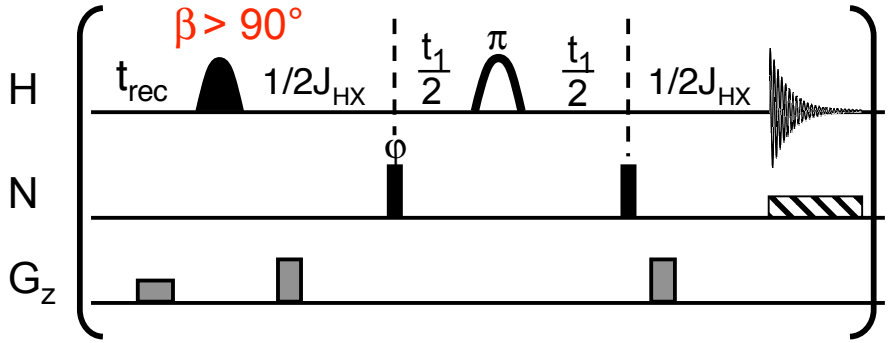


* Schanda & Brutscher, *J. Am. Chem. Soc.* (2005) 127, 8014. Schanda, Kupce & Brutscher, *J. Biomol. NMR* (2005) 33, 199.

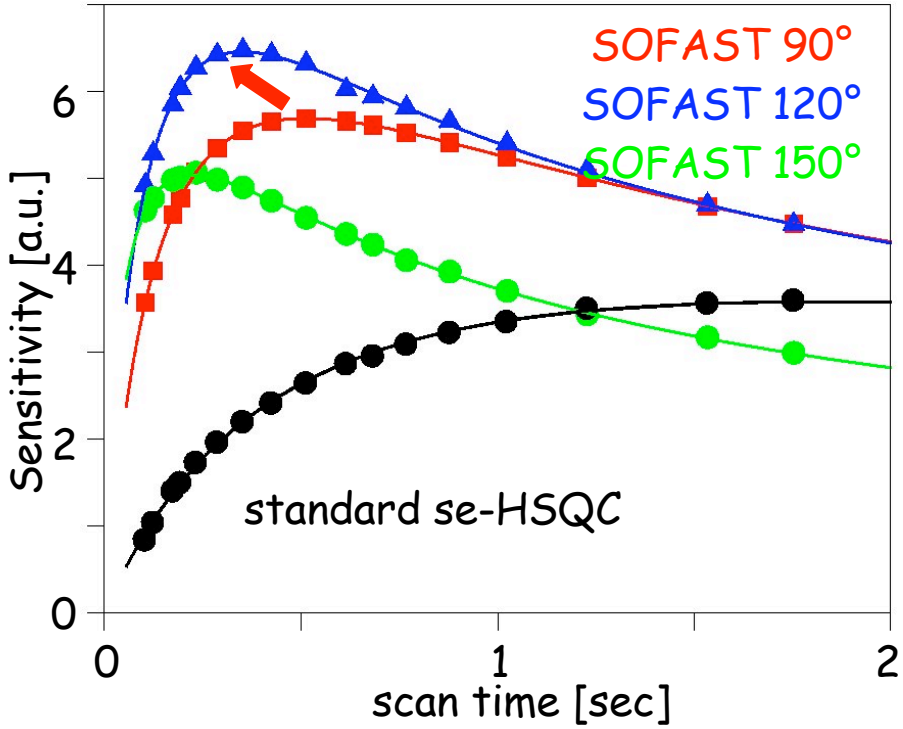
^{1}H-^{15}N correlation experiment: SOFAST-HMQC

Band-**S**elective **O**ptimized-
Flip-**A**ngle **S**hort-**T**ransient



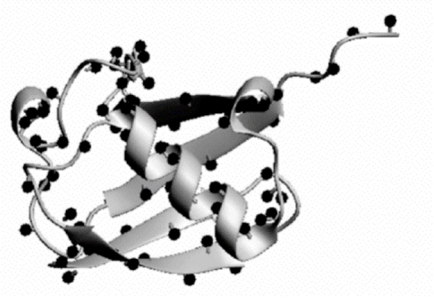
Properties:

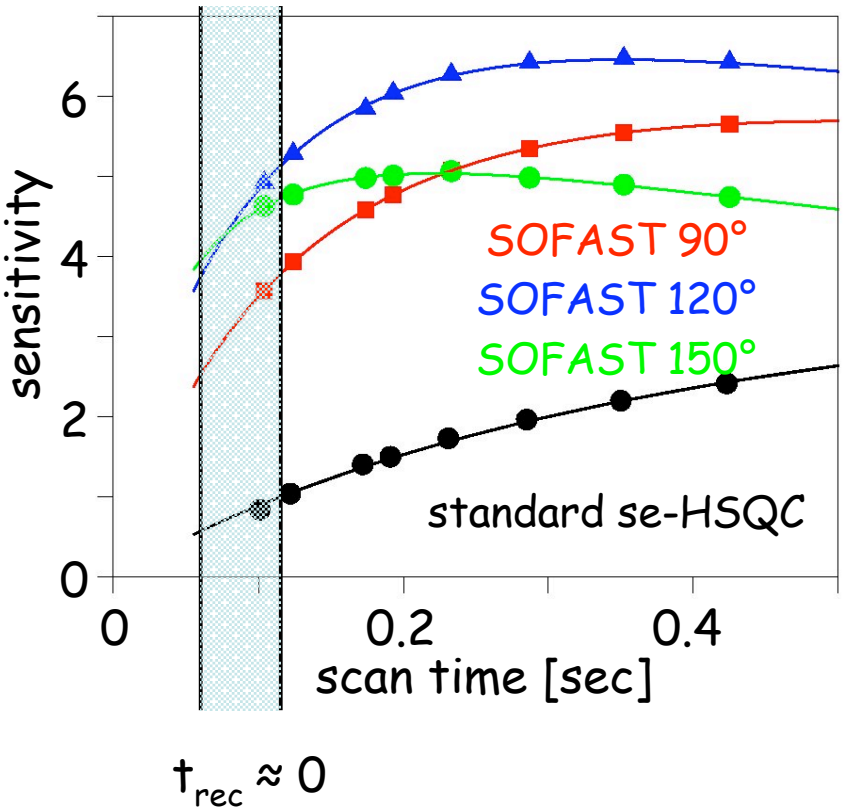
* Selective pulses
* Small number of rf pulses: reduces loss due to B_1 inhomogeneity
* Good water suppression in only one scan
* Partial excitation (Ernst angle)



* Schanda & Brutscher, *J. Am. Chem. Soc.* (2005) 127, 8014. Schanda, Kupce & Brutscher, *J. Biomol. NMR* (2005) 33, 199.

Fast-pulsing regime



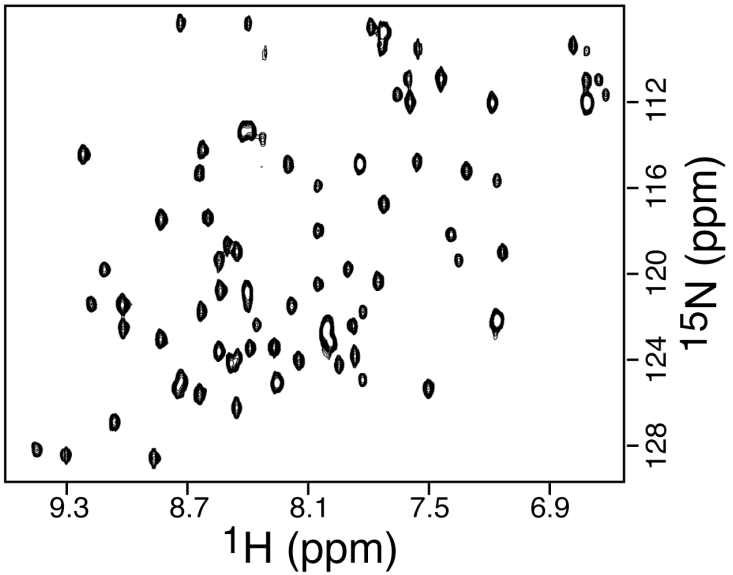


^{15}N-ubiquitin (8.6 kDa, 25°C)

800 MHz, coldprobe

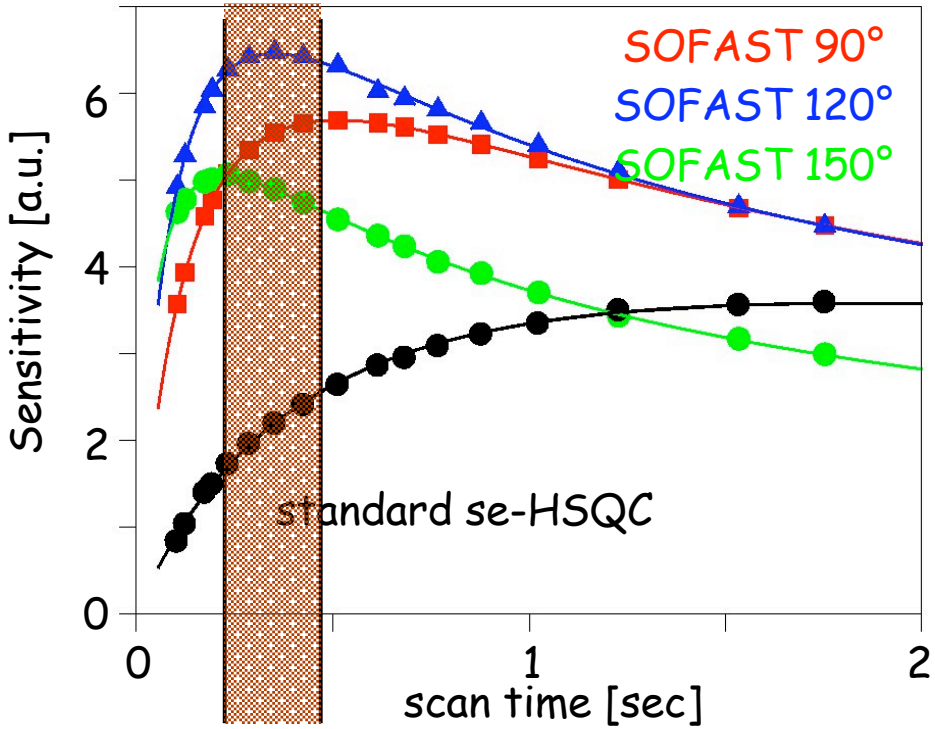
concentration: **~ 0.2 mM**

Exp. time: 4 seconds



140°- SOFAST

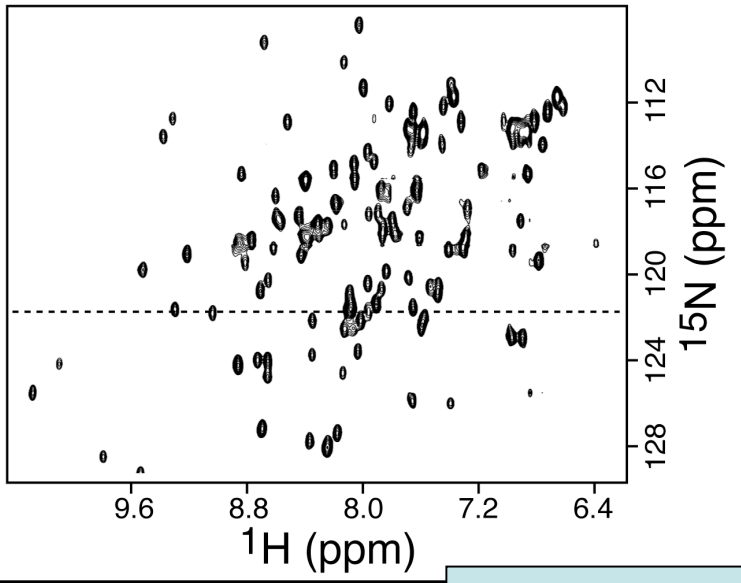
Optimal-sensitivity regime



$t_{rec} \approx 0.2\text{-}0.3$ sec

SOFAST-HMQC performs best in terms of absolute sensitivity

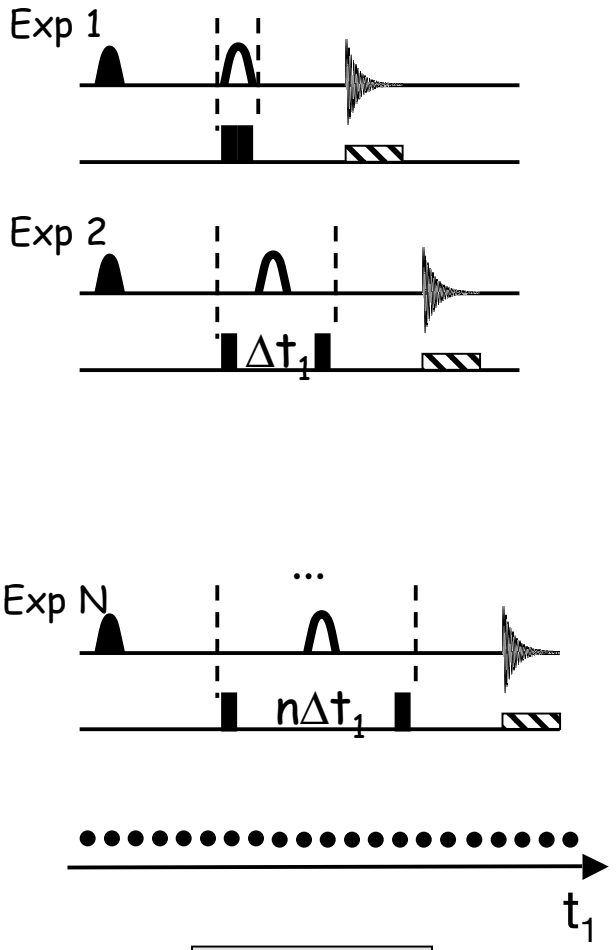
α-lactalbumin (14 kDa, 25°C)
800 MHz, coldprobe
no ^{15}N enrichment, **~ 4.0 mM**
(~ 10 μM ^{15}N-labeled protein**)**
Exp. time: 1 hour



