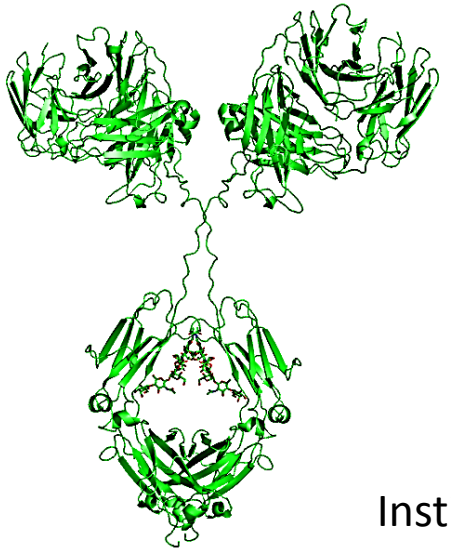
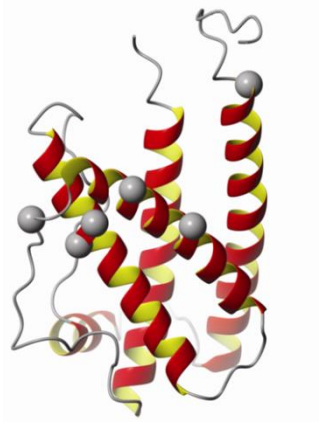
# High-resolution NMR as a Structure Assessment Tool for Protein Therapeutics





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National Institute of Standards and Technology
U.S. Department of Commerce

**BABE, Baltimore, MD**
**October 1, 2014**

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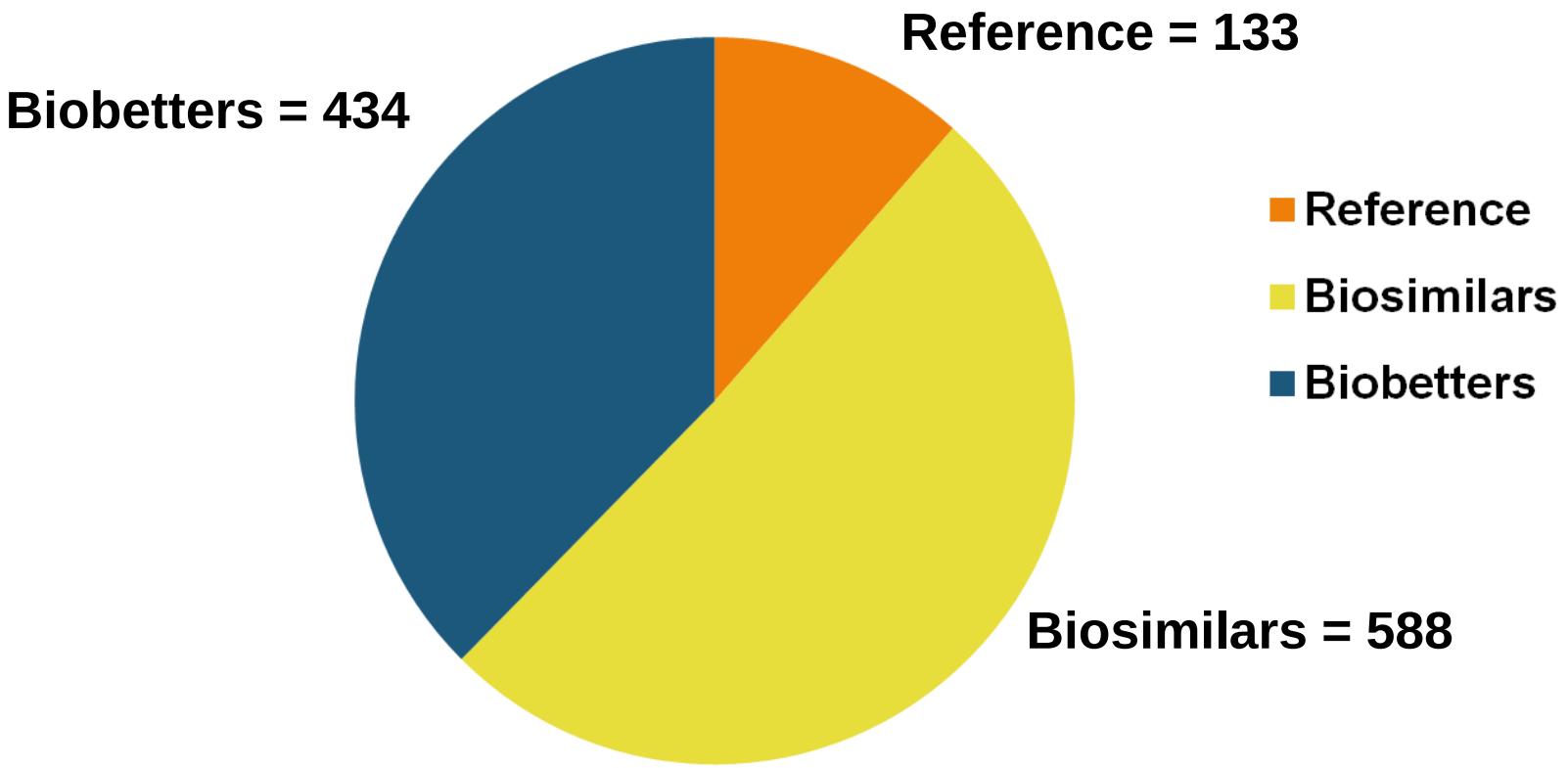
# Biologics in the Pipeline – USA and EU

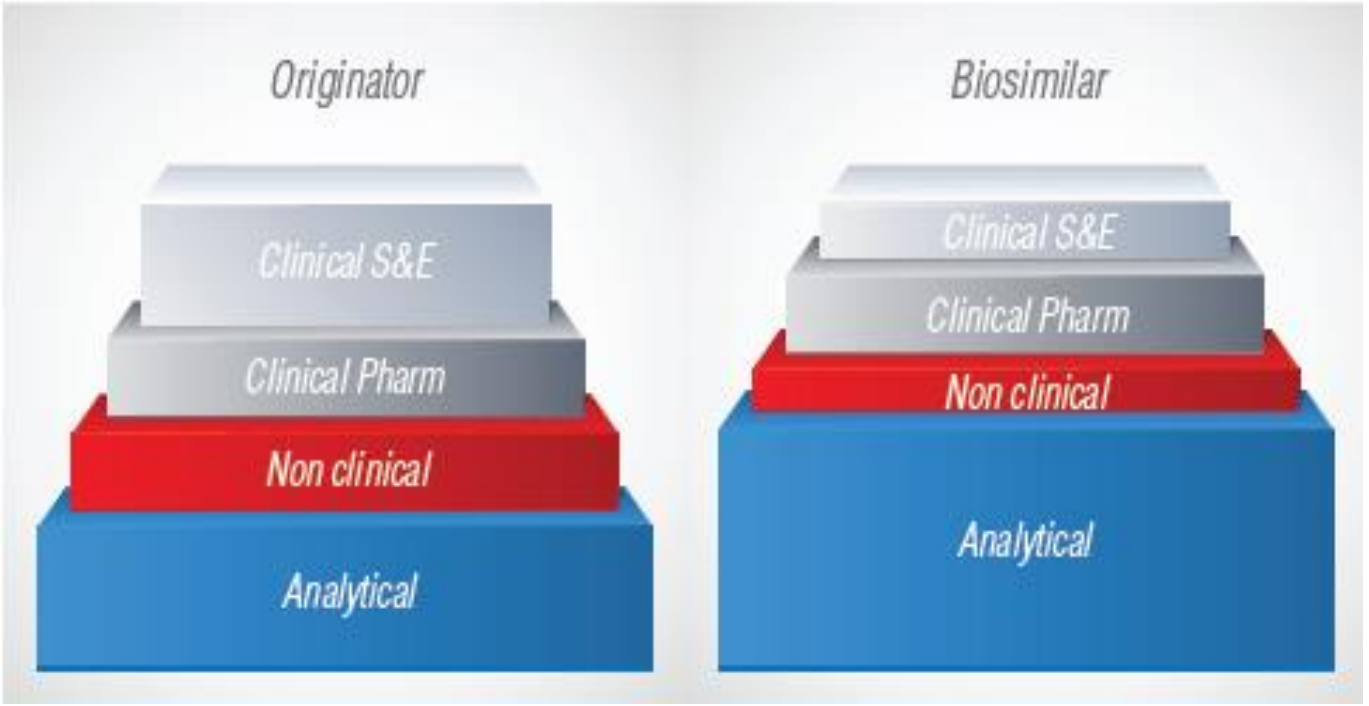



- **Total product entries = 1155**
- **Biologics predicted to reach 20% of Total Pharmaceutical Sales by 2017**

Reference: BioPharma 4/14

**Note, this study only covers recombinant proteins and a few other protein products. Vaccines, blood-derived, cultured cells and tissues and other types of biologics are not included!**

# Higher Order Structure and Biosimilars: A Regulatory Perspective



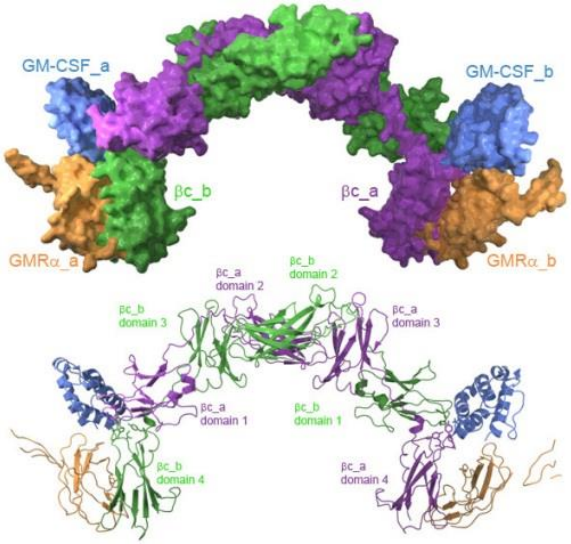

Rigorous analytical comparison of biosimilars (biomanufacturing changes) to their originator drug products obviates the need for extensive (and expensive) animal and clinical trials.

**Higher-Order Structure is the Distinguishing Feature of Protein Therapeutics**

# Higher-Order Structure is the Distinguishing Feature of Protein Therapeutics

"Our current ability to **predict the potency of biologics** would be enhanced if we had improved ability **to measure and quantify** the correct (major) **three-dimensional structure**, aberrant three dimensional structures (**misfolding**), and the **distribution** of different three-dimensional structures".