120°- SOFAST

Can we go even faster ?
Combination with alternative encoding schemes

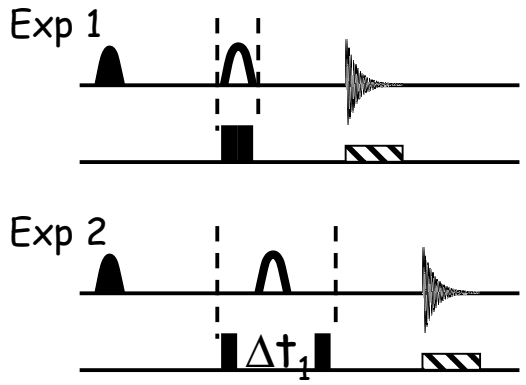
standard ^{15}N labeling



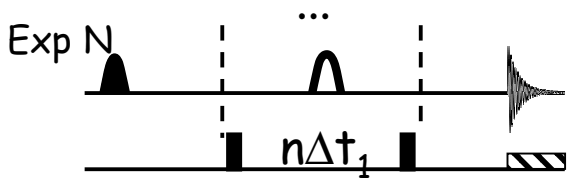
N ≈ 100

Can we go even faster ?
Combination with alternative encoding schemes

standard ^{15}N labeling

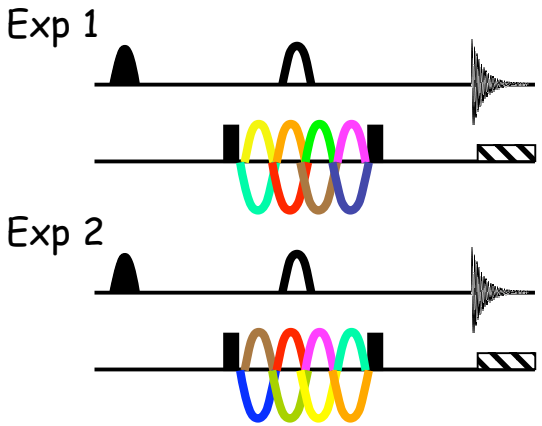


Exp N

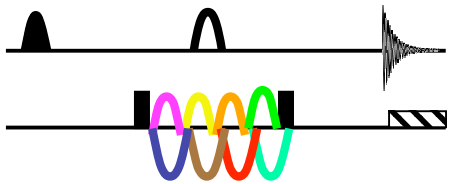


N ≈ 100

Hadamard ^{15}N labeling

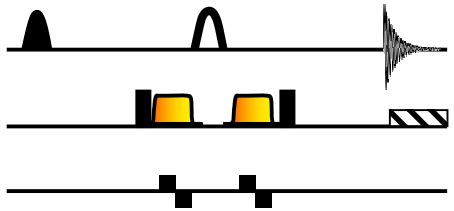


Exp N



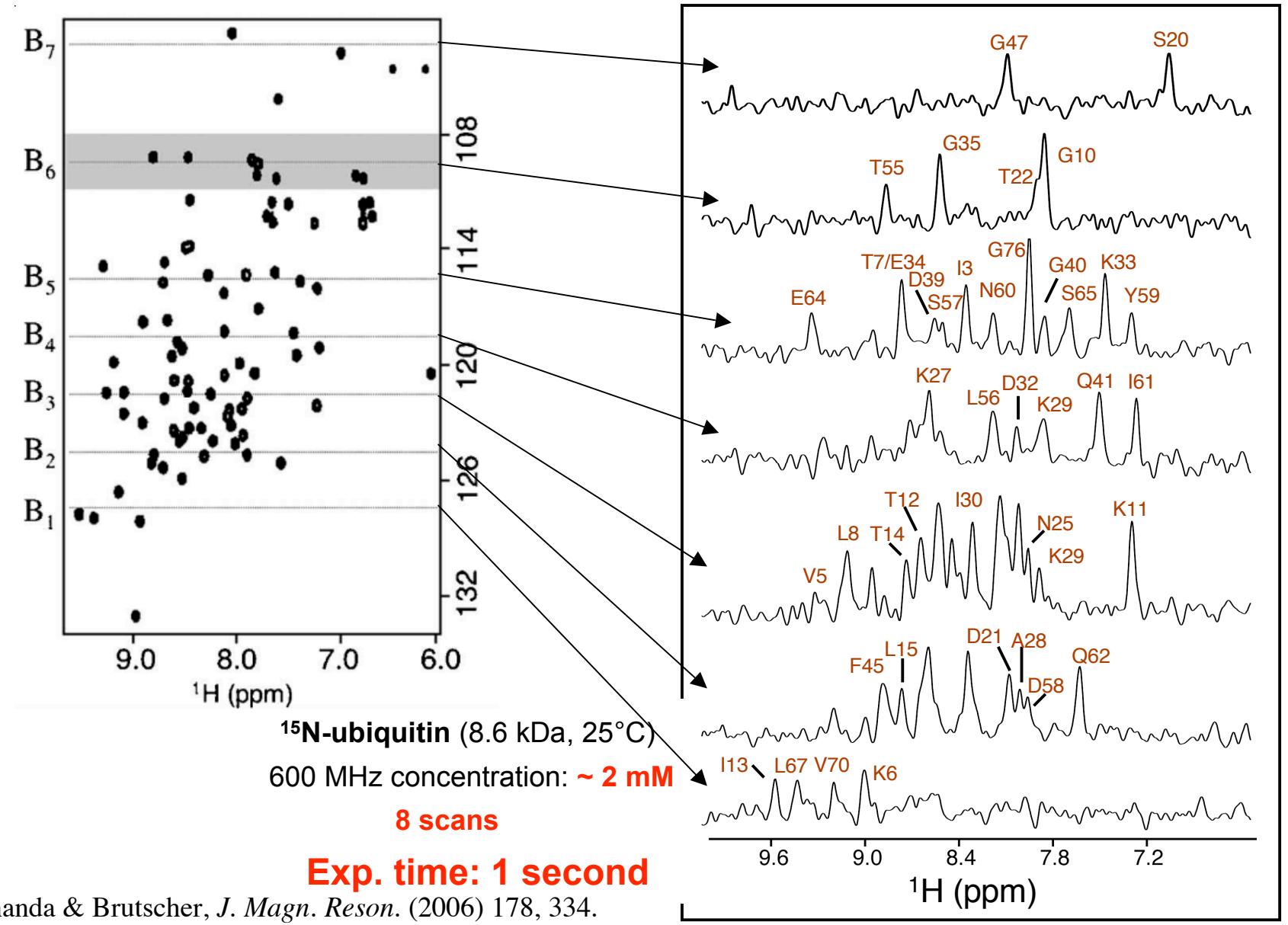
N = 4,8,12,...

Spatial ^{15}N labeling



N = 1 - 4

Hadamard encoded SOFAST HMQC



^{15}N-ubiquitin (8.6 kDa, 25°C)

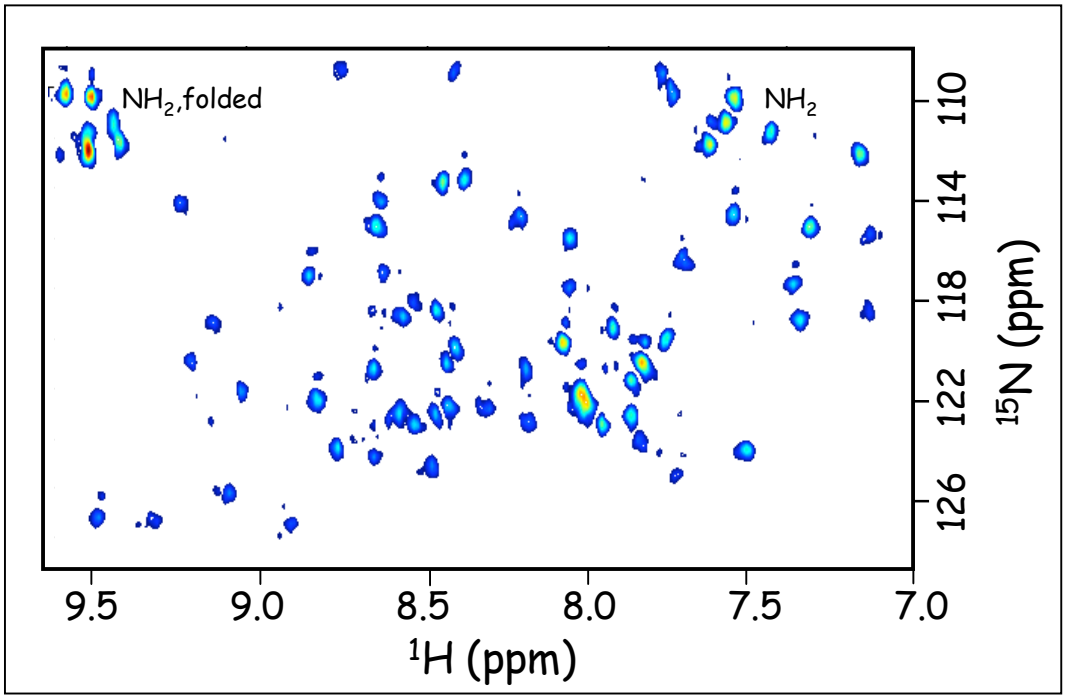
600 MHz concentration: **~ 2 mM**

8 scans

Exp. time: 1 second

Schanda & Brutscher, *J. Magn. Reson.* (2006) 178, 334.

Spatially-encoded SOFAST-HMQC



Total acquisition time:
(8 interleaved scans)
1.4 seconds

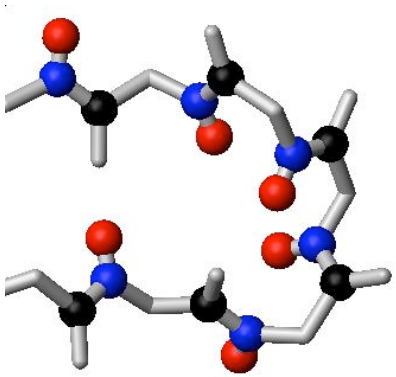
3.4 mM ubiquitin
25°C, pH 6.0
800 MHz,
standard probe

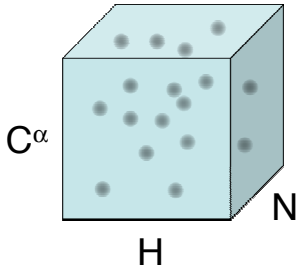
Gal, Schanda, Brutscher & Frydman,
J. Am. Chem. Soc. (2007)129(5):1372-7

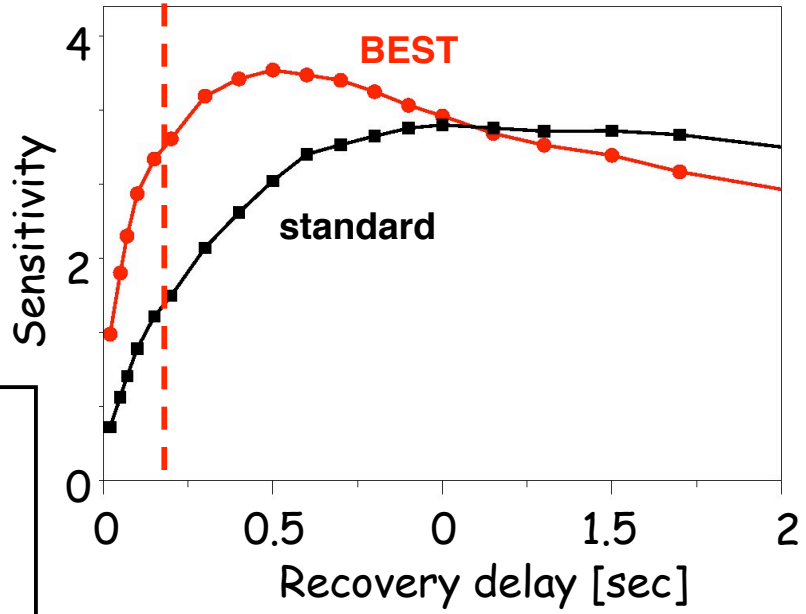
Extension to 3D triple resonance experiments:

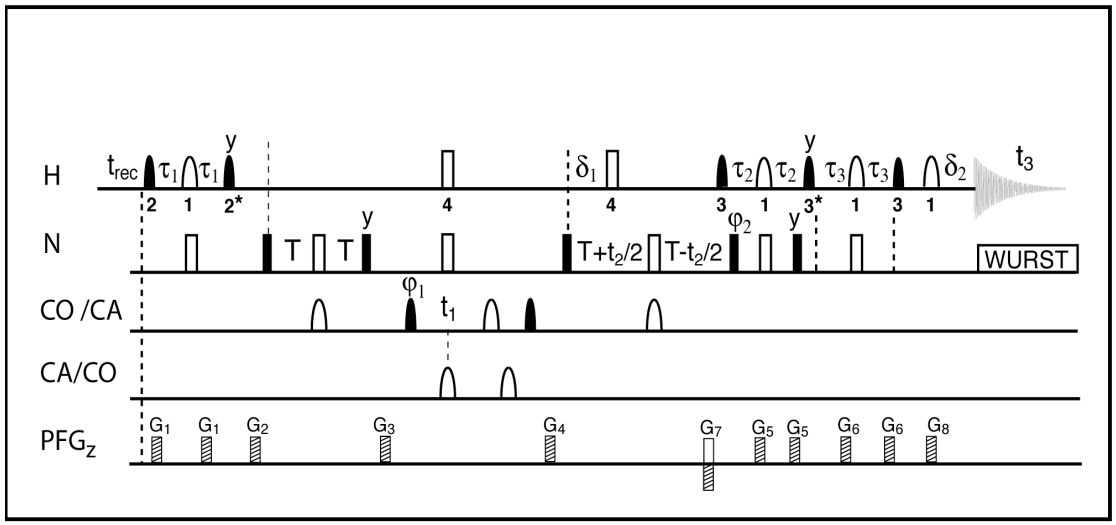
Band-selective Excitation Short Transient Experiments

Example: 3D HNCA

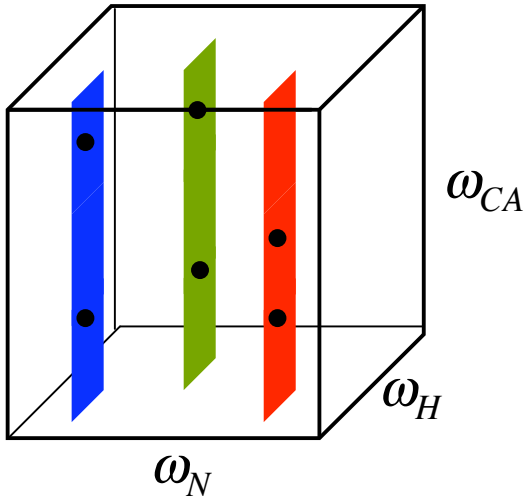


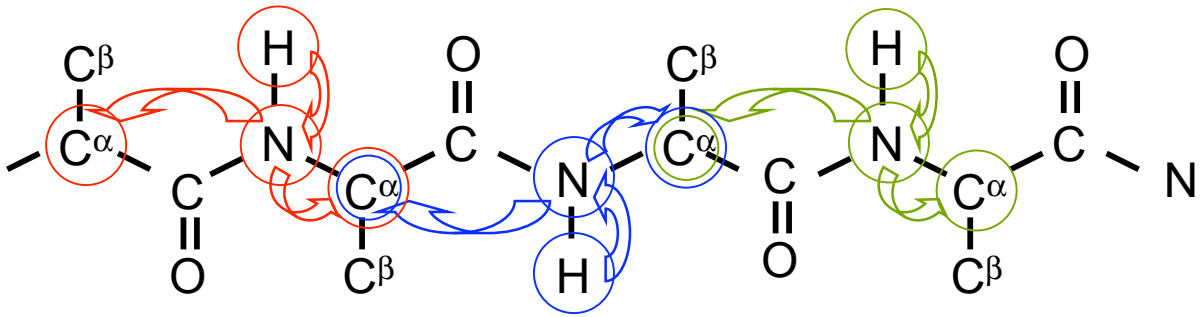




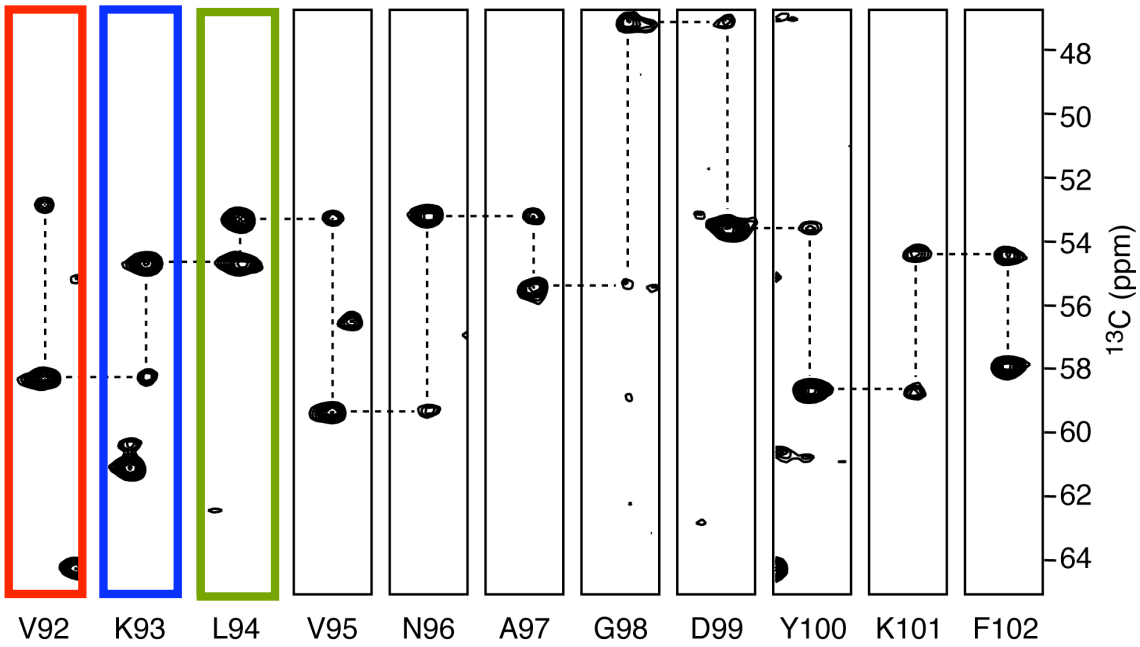


3D HNCA experiment for resonance assignment in a few minutes





H-CA strips extracted from a 3D-BEST HNCA spectrum recorded in **15 min**.



SiR-FP18 (18 kDa)
1.8 mM, 600 MHz,
standard probe

Outline

Part 1: Methods for fast multidimensional NMR

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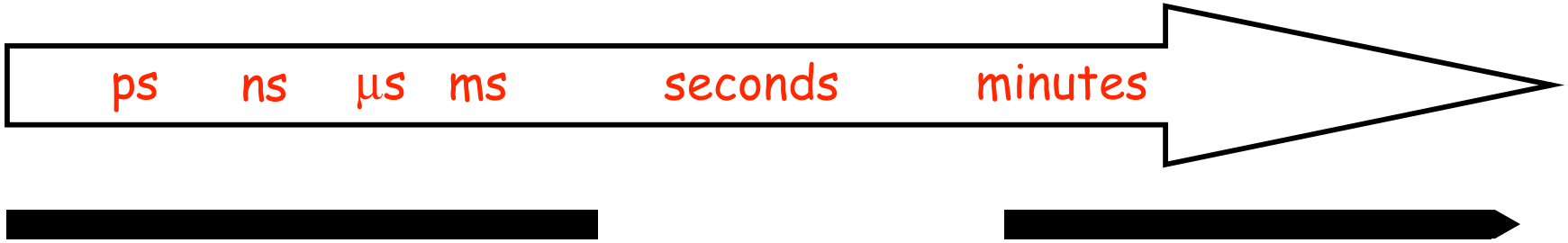
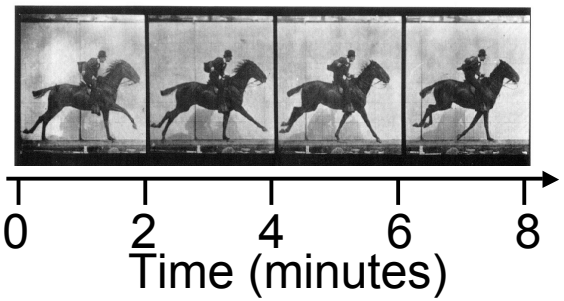
Application to the study of protein folding and unfolding

HET-SOFAST NMR: a fast tool for characterizing protein structure

Dynamics in proteins studied with atomic resolution by NMR



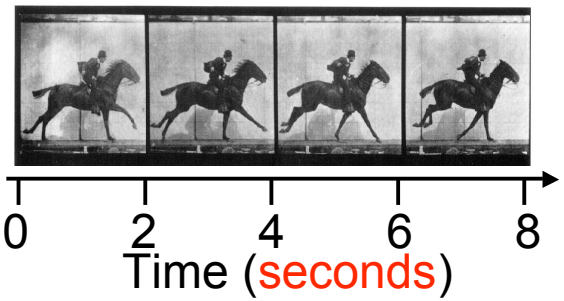


Dynamics in proteins studied with atomic resolution by NMR

Steady-state NMR

Standard real-time NMR

SOFAST real-time 2D NMR



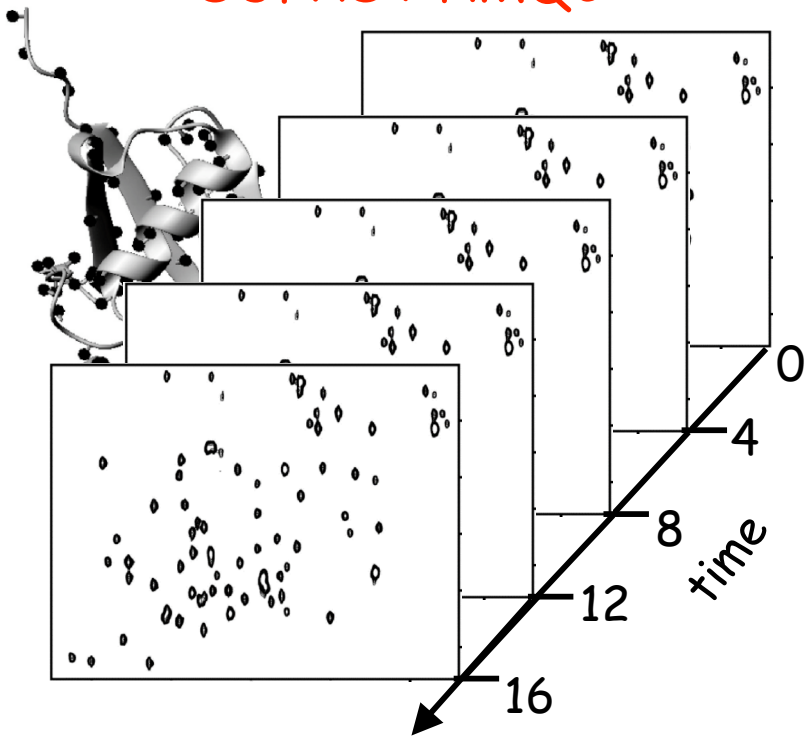
Protein (un)folding, H/D exchange,...

SOFAST real-time 2D NMR

Required tools

Fast data acquisition

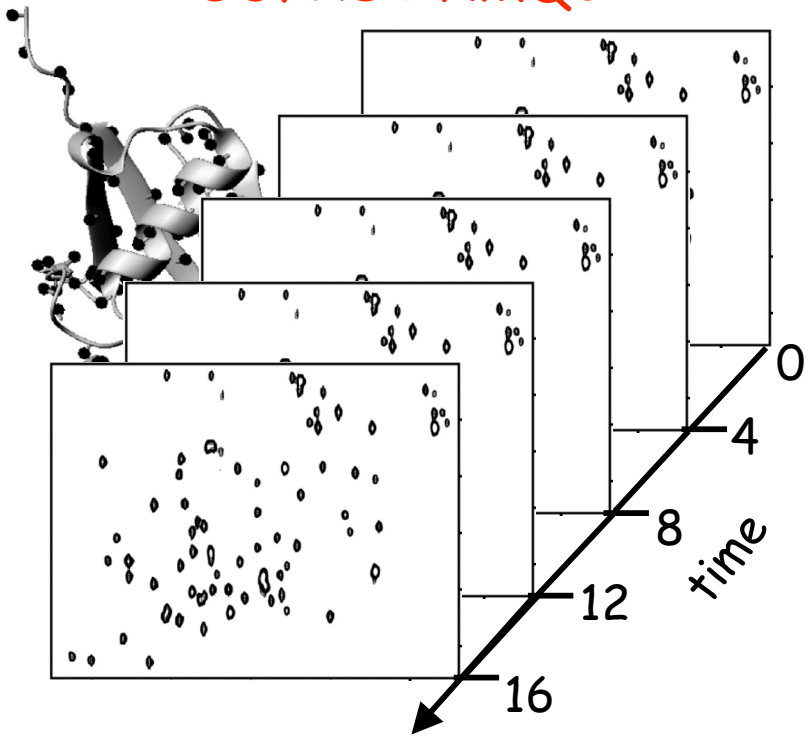
SOFAST HMQC



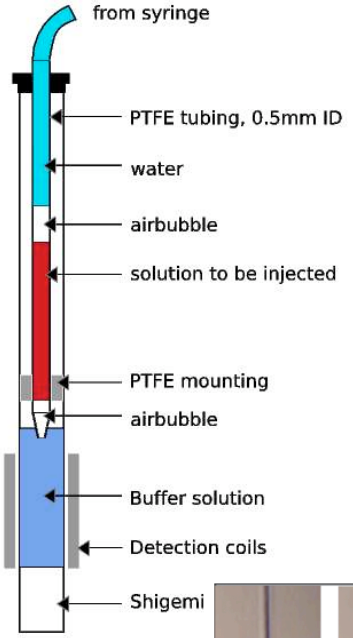
SOFAST real-time 2D NMR

Required tools

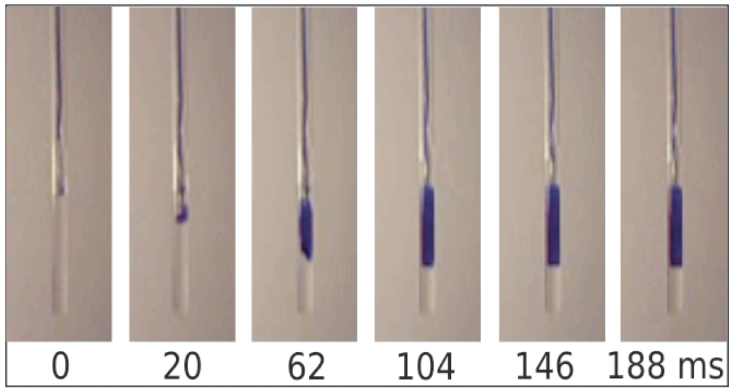
Fast data acquisition
SOFAST HMQC



Initiation of the kinetic event

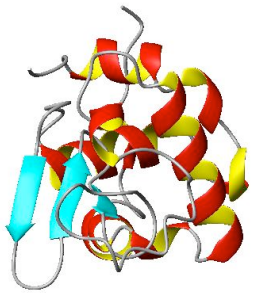


Fast mixing inside the spectrometer within ca. 100 ms



Application I

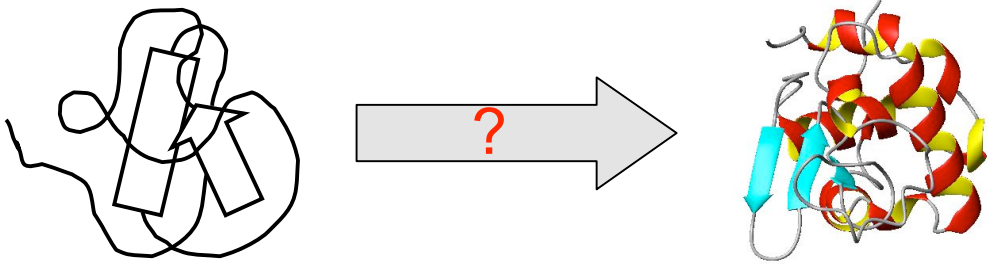
Folding of α-lactalbumin followed in real time