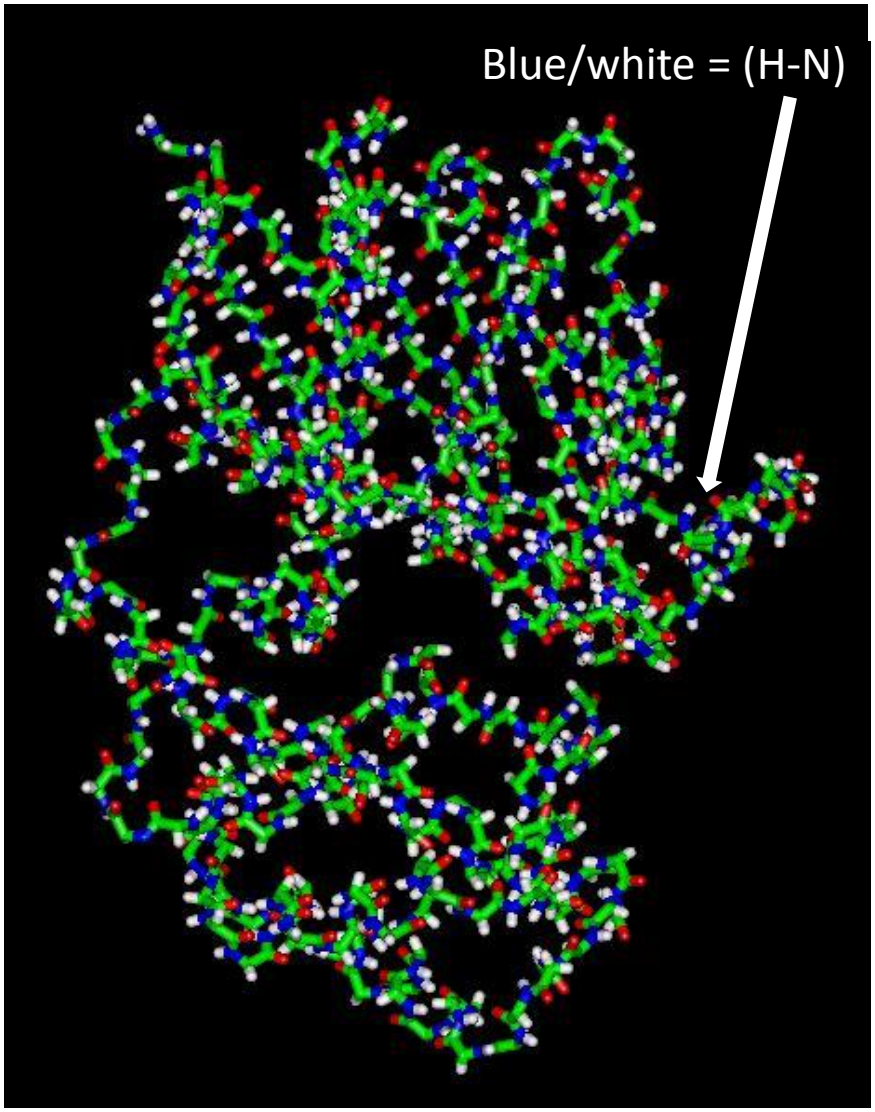
Steven Kozlowski, M.D. CDER, FDA (Congressional Testimony, 2009)

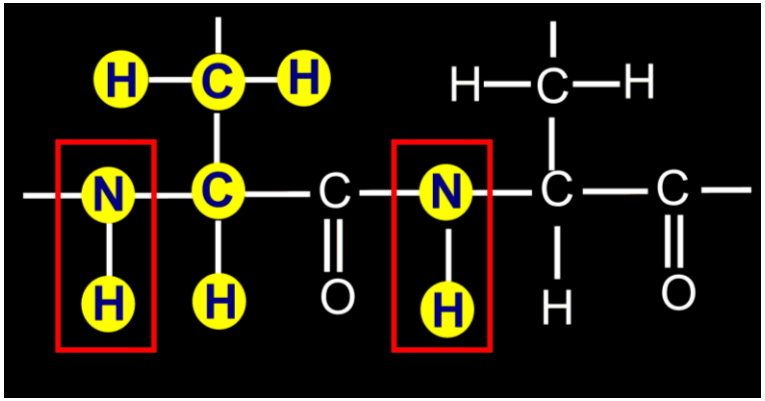



Receptor Bound GM-CSF

**What are the 'best' measurements? What is the confidence of the measure?**

# NMR is the only Spectroscopy that can provide Amino Acid Specific Assignment of Signals




$^{1}$HN-$^{15}$N Amide Correlation for each amino acid in a protein

Sequence Specific Assignment of Signals - Standard NMR methods and stable isotope ($^{15}$N, $^{13}$C) labeling

# Application of NMR Spectral 'Fingerprinting' to Biologics

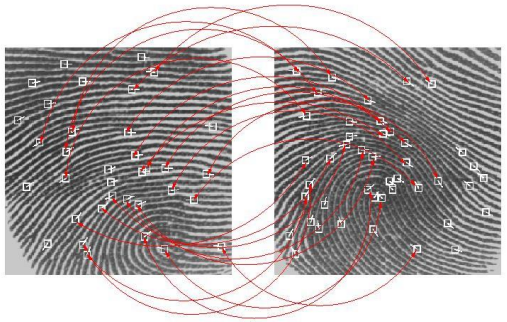
'Real World Applications': Method must be robust and applicable to formulated protein biologic drug products

Isotope Labeling ($^{15}$N-labeling) while cheap – is **NOT** an option

NMR data collected using isotopes at natural abundance

$^{15}$N = 0.37 %

$^{13}$C = 1.11%

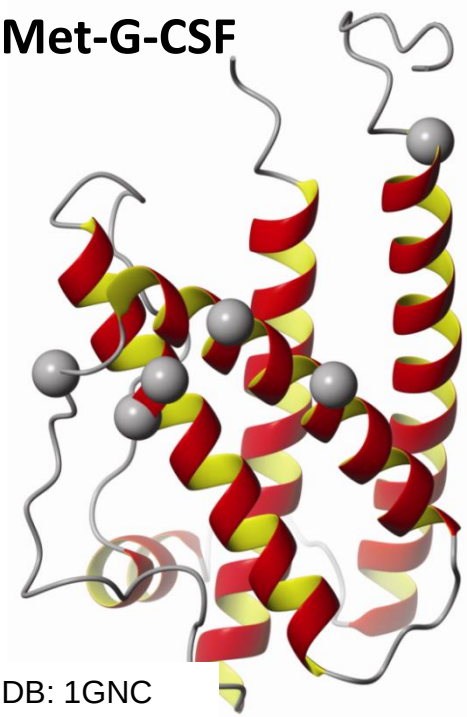


Must be Sensitive: **<u>NMR Cryoprobe Technology</u>**:

S/N > 6,000:1 @ 600 MHz; > 10,000:1 @ 900 MHz

# $^{1}H$-$^{15}N$ HSQC NMR spectrum – 'Ideal Protein Fingerprint'

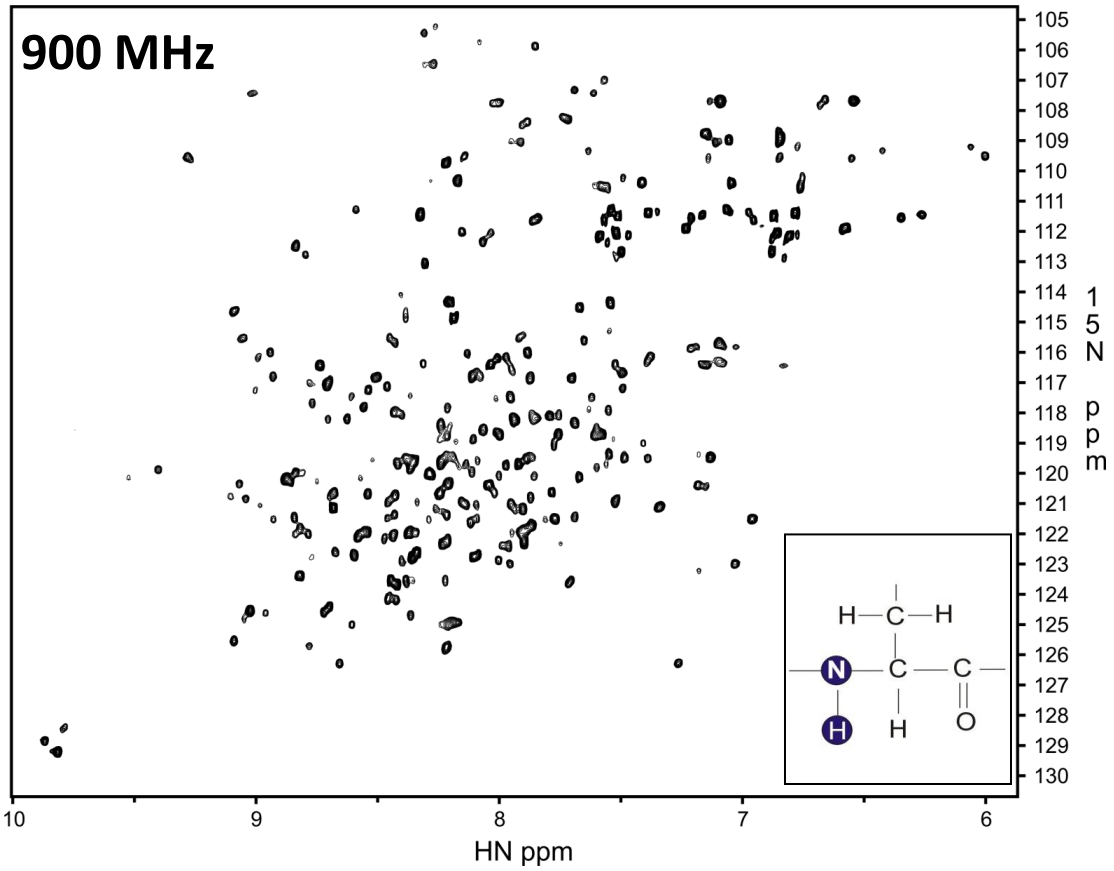
**rh-Met-G-CSF**



PDB: 1GNC

**Rh-Met-G-CSF:** 175 amino acids; non-glycosylated (18.8 kDa) ) – used in cancer patents with neutropenia. **First Accepted FDA Biosimilar Application**

**$^{15}N$-labeled met-G-CSF 'System Suitability' Sample**




0.2 mM Protein; Experimental time: 2.5 hrs

# NMR Method Validation through an Inter-laboratory Round Robin Study

Round robin study on the comparability of NMR spectral 'finger prints' obtained using HSQC type NMR experiments

- **4 Sites in North America and Europe**
  FDA; Health-Canada; MPA-Sweden; NIST
- **4 Fields**
  500, 600, 700 and 900 MHz
- **Different Instrument vintages**
- **2 Vendors**
  Bruker Biospin, Varian/Agilent

FDA

LÄKEMEDELSVERKET
MEDICAL PRODUCTS AGENCY

Health Canada Santé Canada

NIST
National Institute of Standards and Technology
U.S. Department of Commerce

# Inter-laboratory Round Robin Study 1st Round

Goal:

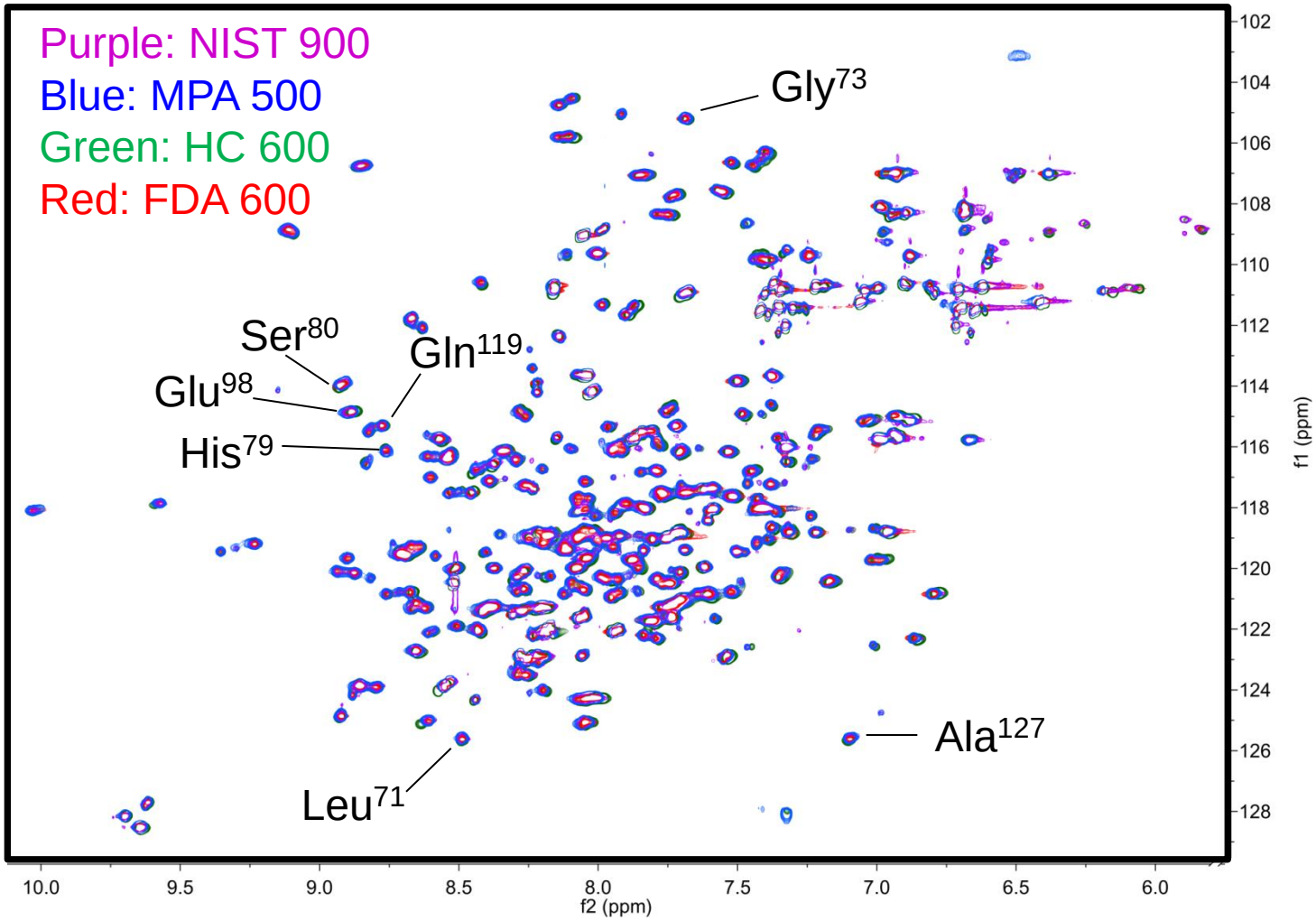
**Establish the robustness of NMR spectral 'finger prints' as structure test for protein therapeutic quality assessment.**

- Compare results using a system suitability sample (recombinant, human $^{15}$N-labeled G-CSF) – prepared by one lab (Health Canada) and distributed to all participating sites.

- Assess comparability of performance of the NMR measurement – quality of the data - across the different instrument manufacturers, field strength and pulse sequences experiments.

- Establish recommendations for data acquisition, processing and analysis for comparability applications.

# Visual overlay of $^{1}H$-$^{15}N$ HSQC NMR spectra of $^{15}N$-labeled met-G-CSF 'System Suitability' Sample



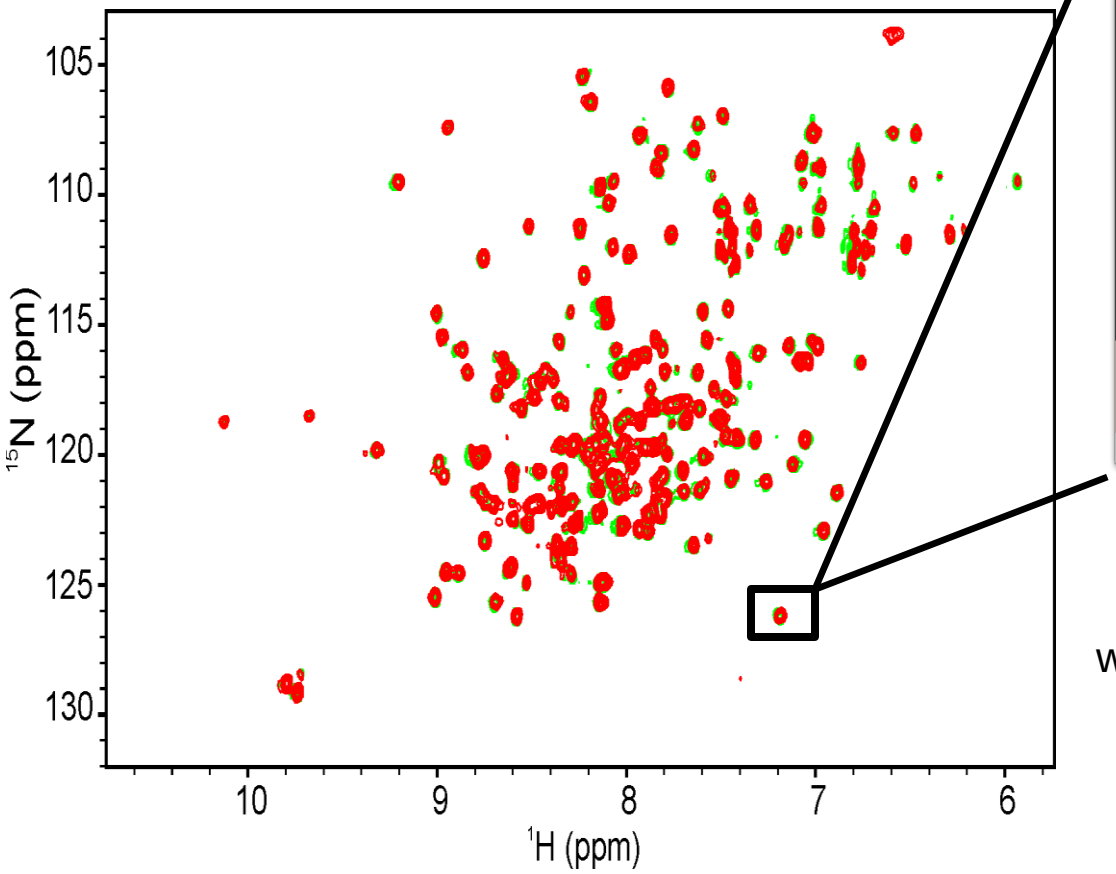

Spectral 'Finger prints' are remarkably consistent across all labs.

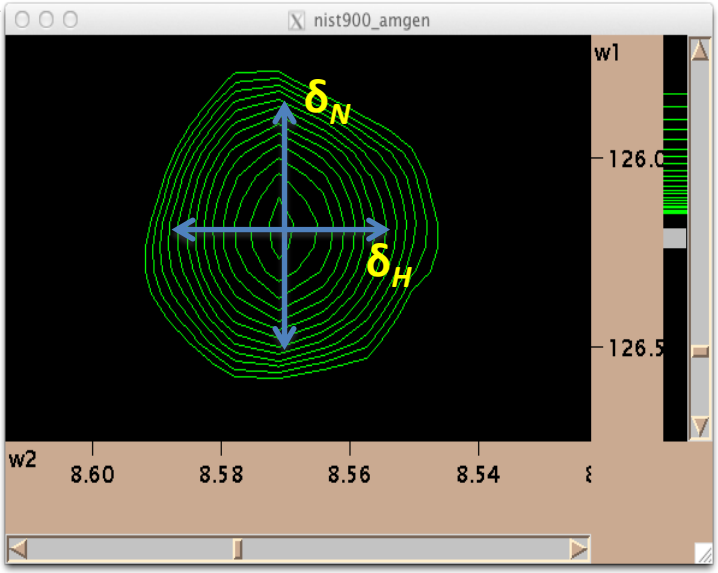
# Establishing limits of experimental variation across fields Using the 'System Suitability' Samples

| | | Four fields RMSD (ppm) | | Six fields RMSD (ppm) | | Total number of backbone resonances | Peak-picked backbone resonances |
|---|---|---|---|---|---|---|---|
| | | Average | Largest deviation | Average | Largest deviation | | |
| $^{15}$N-met-G-CSF | $^{1}$H | **0.003** ± 0.002 | **0.006** (Gln$^{67}$) | **0.006** ± 0.004 | 0.020 (Gln$^{70}$) | 160 | 127 (79%) |
| | $^{15}$N | **0.011** ± 0.006 | **0.034** (Met$^{121}$) | **0.023** ± 0.017 | 0.108 (Ser$^{62}$) | | |
| SH3 domain | $^{1}$H | **0.003** ± 0.001 | **0.008** (Lys$^{26}$) | **0.005** ± 0.003 | 0.011 (Glu$^{17}$) | 59 | 59 (100%) |
| | $^{15}$N | **0.011** ± 0.006 | **0.024** (Glu$^{17}$) | **0.028** ± 0.017 | 0.070 (Ser$^{36}$) | | |