α-lactalbumin 14 kDa
Native state pH 8

Application I

Folding of apo α-lactalbumin followed in real time



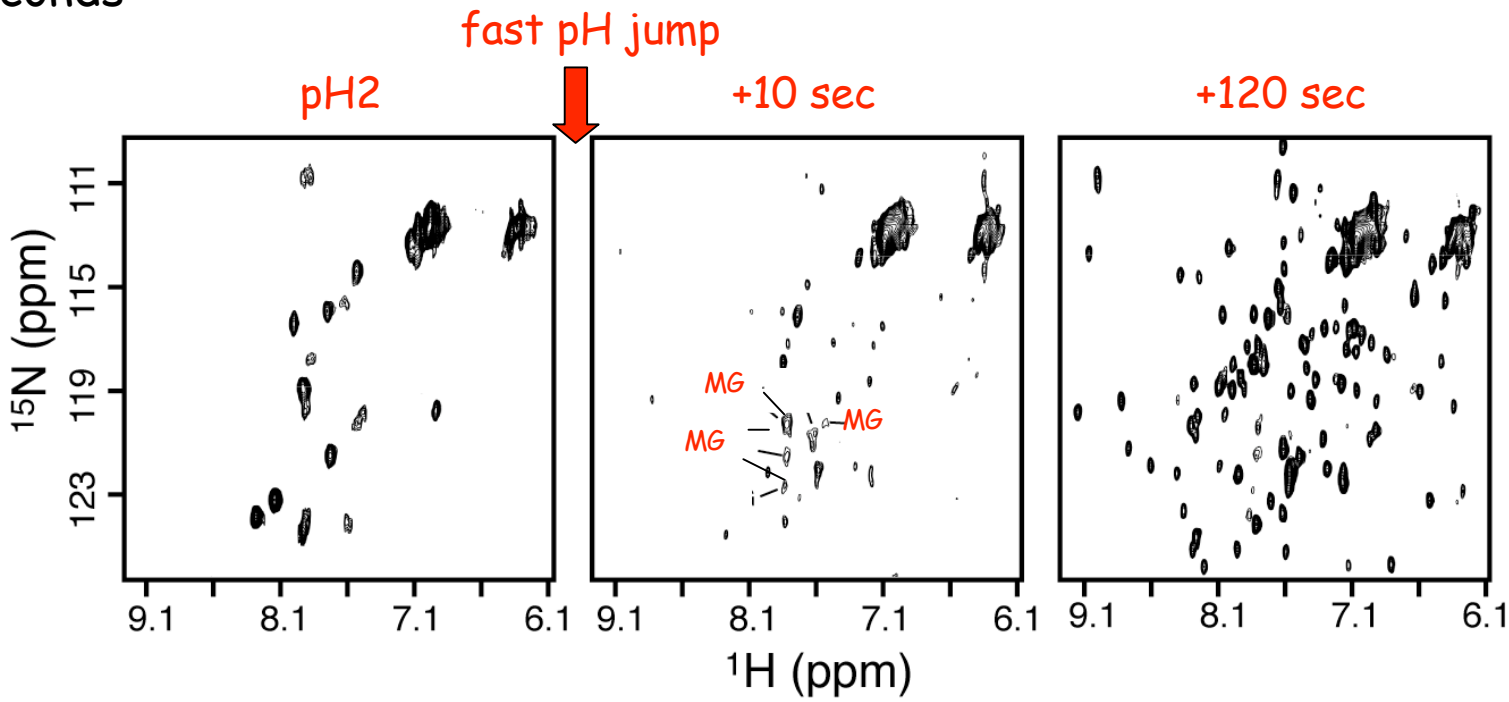
"Molten Globule"
pH 2

α-lactalbumin 14 kDa
Native state pH 8

Application I

Folding of apo α-lactalbumin followed in real time

2D snapshots of the
folding process
every 10 seconds



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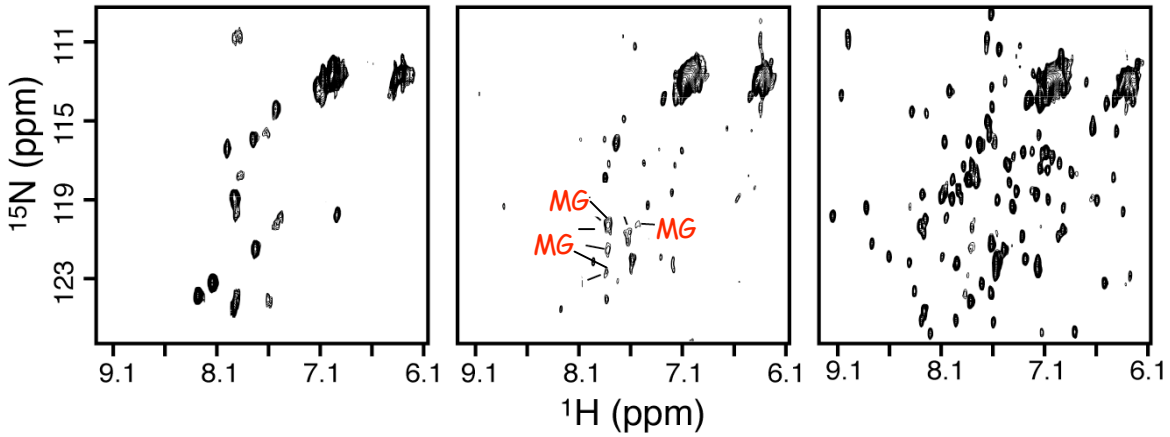
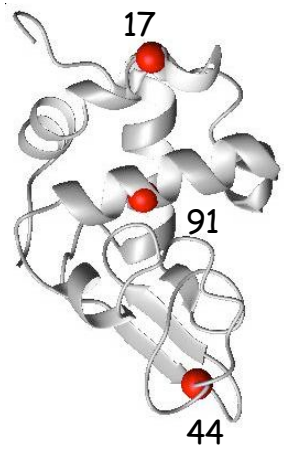
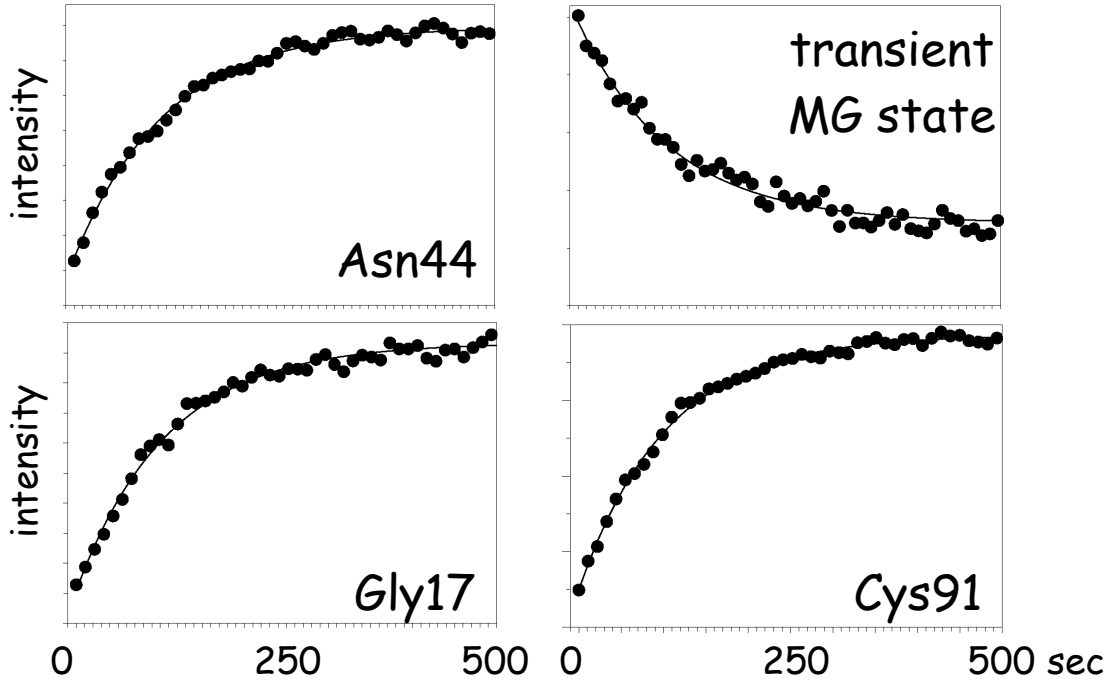
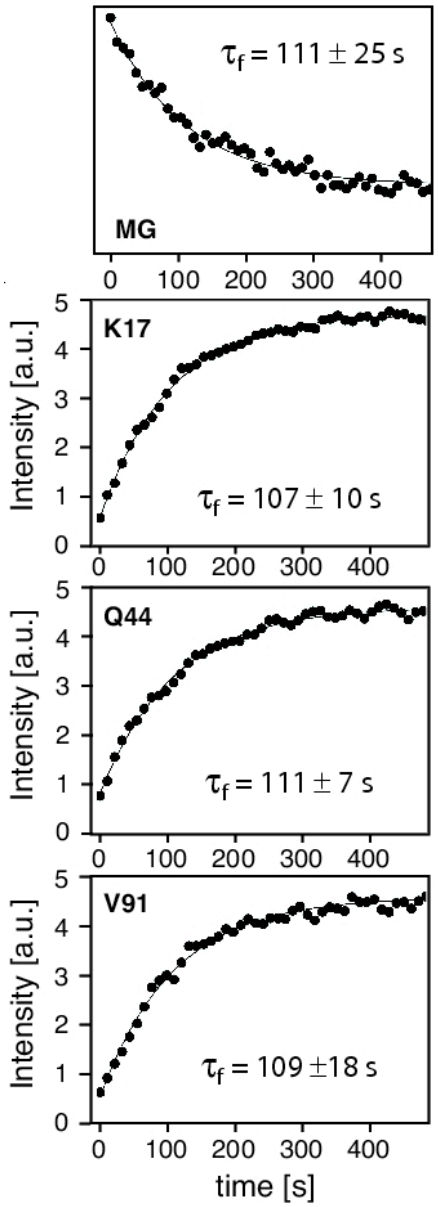


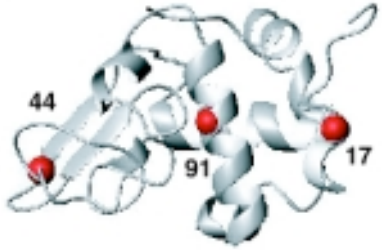


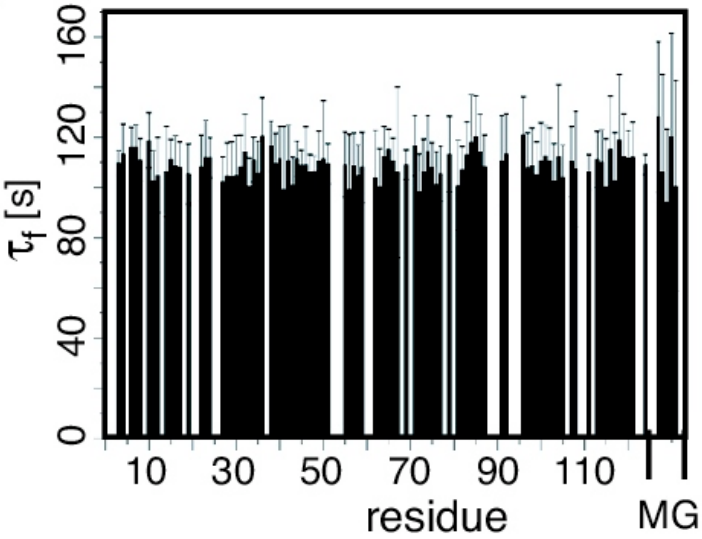


Application I

Folding of apo α-lactalbumin followed in real time







No intermediate peaks observed

Same rate of disappearance of molten globule state and appearance of native state

Conclusion:

A single transition state ensemble controls folding

more folding...

Folding of an amyloidogenic protein

Unfolded → Intermediate(s) ? → Folded

↓ ?

Fibrils

Work in progress

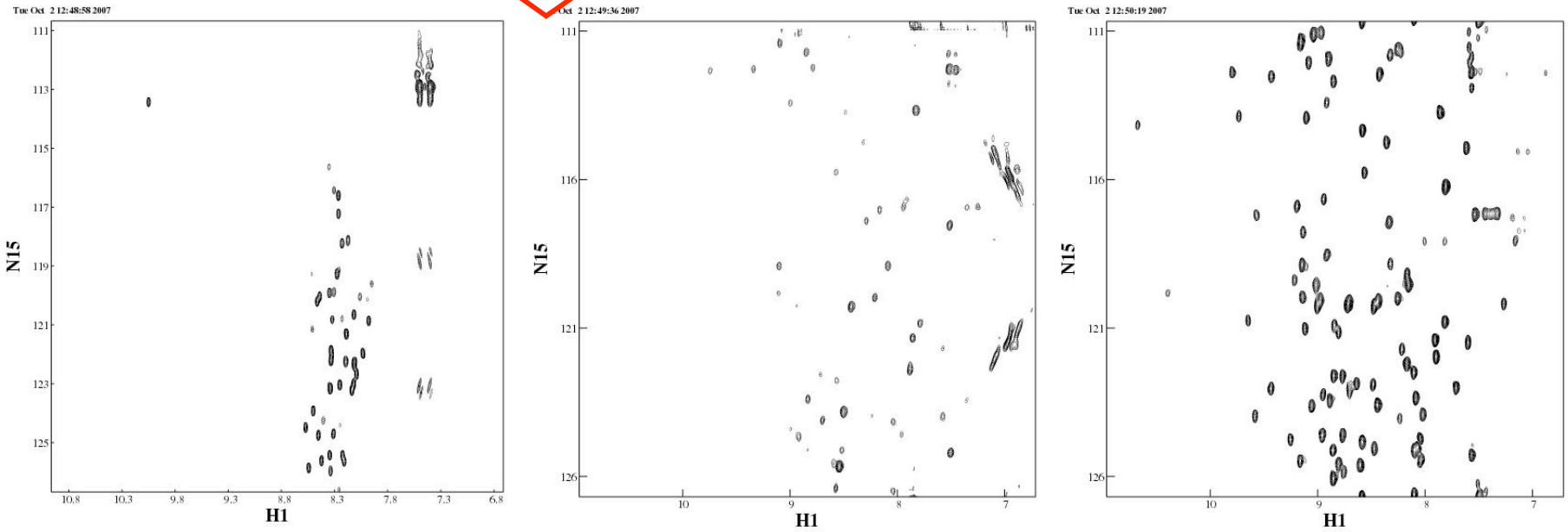
SOFAST-HMQC spectra recorded during refolding every 15 sec

pH jump

Unfolded, pH 2

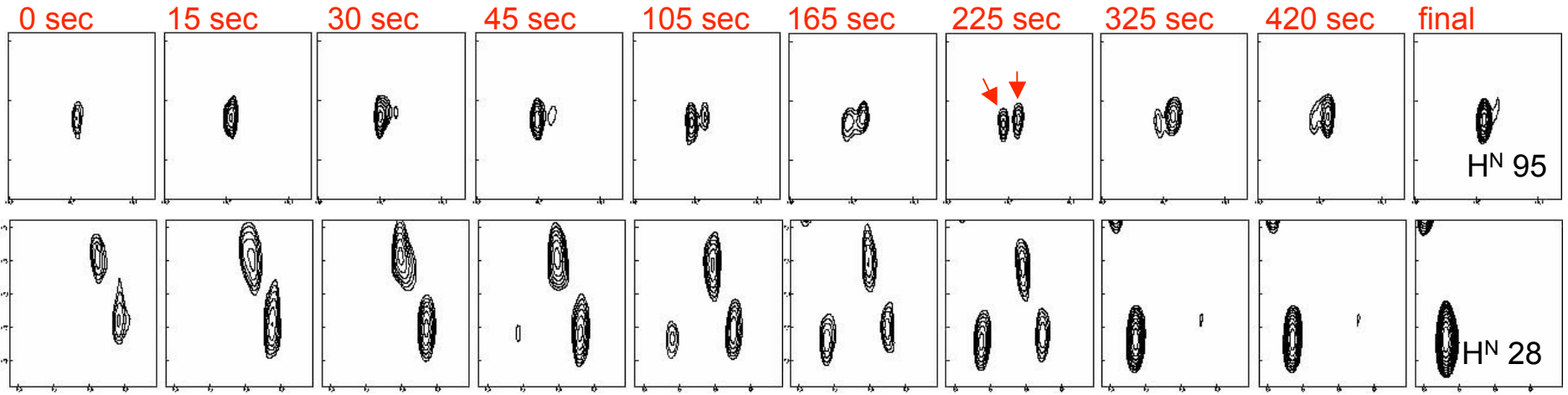
t=0 seconds

Final spectrum



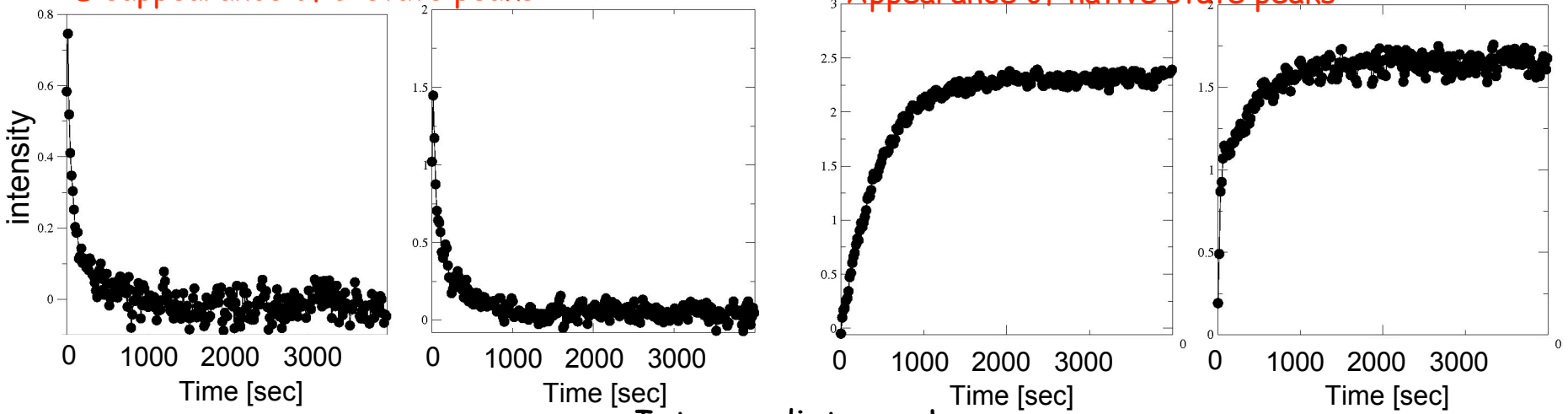
more folding...