**Four Fields**: NIST900, NIST600, FDA500, MPA600
**Six Fields:** NIST900, NIST600, FDA500, MPA600, **HC600, HC700**

# Comparability Assessment of the $^{1}H$-$^{15}N$ HSQC Spectra: CCSD= "combined chemical shift difference"



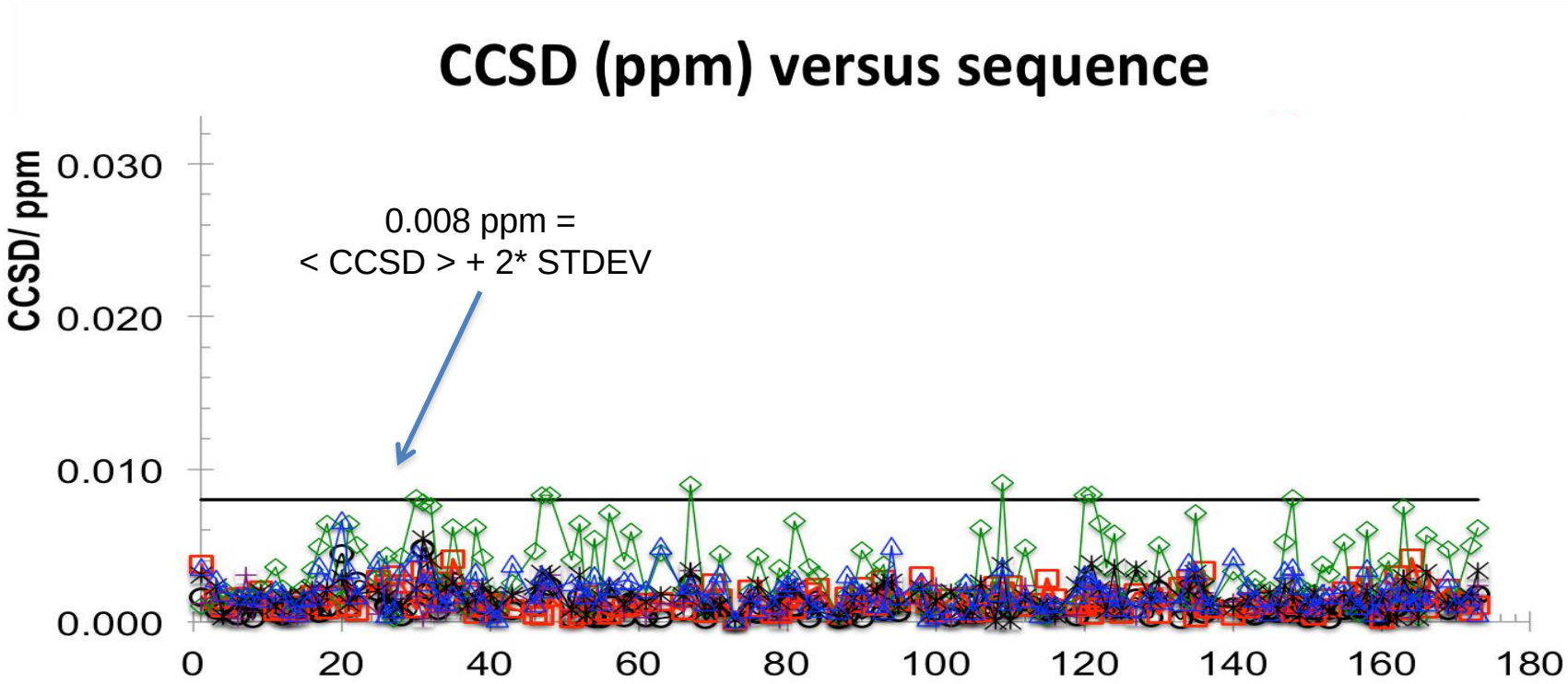

$$\sqrt{[0.5*(\delta_H^2+(\alpha * \delta_N)^2)]}$$

where $\delta_H$ and $\delta_N$ denote deviations from innovator product in $^{1}H$ and $^{15}N$ dimensions.

$$\alpha = 0.1$$

some references use
$\alpha = 0.14$ (0.20 for Gly)

# CCSD Analysis for Comparability: Measurement Variation Observed for the $^{15}$N-GCSF 'System Suitability' Sample




Small lab-to-lab variations, Health Canada shows temperature variation.
Reference = AVERAGE (FDA500, NIST900, NIST600, MPA600)

**HC Data shows sensitivity of temperature offset on $^{15}$N-GCSF shifts**

# Keys to Acquisition and Processing for Comparability

## Spectral Resolution:

- Data acquired with comparable resolution calibrated to instrument
- Data processed using the same functions & parameters
- Cross-peaks picked with a common method

**How well can peak positions be can determined sets the precision of the spectral comparison**

## Signal to Noise

- Experiments are acquired across labs and platforms using comparable S/N in acquisition

**Determines the threshold of detection and correlates with the precision of 'point to point' comparison methods.**

# Inter-laboratory Round Robin Study 2nd Round

Goal:

**Assess NMR as a tool for testing comparability of protein therapeutic structure**

- Lot to Lot comparisons
- Manufacturing change
- Biosimilar assessment

Compare results using commercially available Filgrastim products:

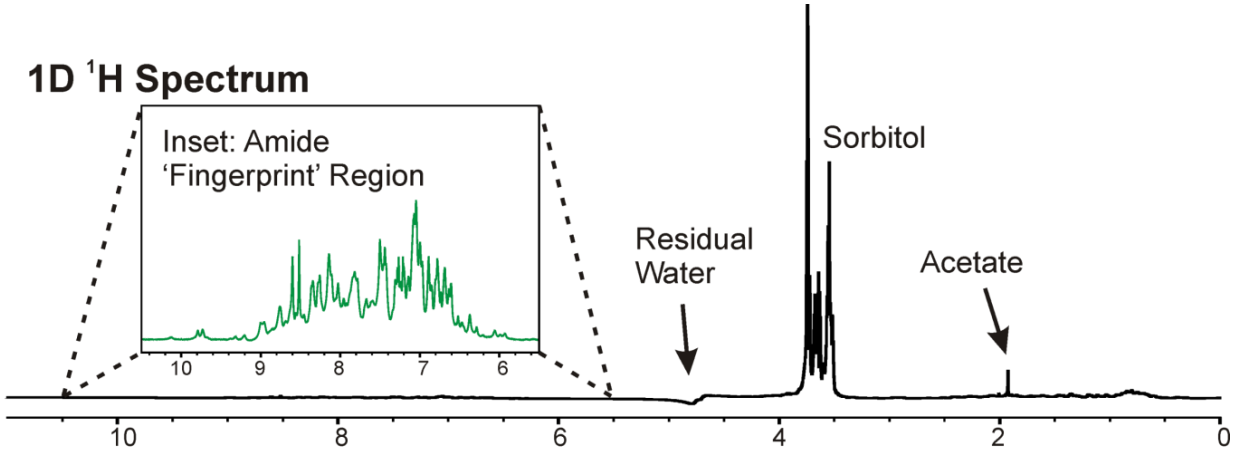
Innovator: Neupogen® (Amgen)

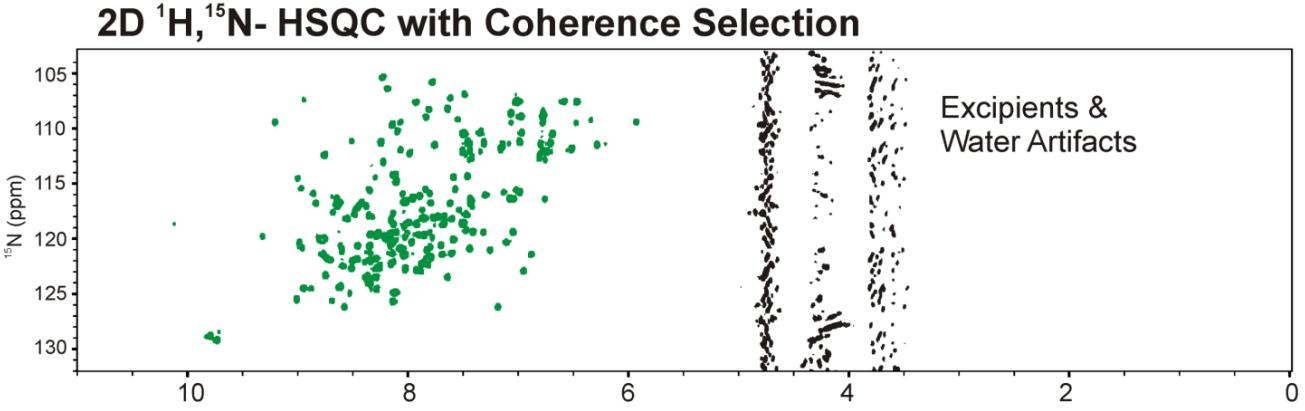
Follow-ons: Neukine® (Intas Biopharmaceuticals), Nufil Safe™ (Biocon), Grafeel™ (Dr. Reddy's Laboratories)

formulate drugs concentrated to 1 mM drug substance by one lab (FDA) and distributed to the participating sites.

# Natural Abundance $^{1}H$-$^{15}N$ HSQC NMR spectra of Formulated Filgrastim Products

## Example: Formulated NUFIL Safe™

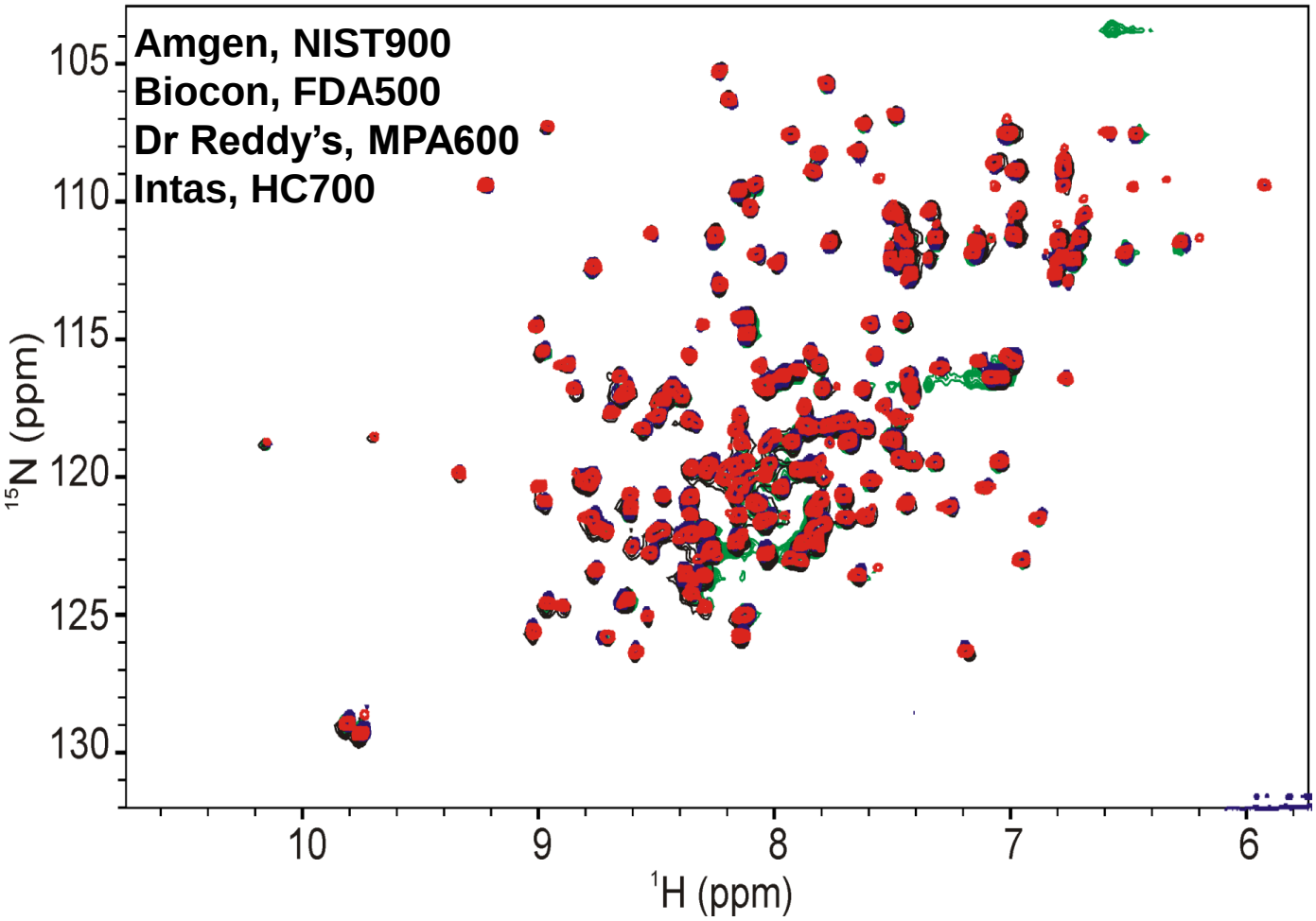



## Formulations

| Drug Name<br>Company | Neupogen<br>Amgen | NUFIL Safe™<br>Biocon |
|---|---|---|
| G-CSF (µg) | 300† | 300† |
| Acetate (mg) | 0.59 | 0.295 |
| **Sorbitol (mg)** | **50** | **25** |
| Polysorbate 80 (mg) | 0.04 | [-] |
| Polysorbate 20 (mg) | [-] | 0.02 |
| Sodium (mg) | 0.035 | 0.018 |
| $H_2O$ injection (ml) | 1 | 0.5 |
| pH | N.A. | 4.0 |

# Overlay of $^{1}H$-$^{15}N$ HSQC NMR spectra of 4 Filgrastim products recorded at 4 sites with 4 magnetic fields




Nearly identical 'finger print' map between the 4 samples/instruments/magnetic fields using comparable acquisition and processing parameters

# Principal Component Analysis of 2D NMR Data

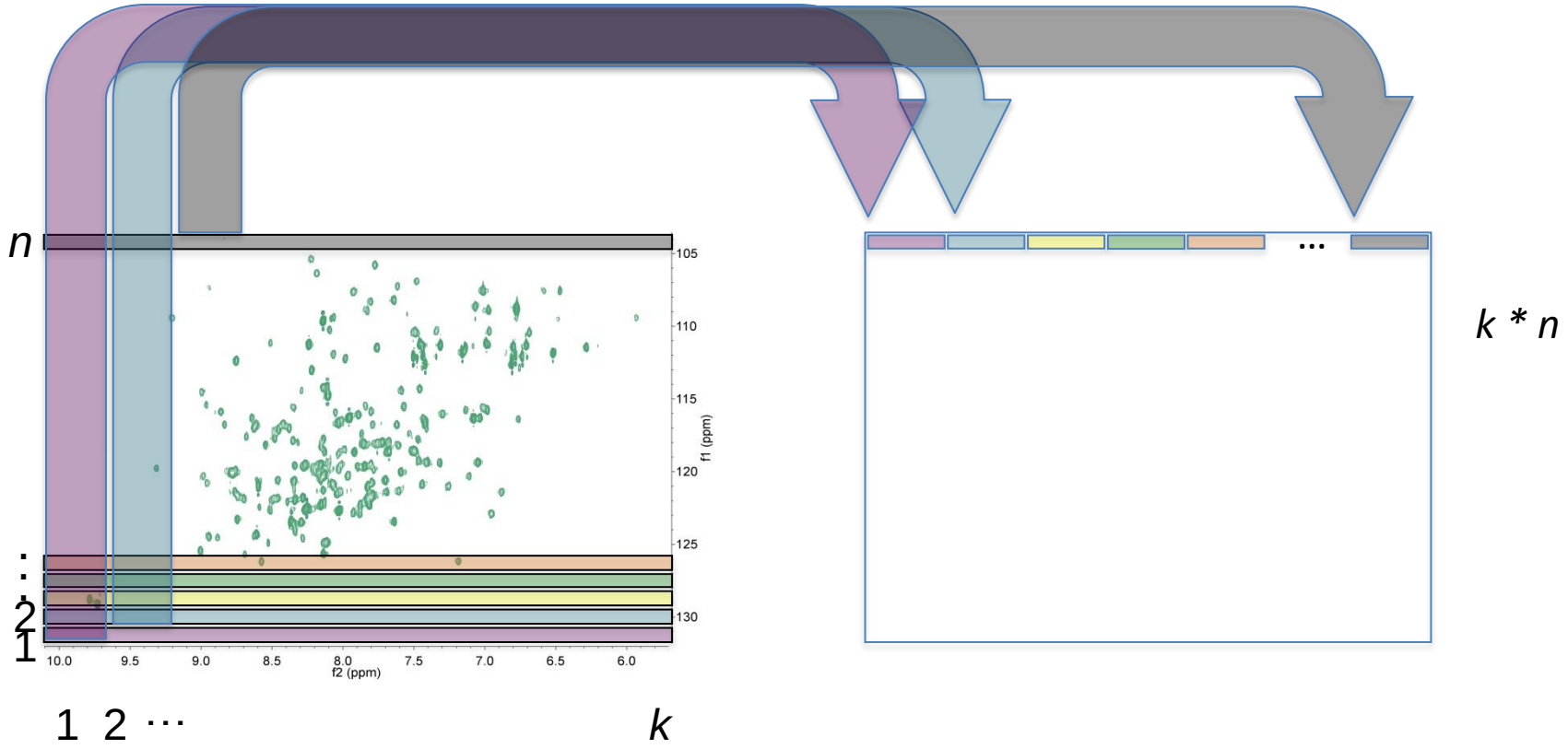
PCA analysis and *k*-NN algorithms can be used to assess degree of similarity and cluster data

- It is un-biased.
- It is fast and does not require chemical shift assignments.
- It can handle crowded regions, where resonances overlap.
- It can be applied to higher dimension NMR spectra.

Q-residuals and $T^2$ statistics guide when choosing appropriate models that describe the data accurately.

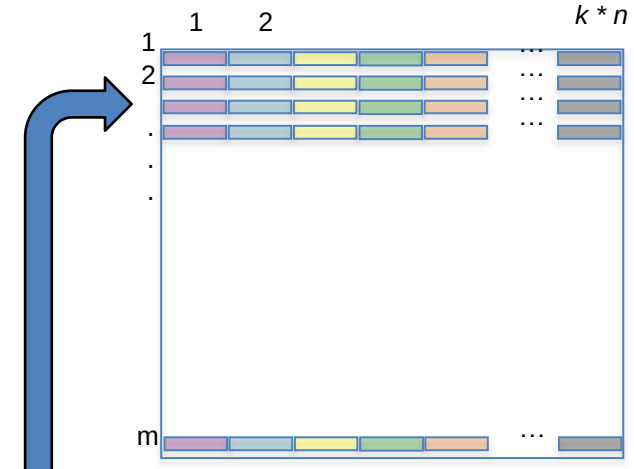
# Statistical Analysis by Creating 2D Matrices using the 1D Traces from the $^{1}H$-$^{15}N$ HSQC spectra

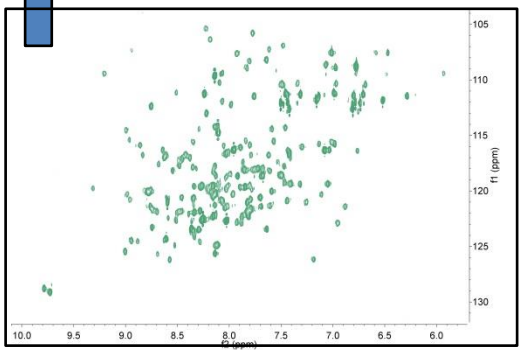
**One 2D map, (*k***n*)** → **One single row vector**