Folding of an amyloidogenic protein



Disappearance of I-state peaks

Appearance of native state peaks



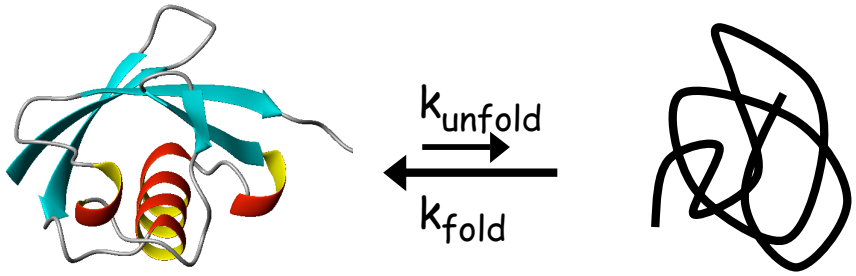
Intermediate peaks

Multiexponential intensity changes

Site-specific analysis of kinetic behavior will reveal nature of these states.

Application II

(Un)folding under native conditions studied by H/D exchange

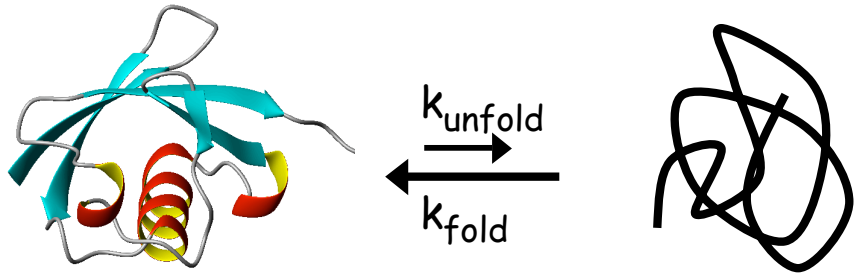


folded

Unfolded
minor population
"NMR-invisible"

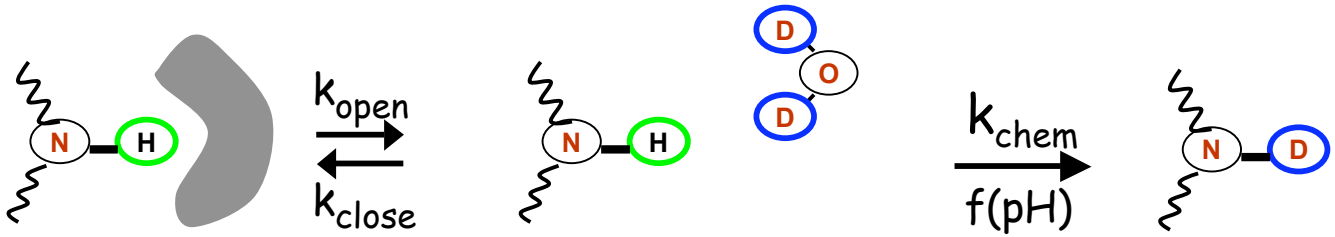
Application II

(Un)folding under native conditions studied by H/D exchange



folded

Unfolded
minor population
"NMR-invisible"



protected
(folded)

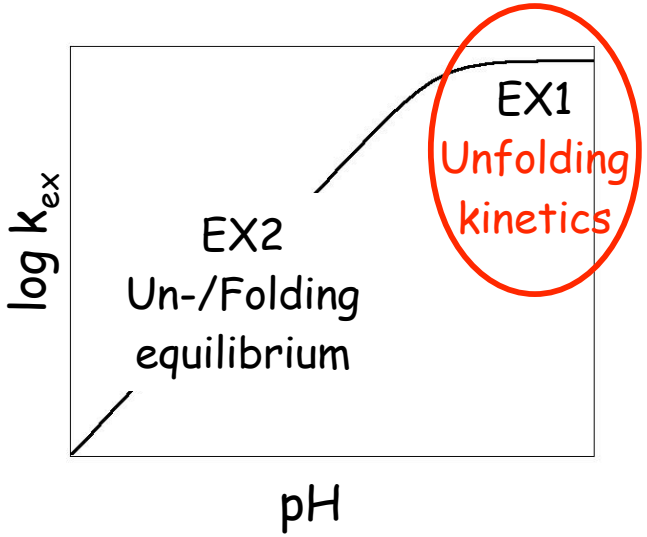
unprotected
(exchange competent)

exchanged

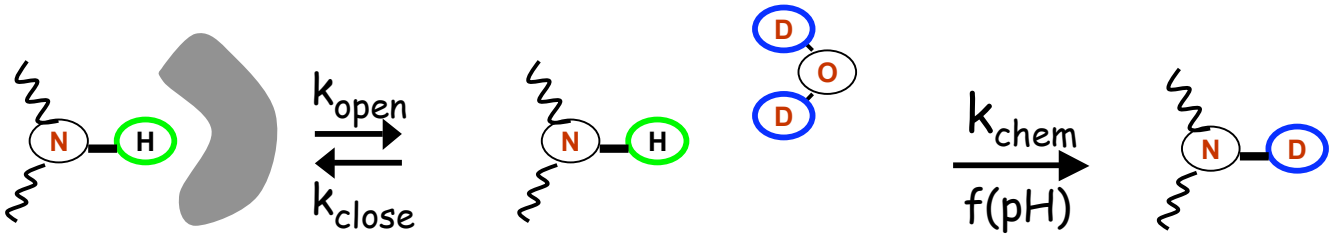
H/D exchange gives access to the "invisible" manifold of partially unfolded conformations under native conditions

Application II

Fast H/D exchange gives insight into unfolding



At high pH the observed exchange rates directly reflect (partial) unfolding rates



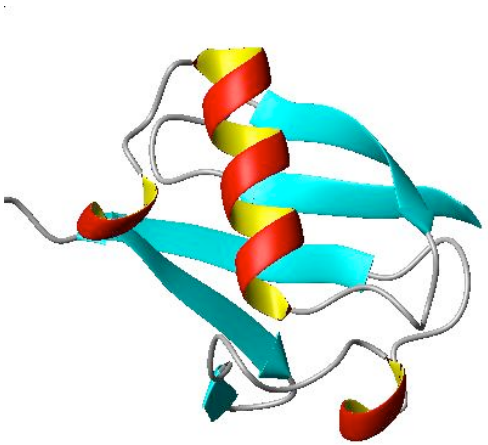
protected (folded)

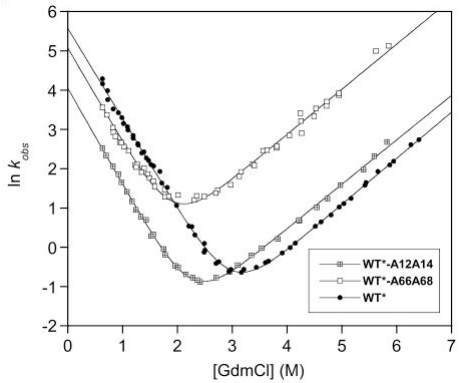
unprotected (exchange competent)

exchanged

Application II

Unfolding of ubiquitin studied by EX1 H/D exchange



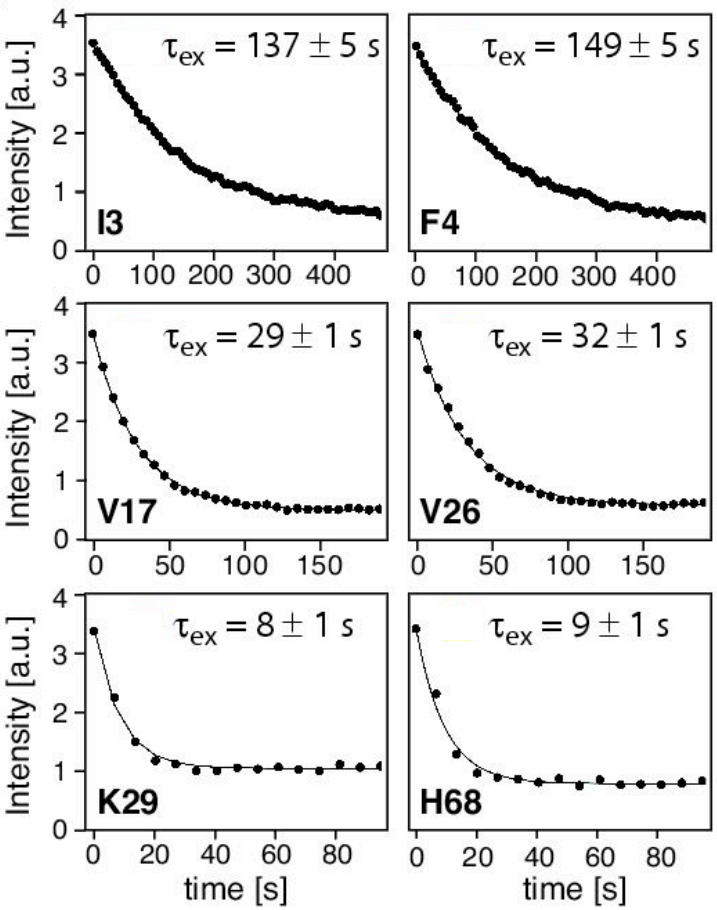


Global unfolding rate determined from fluorescence Chevron plots:

k_{NU}=0.0084 s^{-1} τ_{NU}=119 s

Bofill, R., ..., Searle, M., *J. Mol. Biol.* (2005) 349, 205

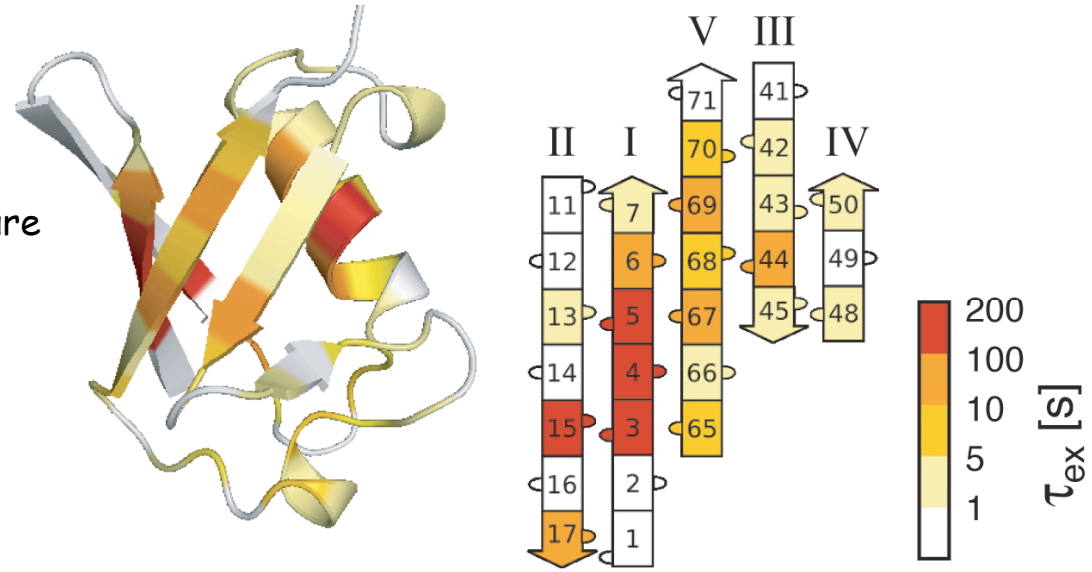
SOFAST HMQC-based H/D exchange experiments on 0.7mM ubiquitin at pH 12, 298 K



Application II

Unfolding of ubiquitin studied by EX1 H/D exchange

EX1 H/D exchange rates plotted on ubiquitin's structure

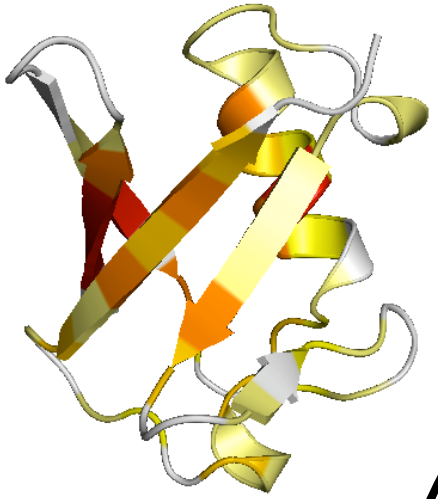


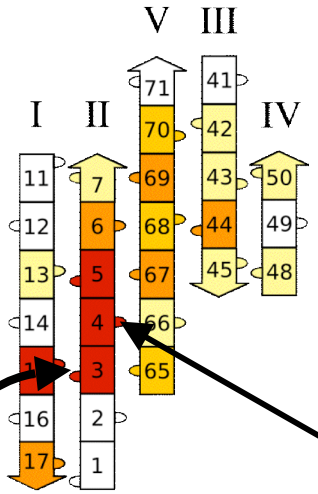
* Unfolding in ubiquitin is a heterogeneous process
* Partial unfolding of the C-terminal face of the β-strand is faster
* Gradual decrease of unfolding rates
* The globular unfolding observed by fluorescence appears to be the unfolding of the N-terminal "folding nucleus"

Application II

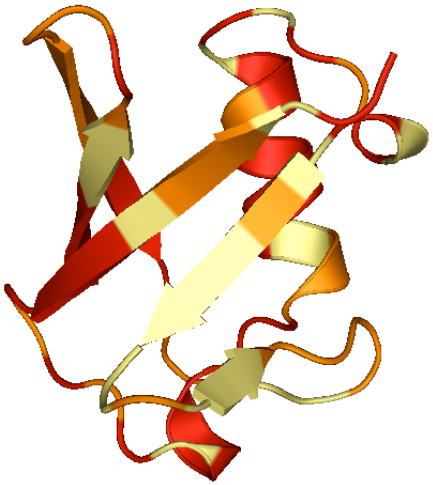
Heterogeneous unfolding behavior of ubiquitin

EX1 H/D exchange





Cold denaturation



Ubiquitin in reverse micelles, -20°C
Babu et al. (2004) Nat Struct Mol Biol 11:352

Most thermostable H-bond: I3 → L15

Cordier and Grzesiek (2002), J.Mol.Biol, 715: 739.