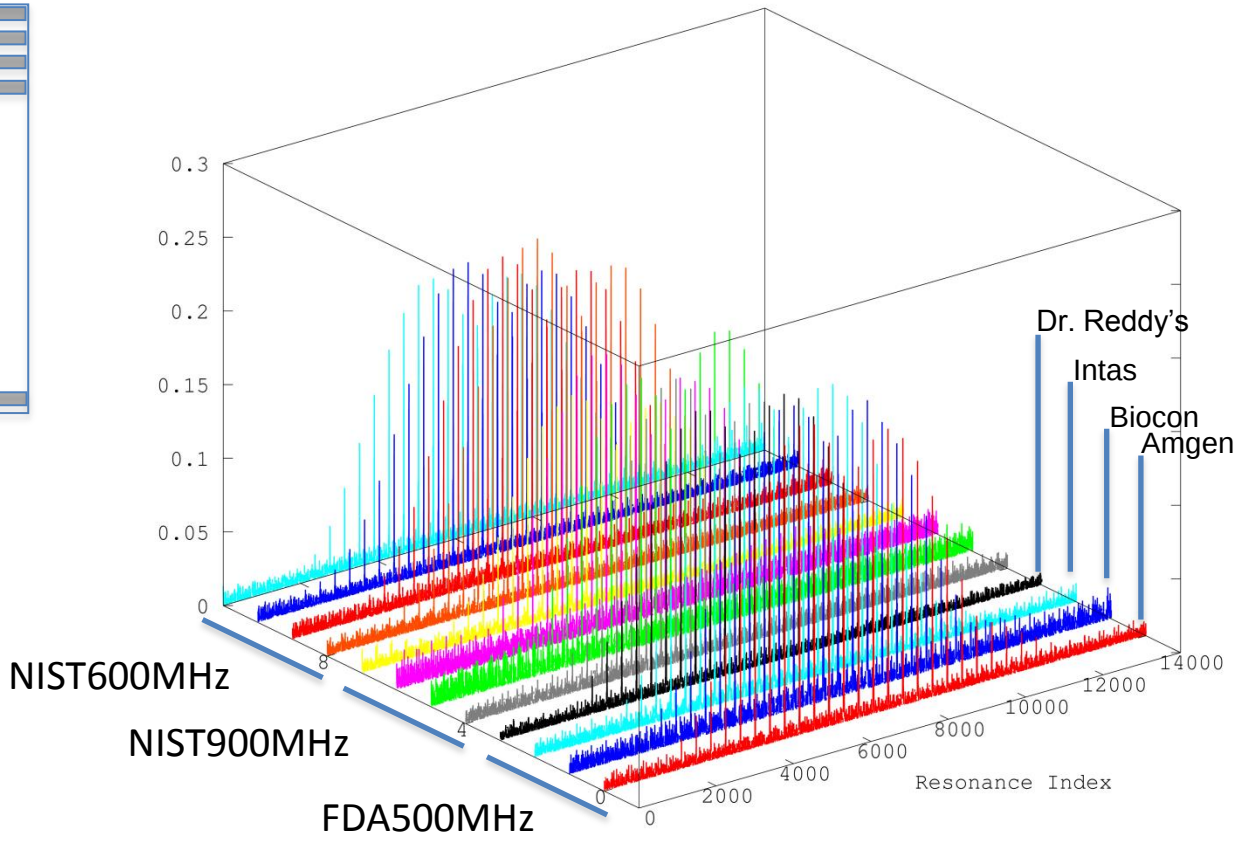



Convert a Series of 2D NMR Datasets into a single 2D matrix

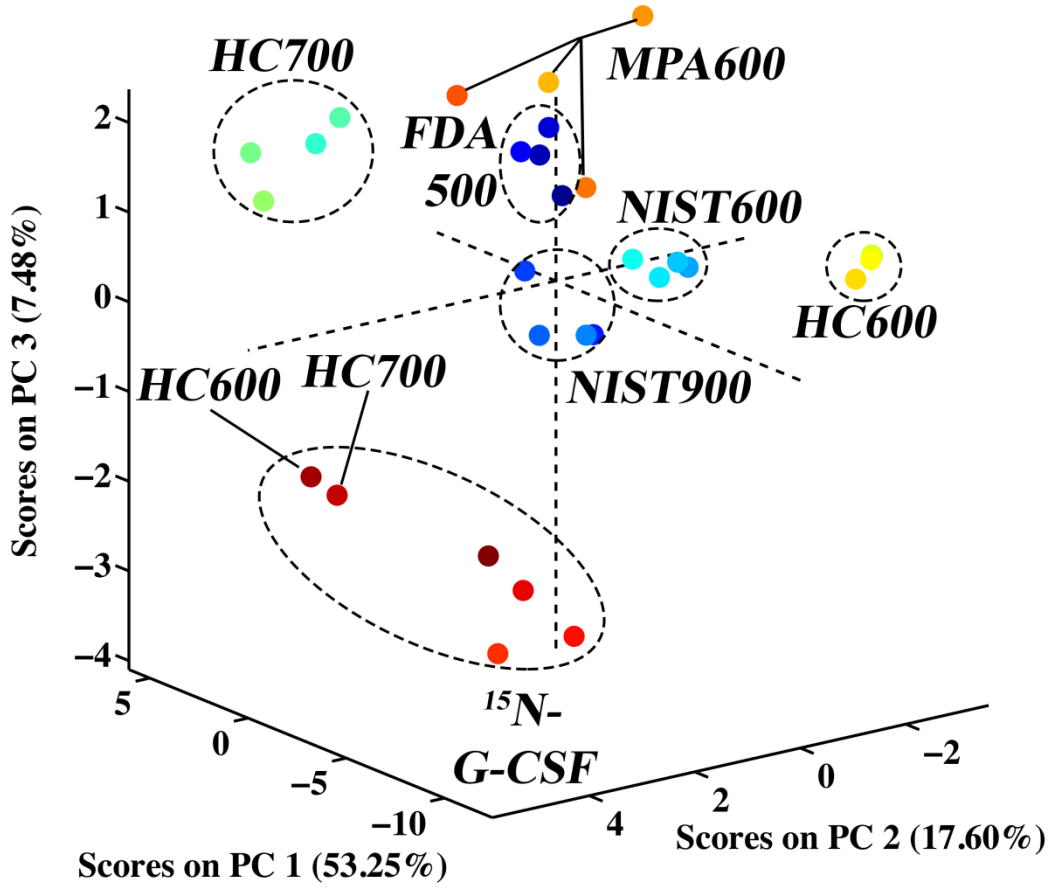
# Example of *k*n*m* matrix using the 1D Traces from the $^{1}H$-$^{15}N$ HSQC NMR spectra



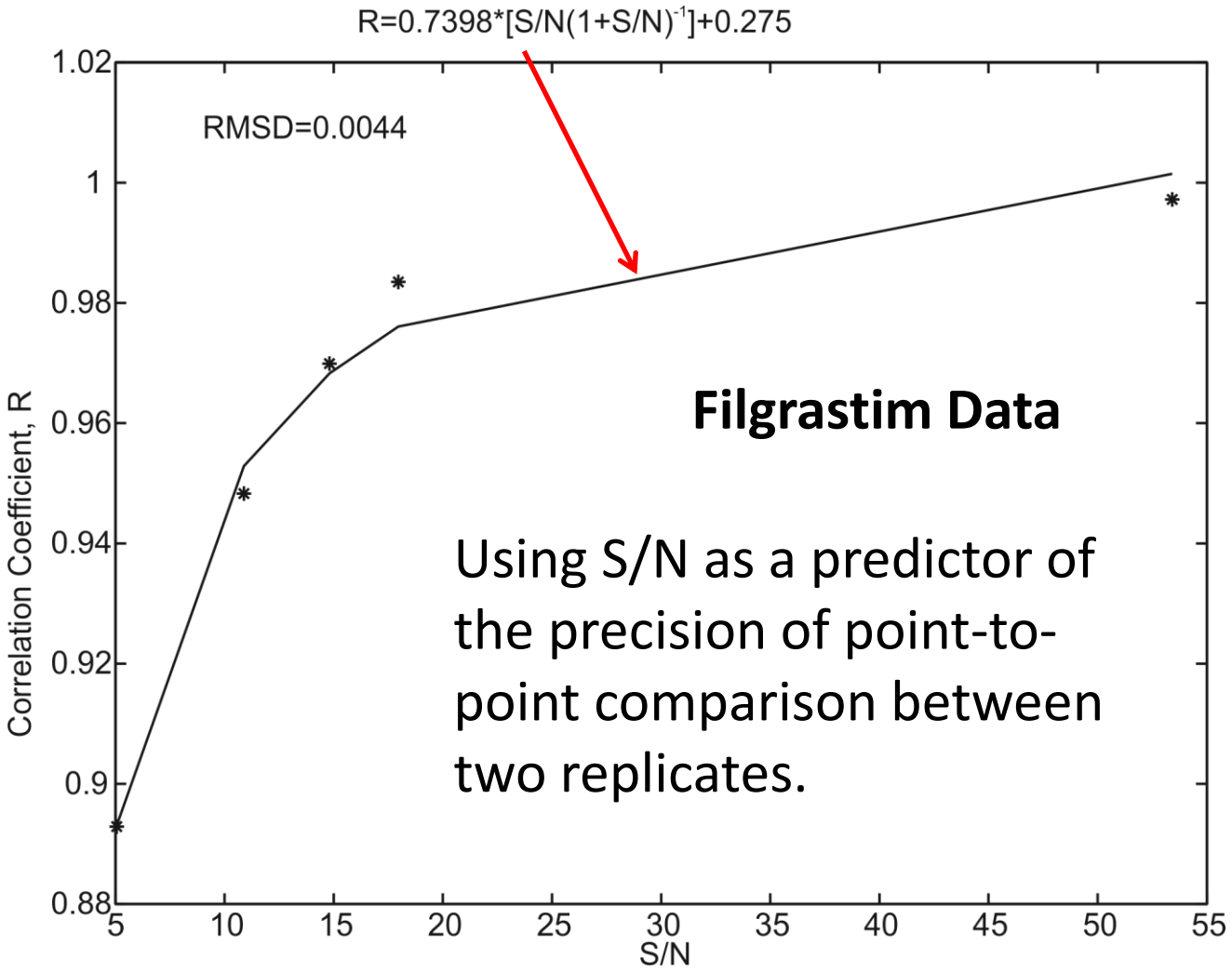


# Principal Component Analysis for Comparability: Filgrastim Products vs the System Suitability Sample




FDA500 (dark blue), NIST900 (light blue), NIST600 (green/yellow), and MPA600 (orange). $^{15}$N-G-CSF are colored red.

# Estimating Spectral Correlation from Signal-to-Noise

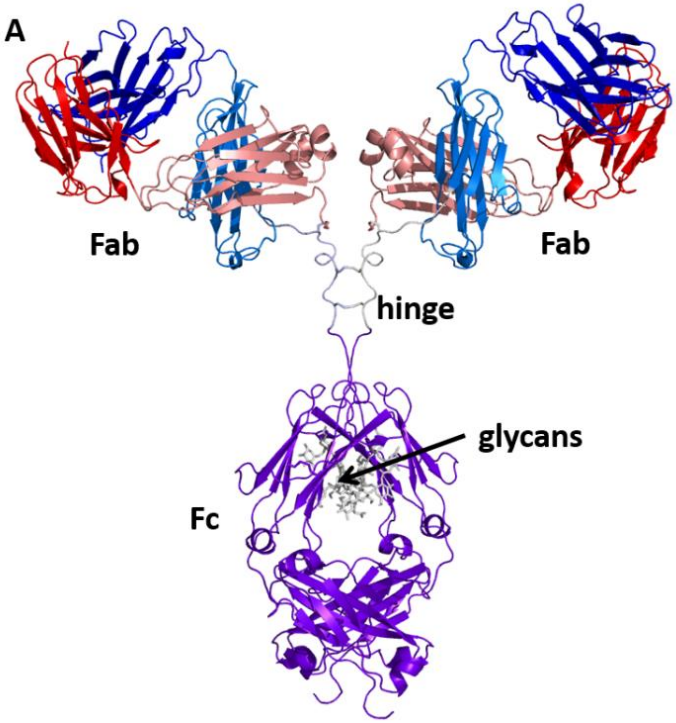



Adapted from equations to estimate S/N in from the signal correlation in a 2 channel Gaussian system. Bershad and Rockmore (1974) IEEE trans. Informatin Theory, p. 112

# What about monoclonal Antibody Drugs?

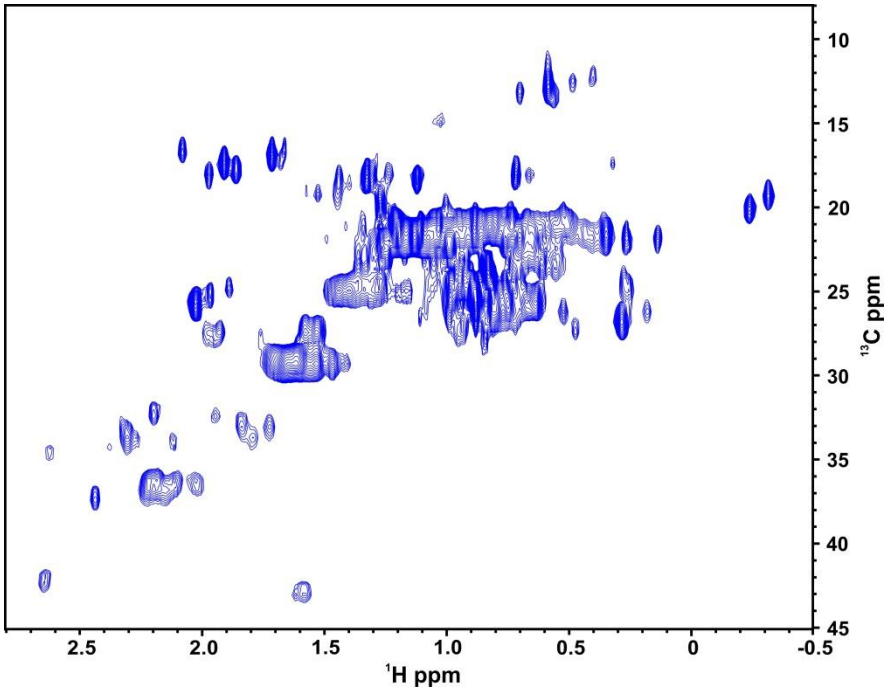
- **General Perception of NMR Spectroscopy of Large Biomolecules**
  - Practical application to biomolecules ~30 kDa or less
  - For applications above 30 kDa, isotope labeling, perdeuteration is required

- **What is the purpose of the measurement? Structure Determination versus Structure Assessment Tool**
  - If desire a spectral map for comparability, the NMR spectral fingerprint becomes an accessible option.



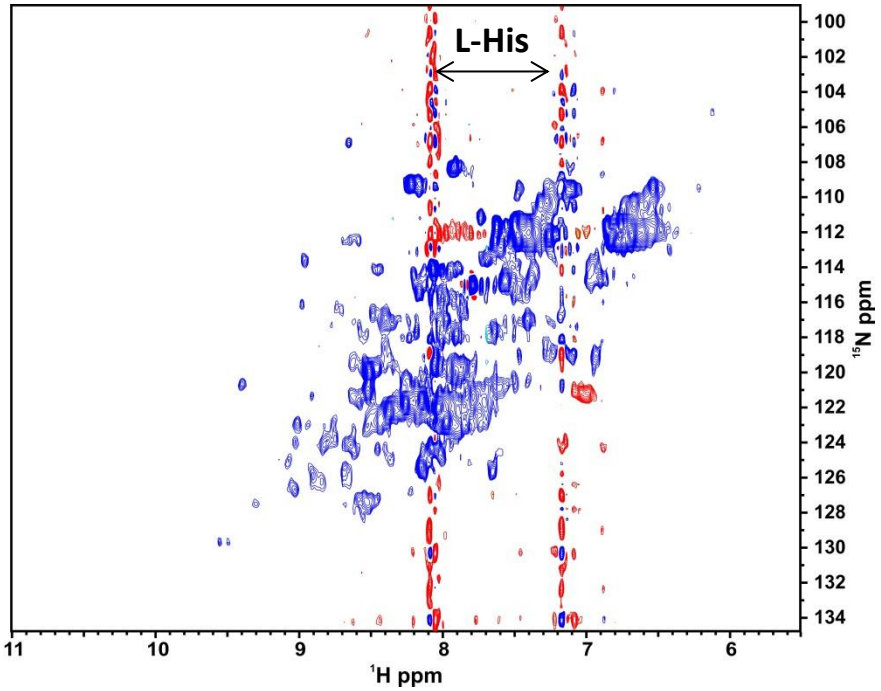
# Intact mAb: Standard Mapping Methods are not Practical at Natural Isotopic Abundance

**$^{1}H$-$^{13}C$ methyl HSQC spectrum**




$^{1}H$-$^{13}C$ methyl HSQC spectrum of the NIST standard mAb at 50 $^{o}C$

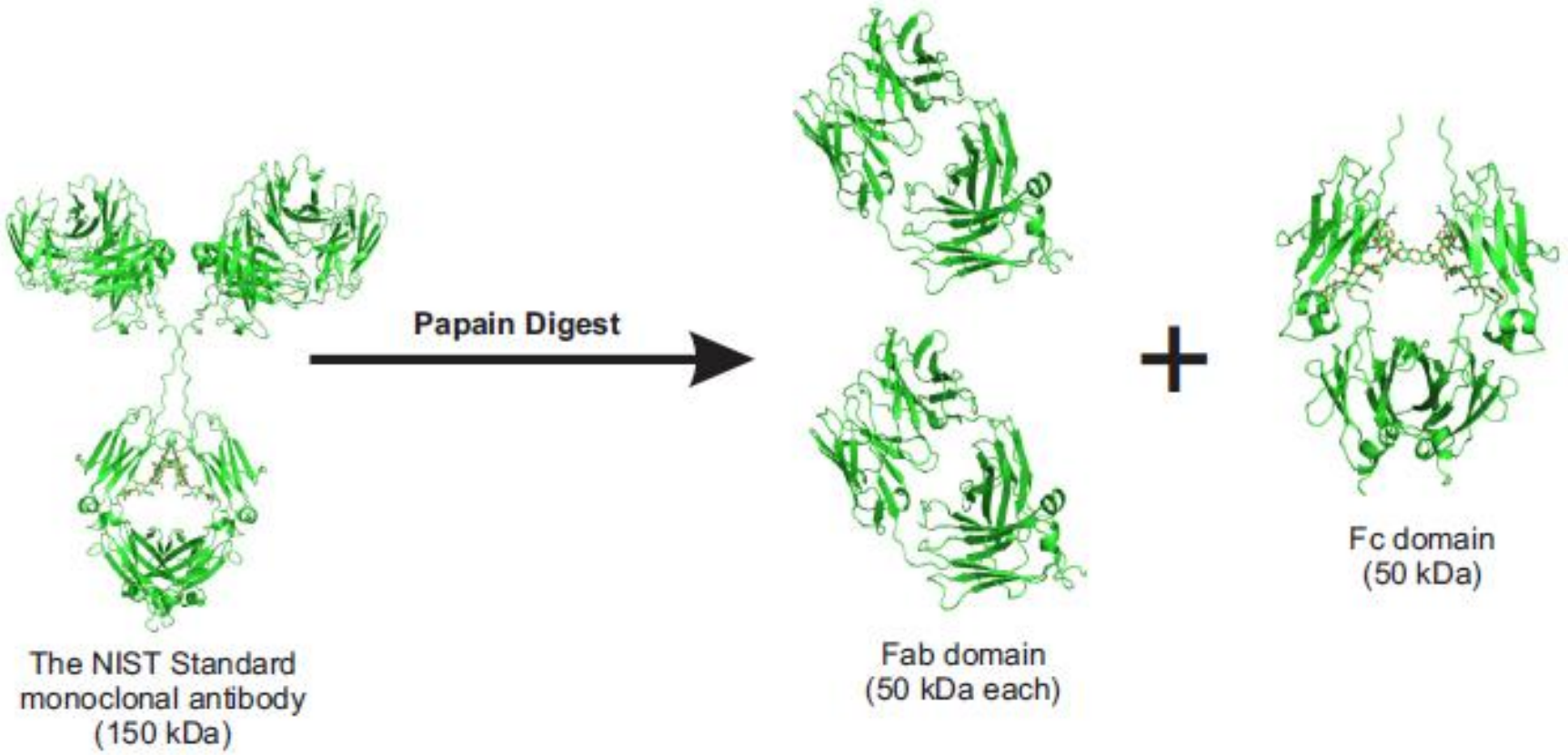
**$^{1}H^{N}$-$^{15}N$ SOFAST-HMQC**




$^{1}H^{N}$-$^{15}N$ SOFAST-HMQC spectrum of the NIST standard mAb at 50 $^{o}C$

**mAb ~ 150 kDa and Dynamic**

# Fab/Fc fragmentation can be accomplished by a simple Selective Protease (Papain digestion)

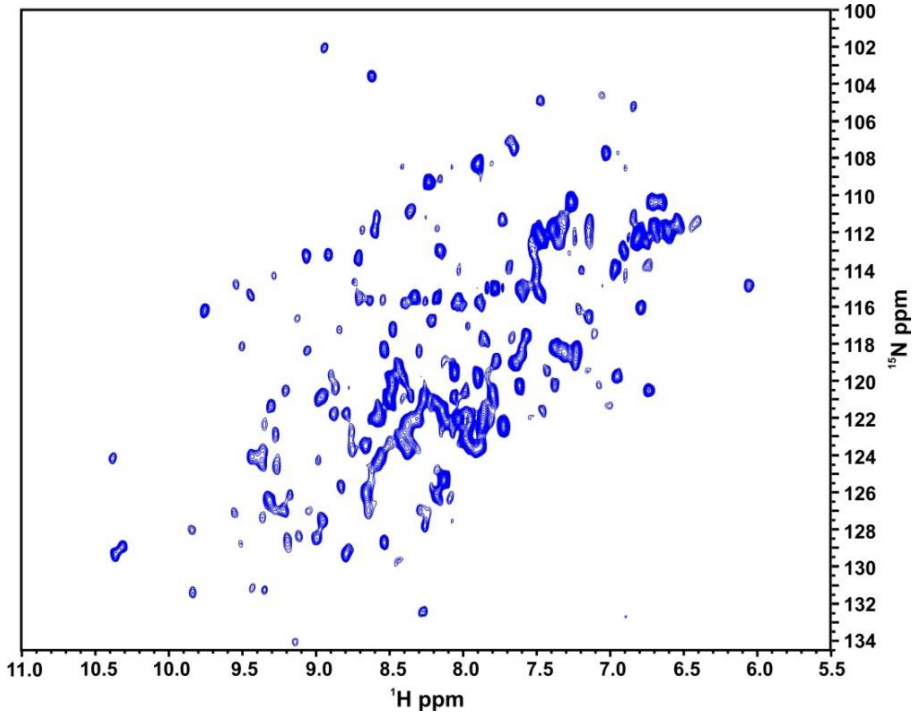



- In mass spectrometry language, let's try a "middle down approach"
- Use the protease Papain to effect cleavage at the hinge region