Biological implication ?

Engineering a disulfide bridge between 4 and 66: 70-80% decrease of activity in signaling proteolysis

Ecker et al. (1989) J Biol Chem 264, 1887

Outline

Part 1: Methods for fast multidimensional NMR

Concepts

Sensitive fast-pulsing experiments: SOFAST HMQC, BEST experiments

Part 2: Applications

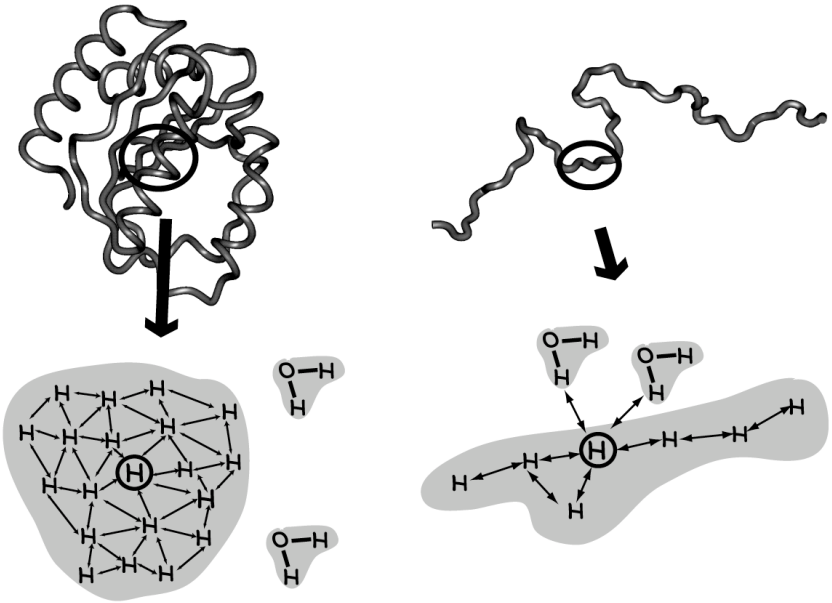
Application to the study of protein folding and unfolding

HET-SOFAST NMR: a fast tool for characterizing protein structure

HET-SOFAST NMR

Amide proton relaxation exploited for structural characterisation

Towards a quantitative measure of structure:
what distinguishes **structured** and **unstructured**
polypeptide chains ?

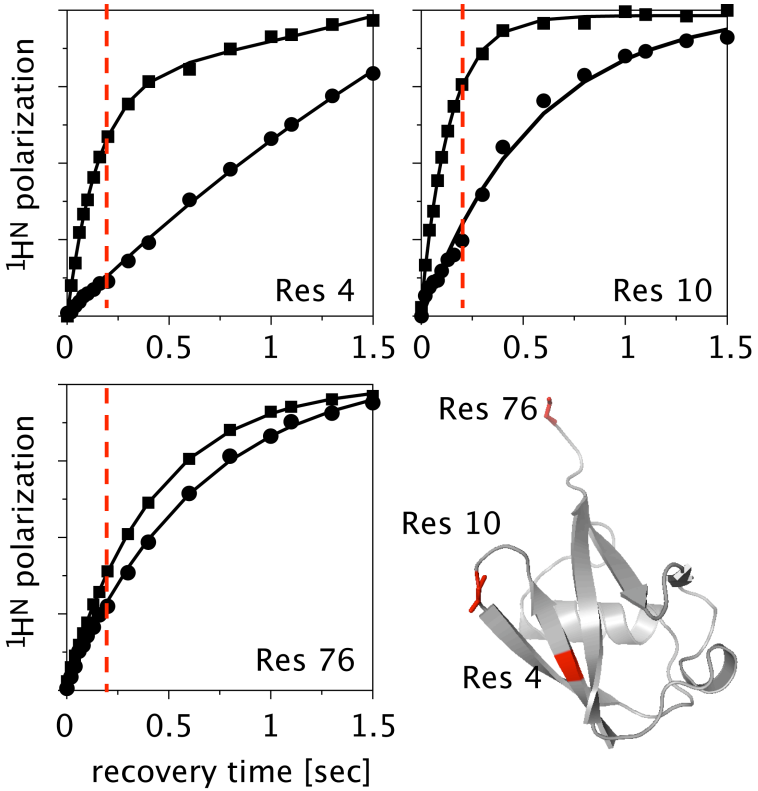


- high proton density
- low water accessibility

- low proton density
- high water accessibility

HET-SOFAST NMR

Amide proton relaxation exploited for structural characterisation



non-selective(●)
selective(■)

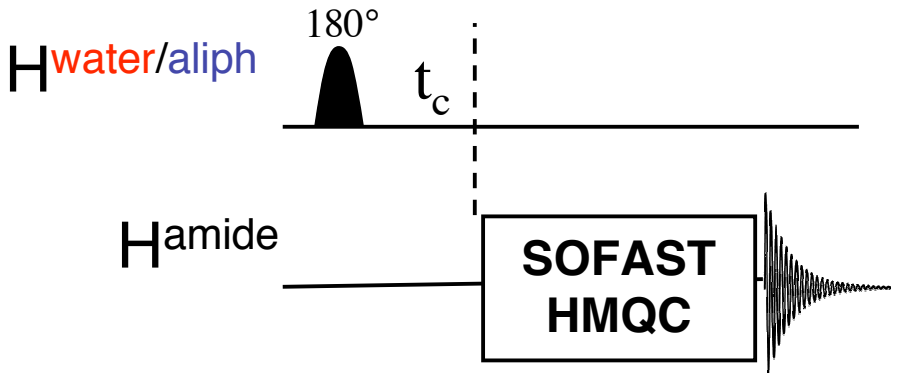
$$\lambda = I^{sat}/I^{ref}$$

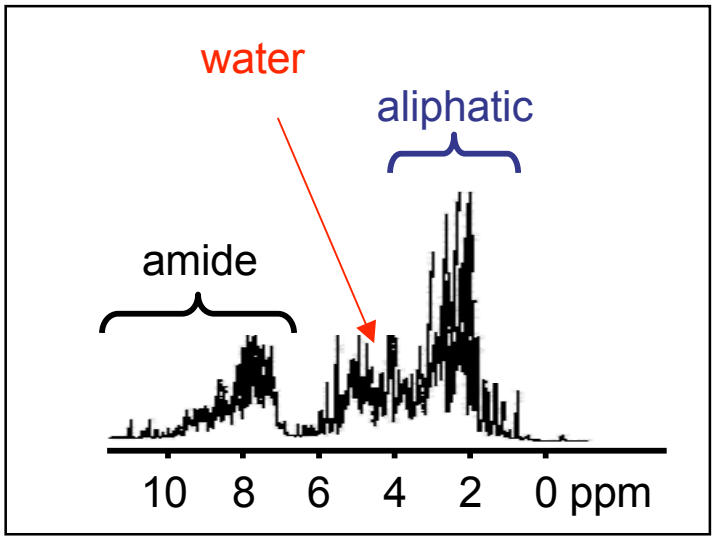
Amide proton relaxation depends on the structural context:

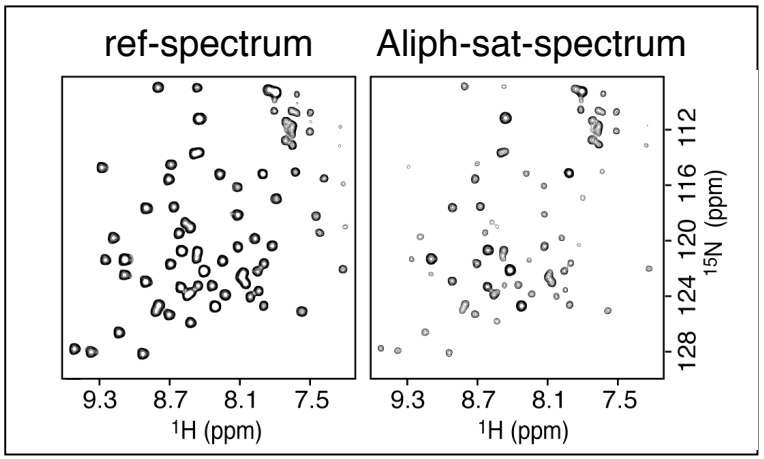
* number of aliphatic protons close in space
* local dynamics
* water exchange of H^N

HET(erogeneity) SOFAST NMR *

Quantification of **proton density** and **water accessibility**
along polypeptide chains





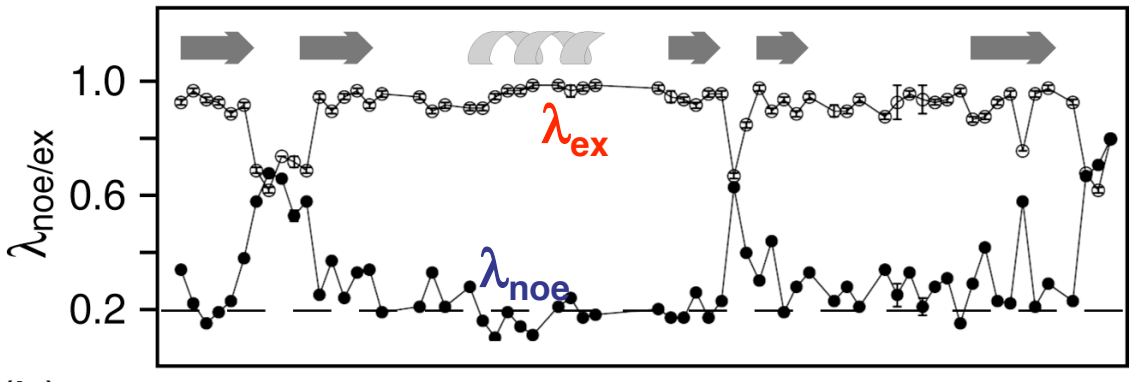


Ubiquitin (2 mM, 25°C, 600 MHz)

Acquisition time: 10 s / spectrum

* Schanda, Forge & Brutscher,
Magn. Reson. in Chem. (2006) 44, S177.

2D HET-SOFAST NMR: characterizing structural compactness for assigned protein samples



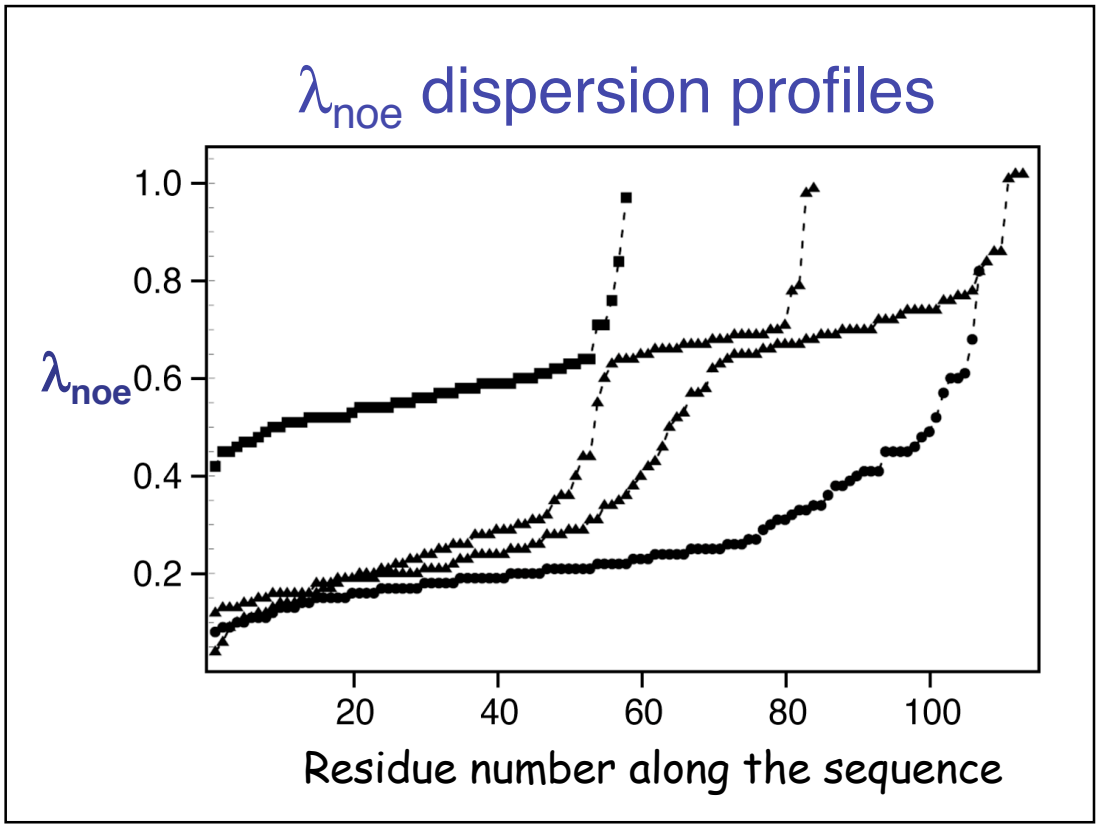
Ubiquitin native state, pH 6.5

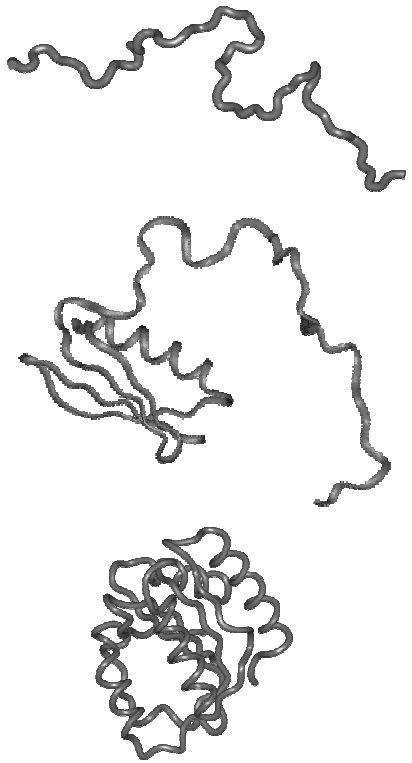
Similar information to heteronuclear NOE
but obtained in significantly reduced experimental time

HET-SOFAST NMR

Structural and dynamic heterogeneities along polypeptide chains

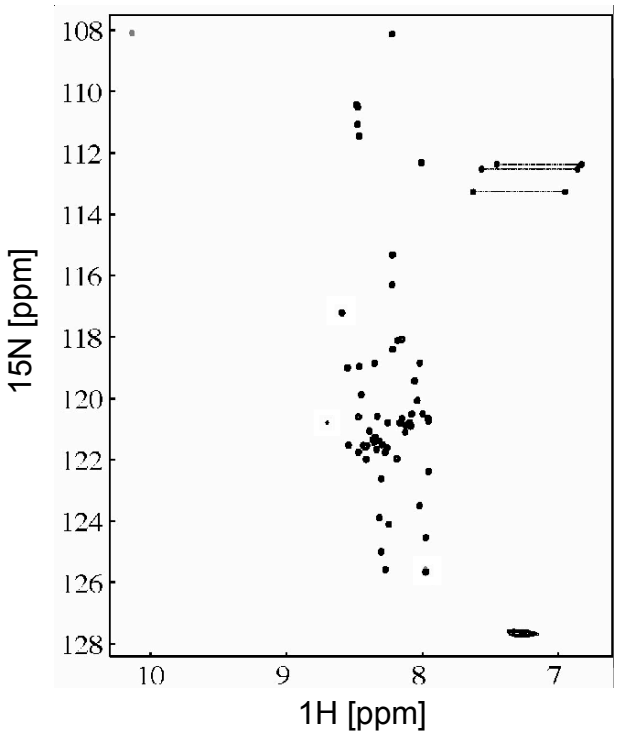
prior to resonance assignment

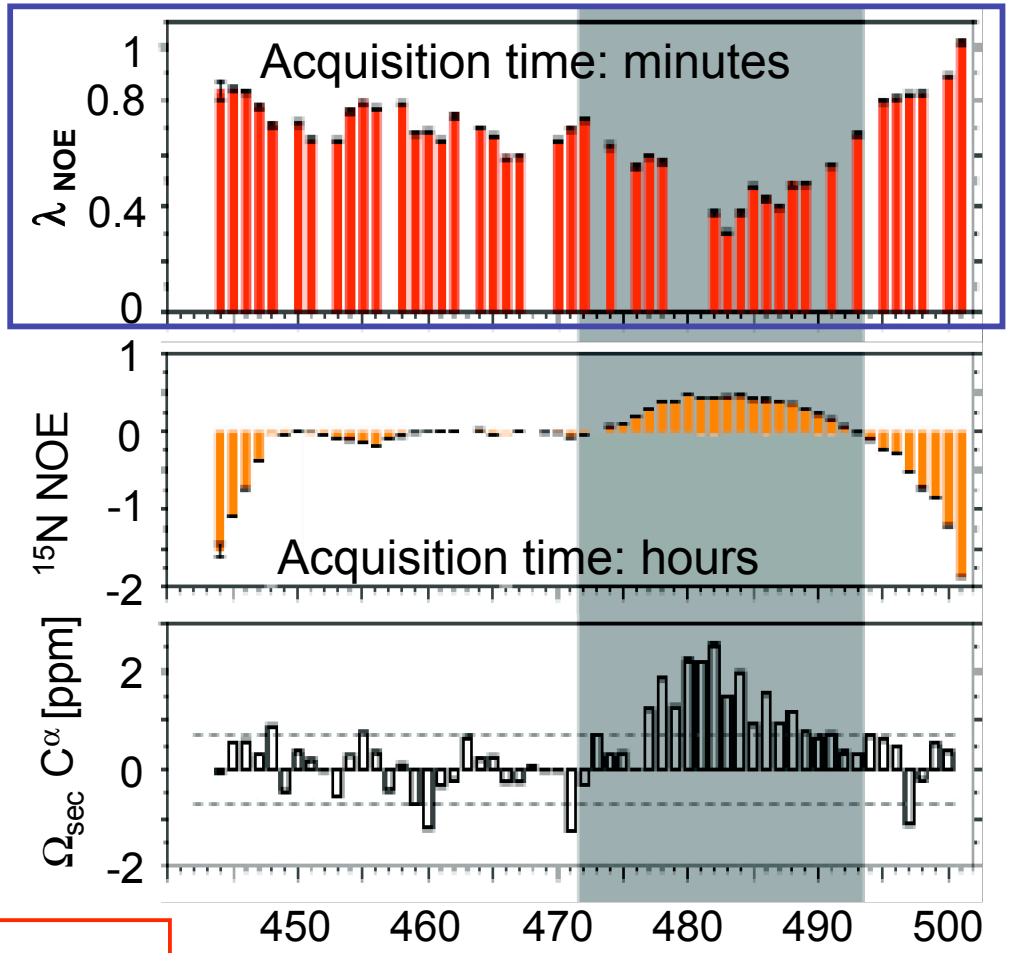




HET-SOFAST NMR

Application: N-tailC, an unstructured protein with a small helical propensity



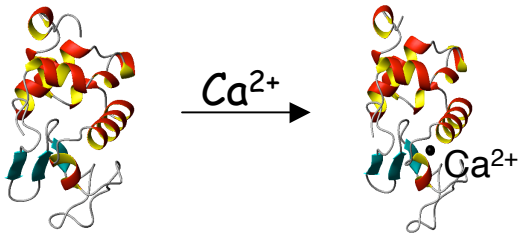


HET-SOFAST NMR allows to detect even partial structural preferences

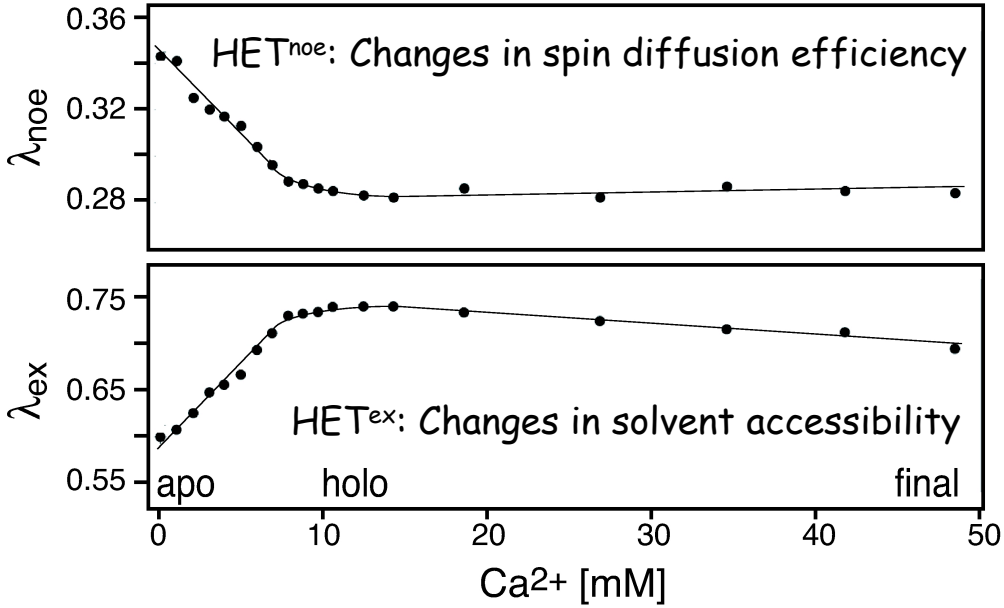
Houben et al
J Virol. (2007) 81(13):6807-16.

HET-SOFAST NMR

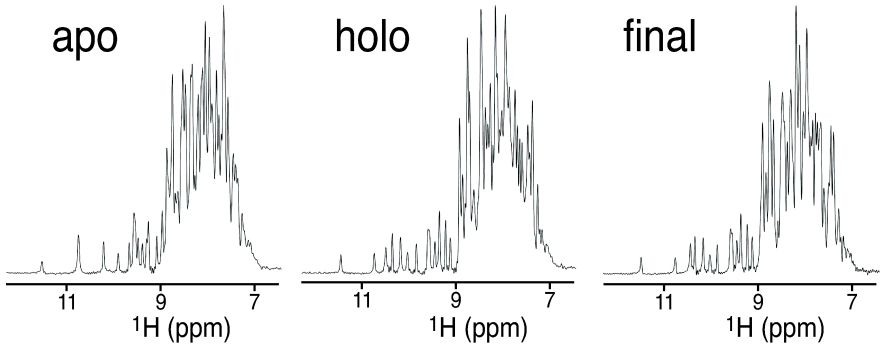
Following subtle structural changes upon metal binding



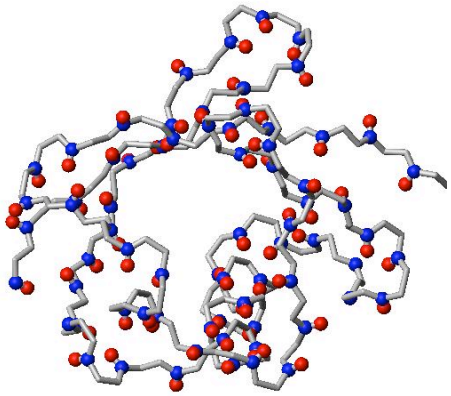
α-lactalbumin
The structure of apo and holo forms are very similar
Metal binding was reported to induce some "structure rigidification"



HET-SOFAST NMR experiments can be used to follow small changes of protein "compactness"



CONCLUSIONS



Longitudinal relaxation optimized experiments (SOFAST, BEST) allow significant acceleration of nD NMR data acquisition

These approaches are fully compatible with alternative sampling methods (Hadamard, spatial encoding, reduced dimensionality,…)

Kinetic reactions in proteins can be probed simultaneously residue by residue on a time scale of seconds

1H longitudinal relaxation can be used as a very sensitive probe for local structure and dynamics (HET-SOFAST NMR)

Merci

Bernhard Brutscher
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Mirjam Falge

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Isabel Ayala
Cécile Giustini

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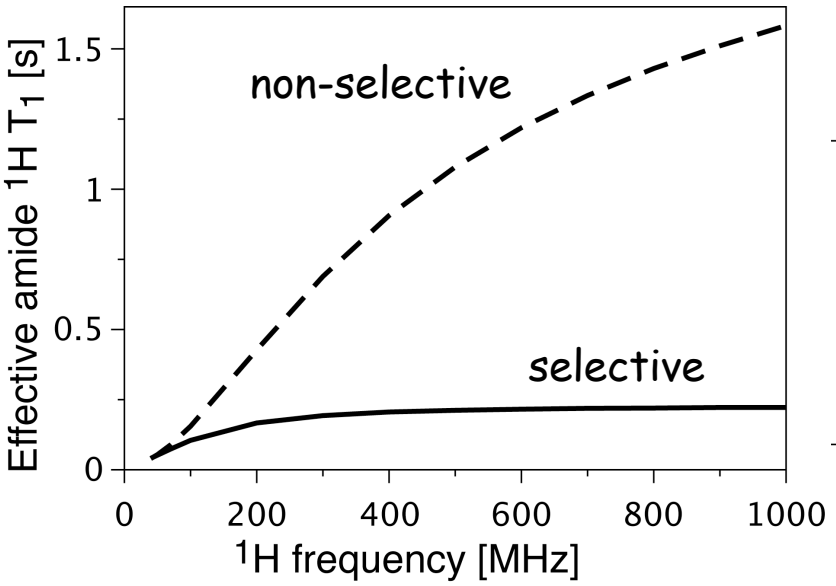
Thank you
Folding
Vincent Forge
Pulses, technical things
Eriks Kupce
Peter Sandor
« Ultrafast NMR »
Lucio Frydman
Maayan Gal

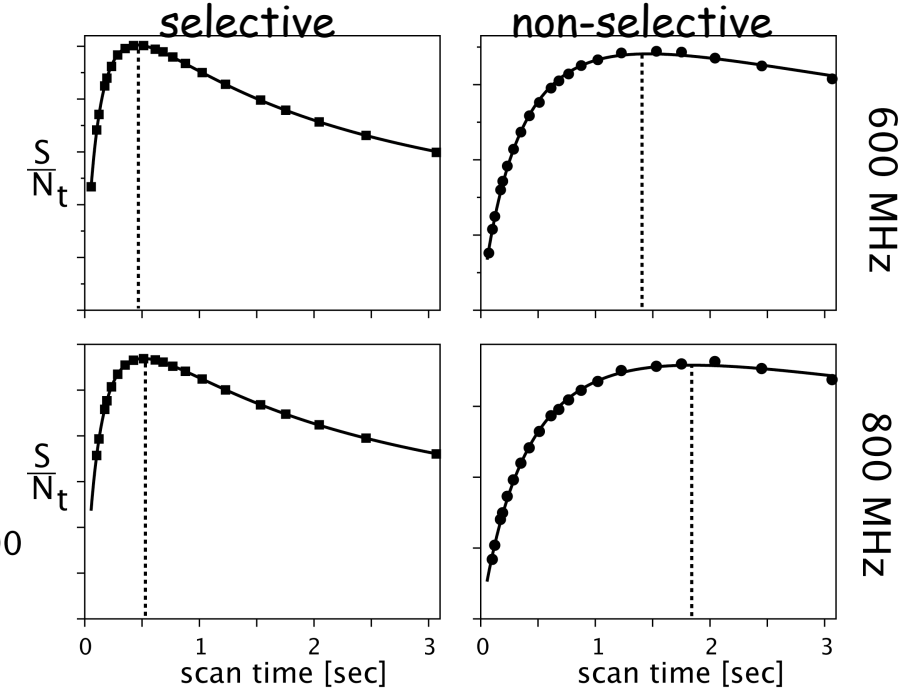
Longitudinal ^{1}H relaxation enhancement