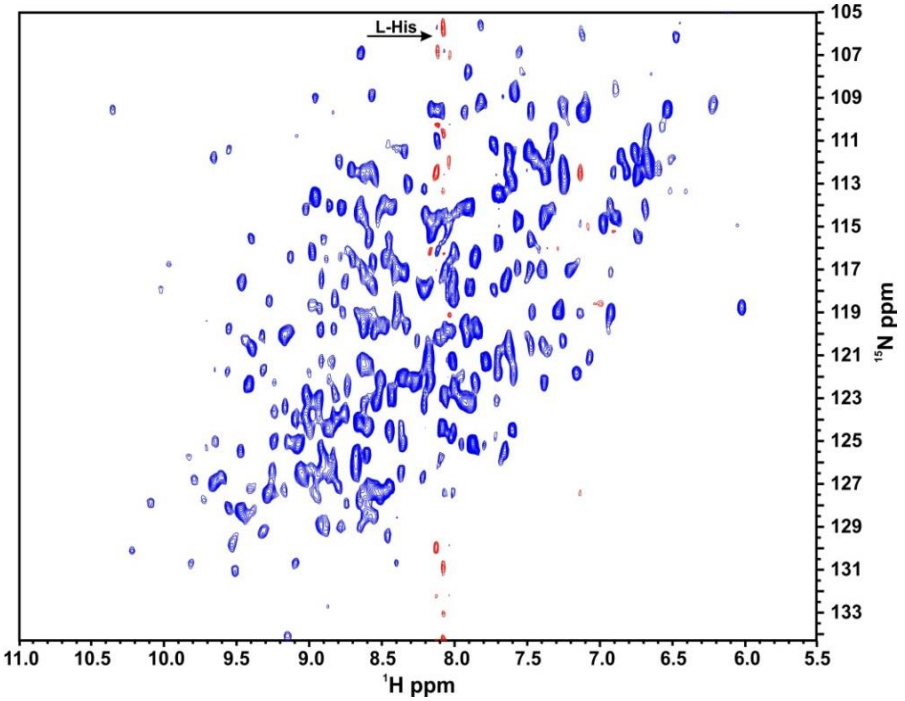
**Goal: Minimal sample manipulation**

# Fc and Fab Domain $^{15}$N-HSQC Fingerprinting

Fc Amide Region



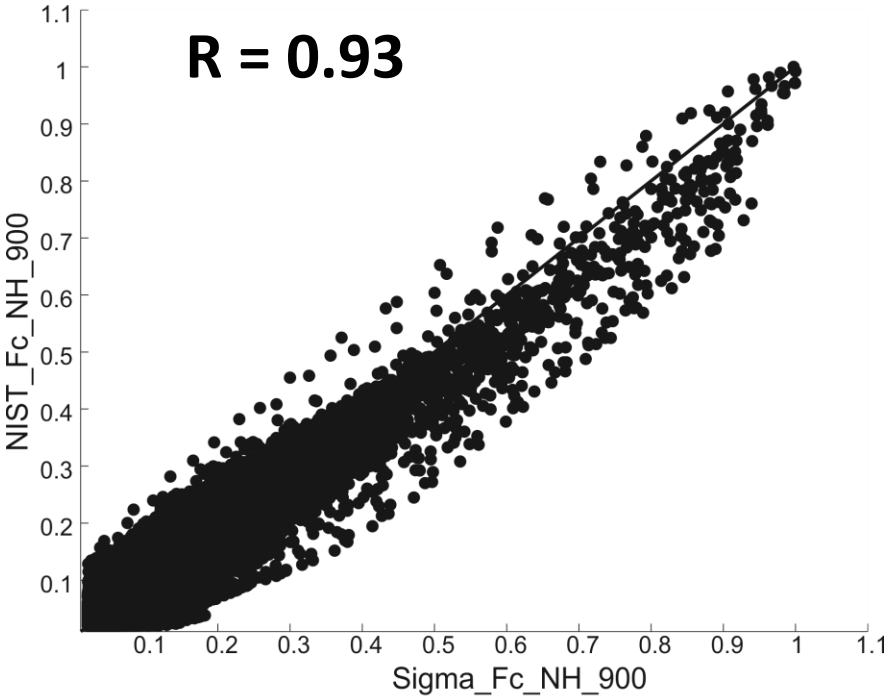

Fab Amide Region

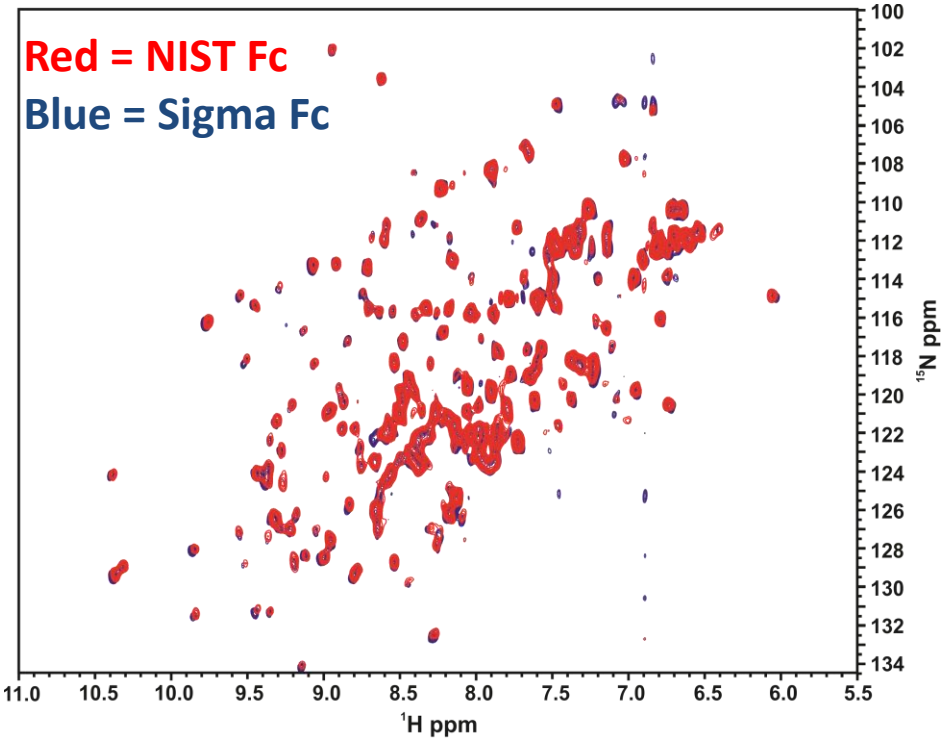



- All data was collected on a **900 MHz** spectrometer at 50° C at a concentration of ~0.5 mM in 25 mM L-histidine (*d3)* , pH 6.0. Total experimental time ~24 hrs using standard SOFAST or BEST pulsing techniques

- A standard spectral fingerprint (HSQC) experiment would take ~ 127 hrs!

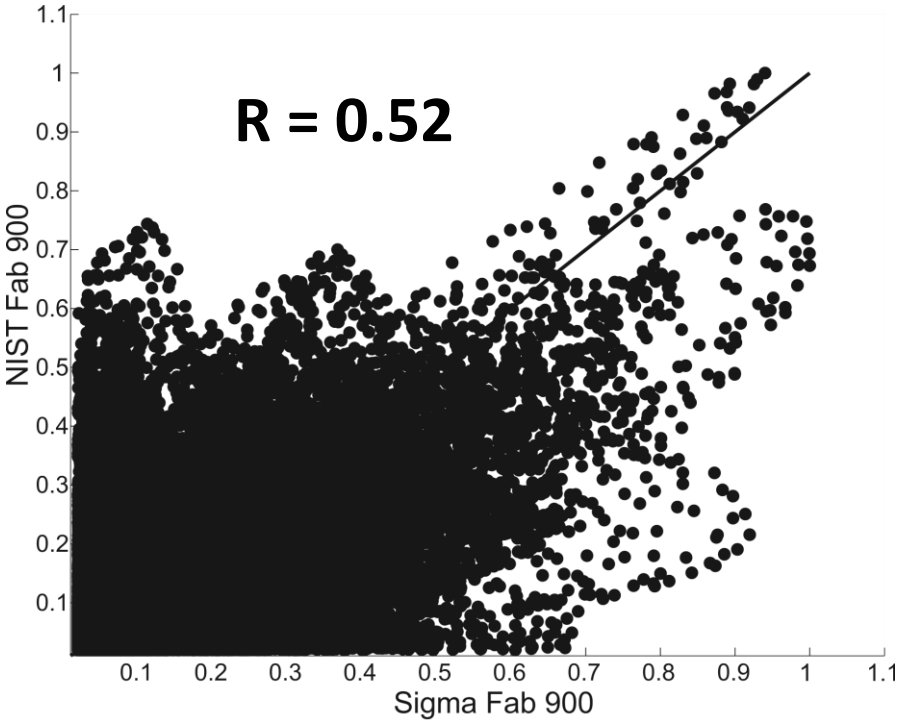
# Fc Region: Comparison of NIST mAb and a IgG1κ poly Ab Spectra (Sigma)

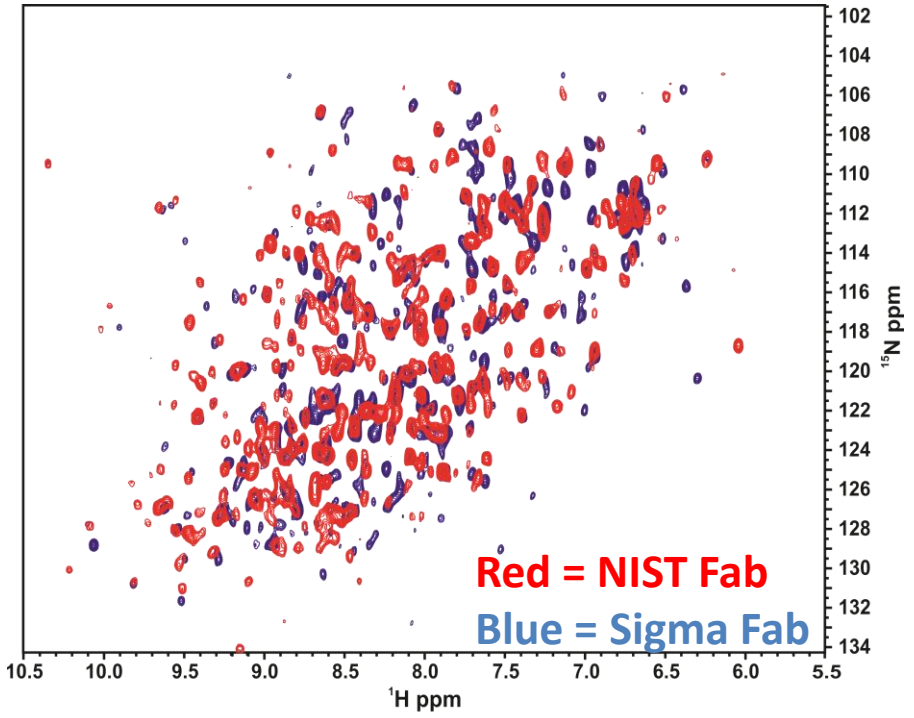



- Visually, the spectra look very similar
- Pearson linear correlation coefficient calculated between normalized datasets

# Fab region: Comparison of NIST mAb and a IgG1κ poly Ab Spectra (Sigma)




While the Fc regions from the NIST and Sigma mAb sample spectra are highly similar, as expected the Fabs are highly dissimilar due to sequence variation

# Summary

**NMR can be used as a high resolution structure probe of primary, secondary and higher-order protein structure**

**NMR methods for comparability assessment:**

- Simple & Robust … Fast
- Lab to Lab Reproducibility
- Natural Abundance (no isotope labeling)
- Tailored Correlations (Signal Filtering/Selection)

**Need to establish standard experimental protocols**

- Don't want to use your NMR as a pH meter or thermometer!

**NMR Fingerprinting of Fc and Fab regions is practical**

- Allows the tracking of structure, including glycosylation
- Applicable at **600 MHz**, the "workhorse" NMR spectrometer
- $^{13}C$ Methyl maps can take less than one hour using NUS

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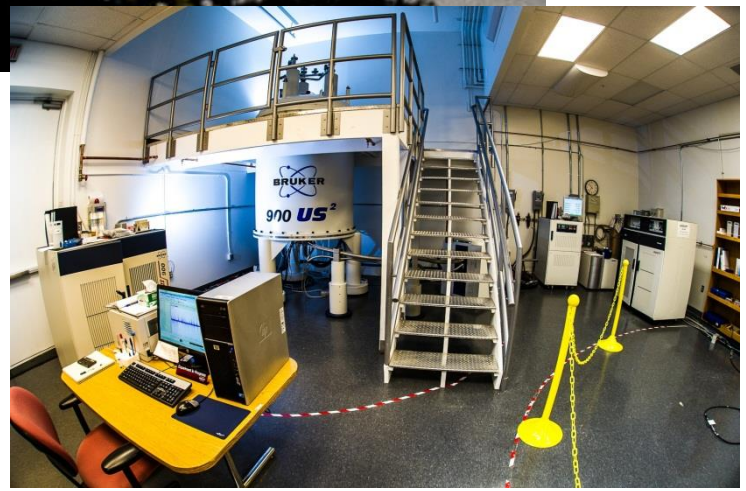
Ian McEwen (MPA-Sweden)

Wagner Group (Harvard)

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