Magnetic field dependence

simulation



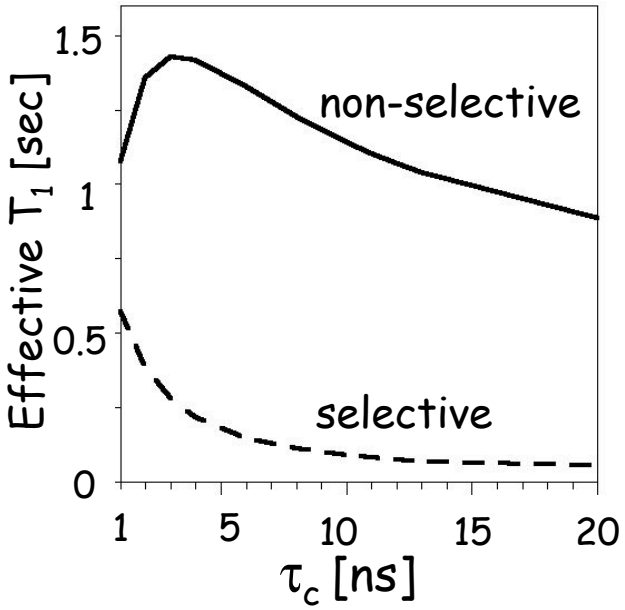
experiment



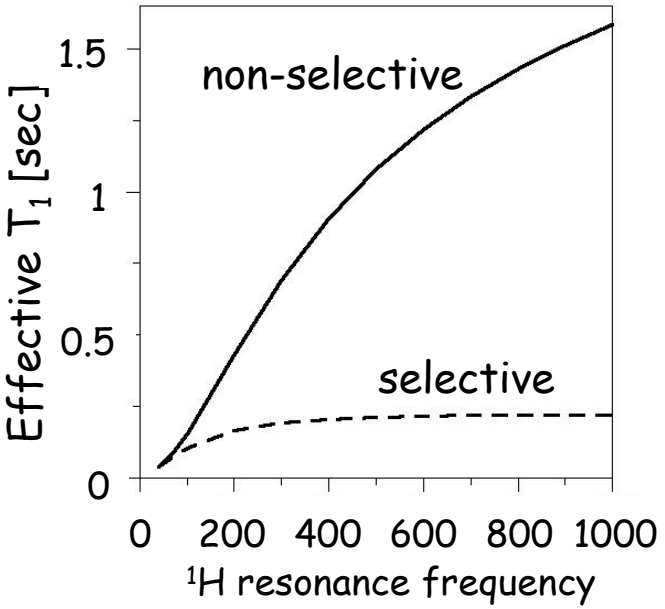
Longitudinal relaxation optimized (selective) experiments become important especially for high field strength

Amide ^{1}H relaxation in proteins: Insight from simulation

Size of the molecule (tumbling rate)

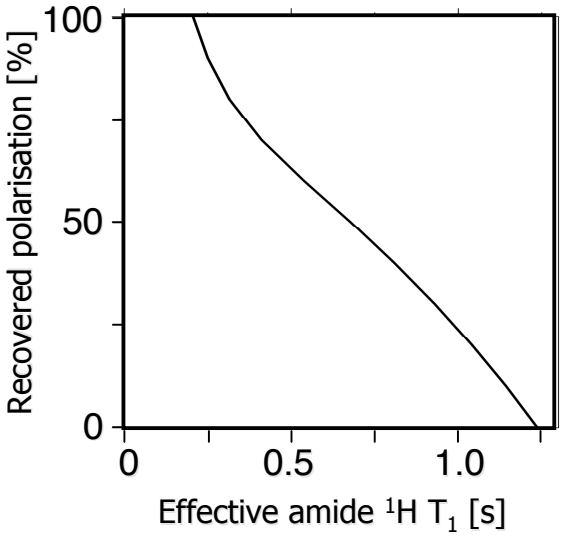


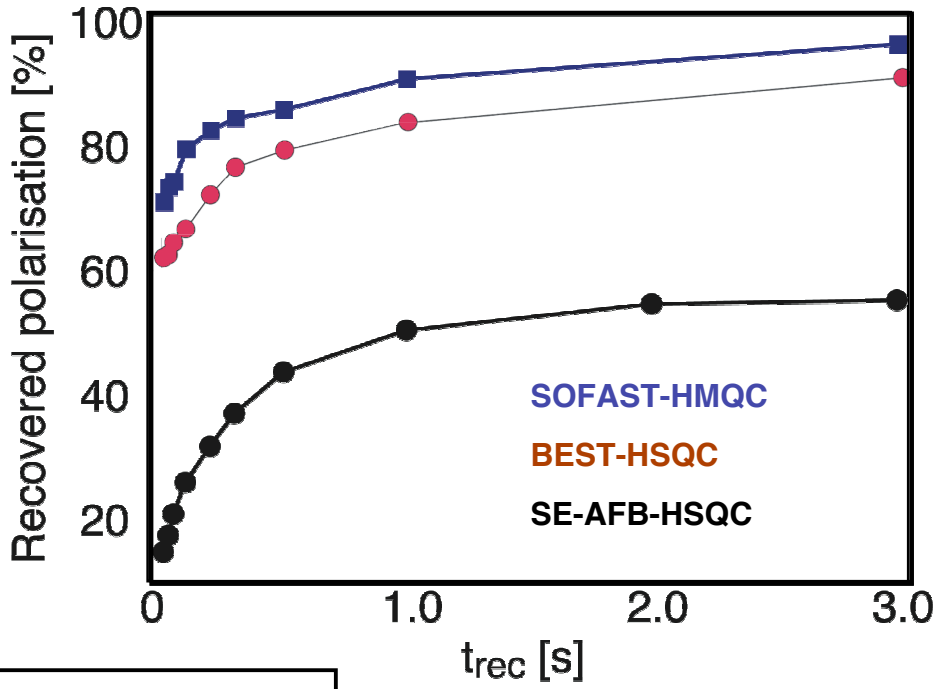
Magnetic field strength

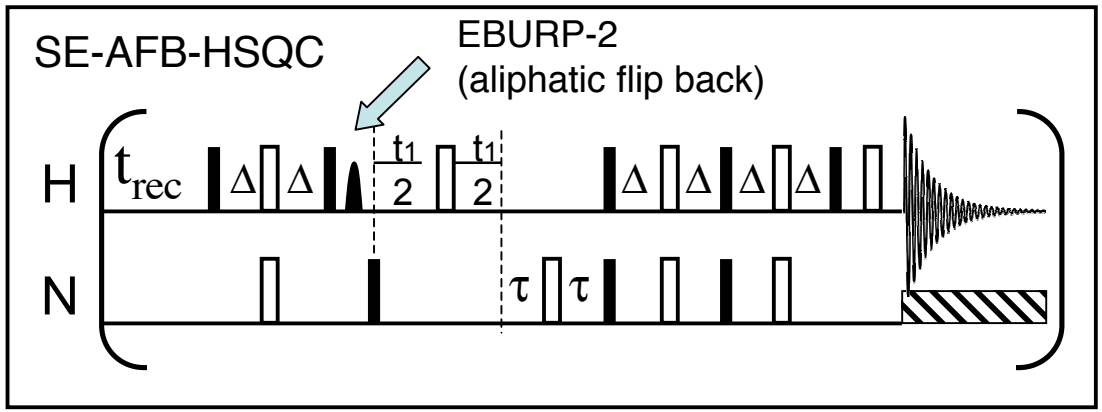


Fast pulsing

Aliphatic ^{1}H polarization recovery





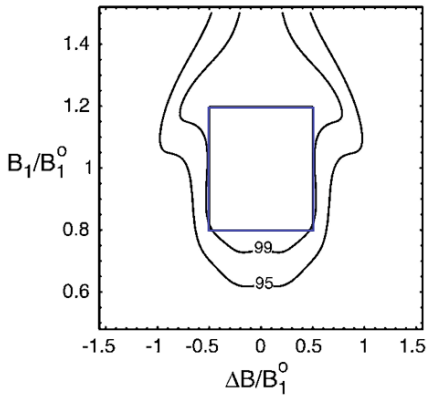


BEST experiments

Choice of selective pulses

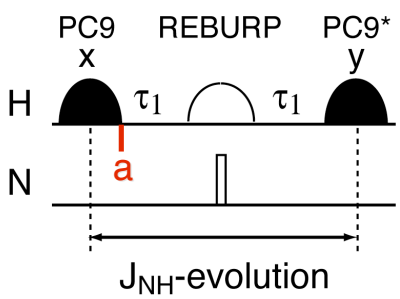
Broadband Inversion Pulses

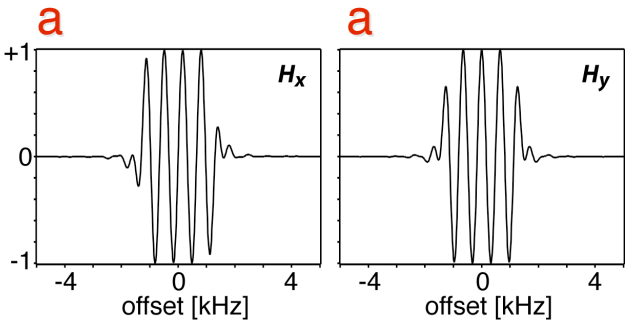
(Shaka & co,JMR 2001,151,269)



Time-optimized INEPT transfer

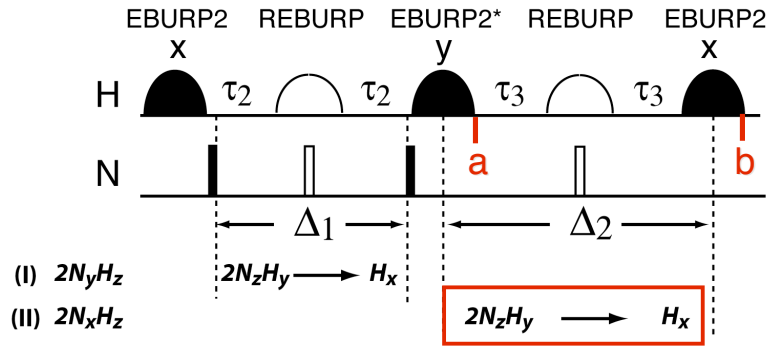
using PC9 (Kupce & Freeman,JMR 1993,102A,122)

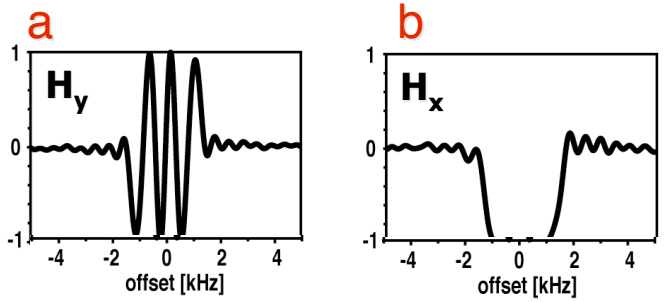




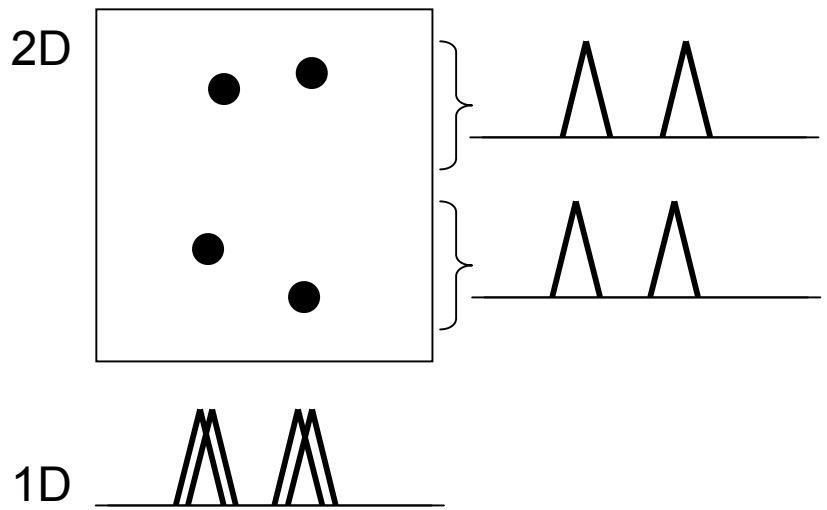
Planar mixing without general rotation pulses

using EBURP2 (Geen & Freeman,JMR 1991,93,93)

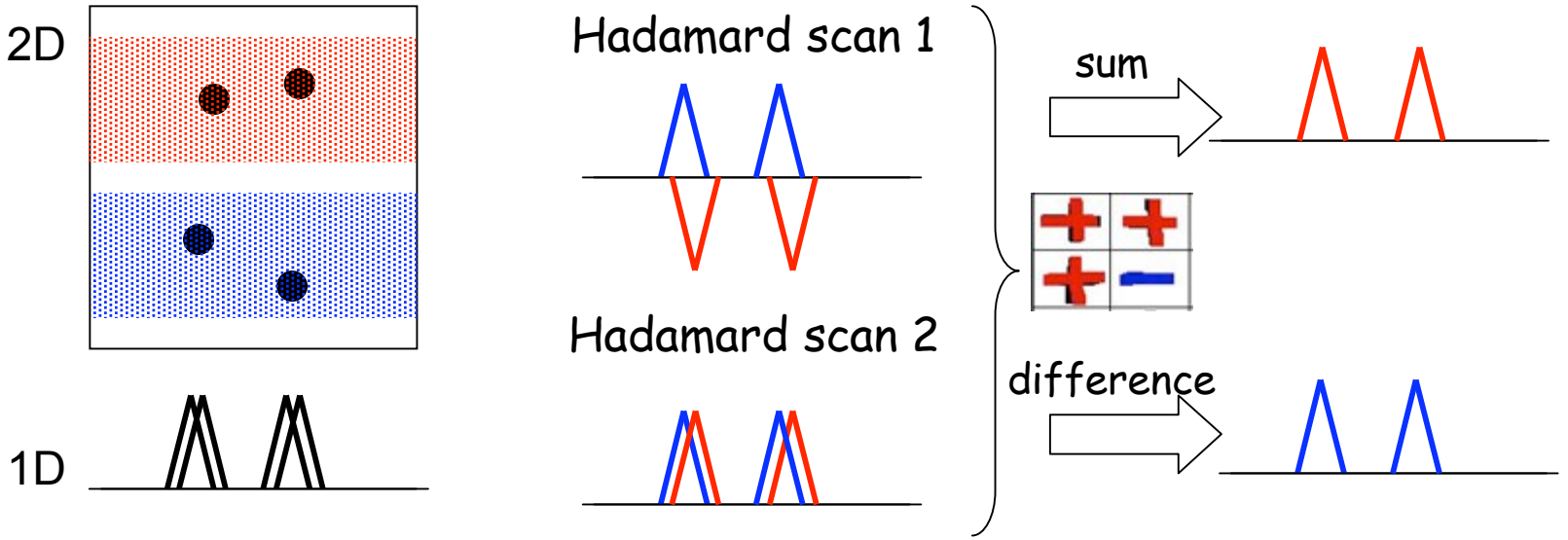




Hadamard encoded SOFAST HMQC



Hadamard encoded SOFAST HMQC



Retreiving the 2D information
is achieved in only 2 scans !