Winter School on Structural Cell Biology

Practical course on liquid NMR spectroscopy of proteins

Learning about the hardware – 20-30 minutes

A) NMR Spectrometer

1) Magnet
2) Console
3) Probehead
    i. Cryoprobe vs. room temperature
    ii. TXI vs. TXO
    iii. ^{1}H, ^{13}C, ^{15}N, ^{19}F, ^{31}P
4) Tubes
    i. 3 mm
    ii. 5 mm
    iii. Shigemmi
    iv. 1.7 mm, 10 mm, shaped tube

B) Before the measurement – **15-20 minutes**

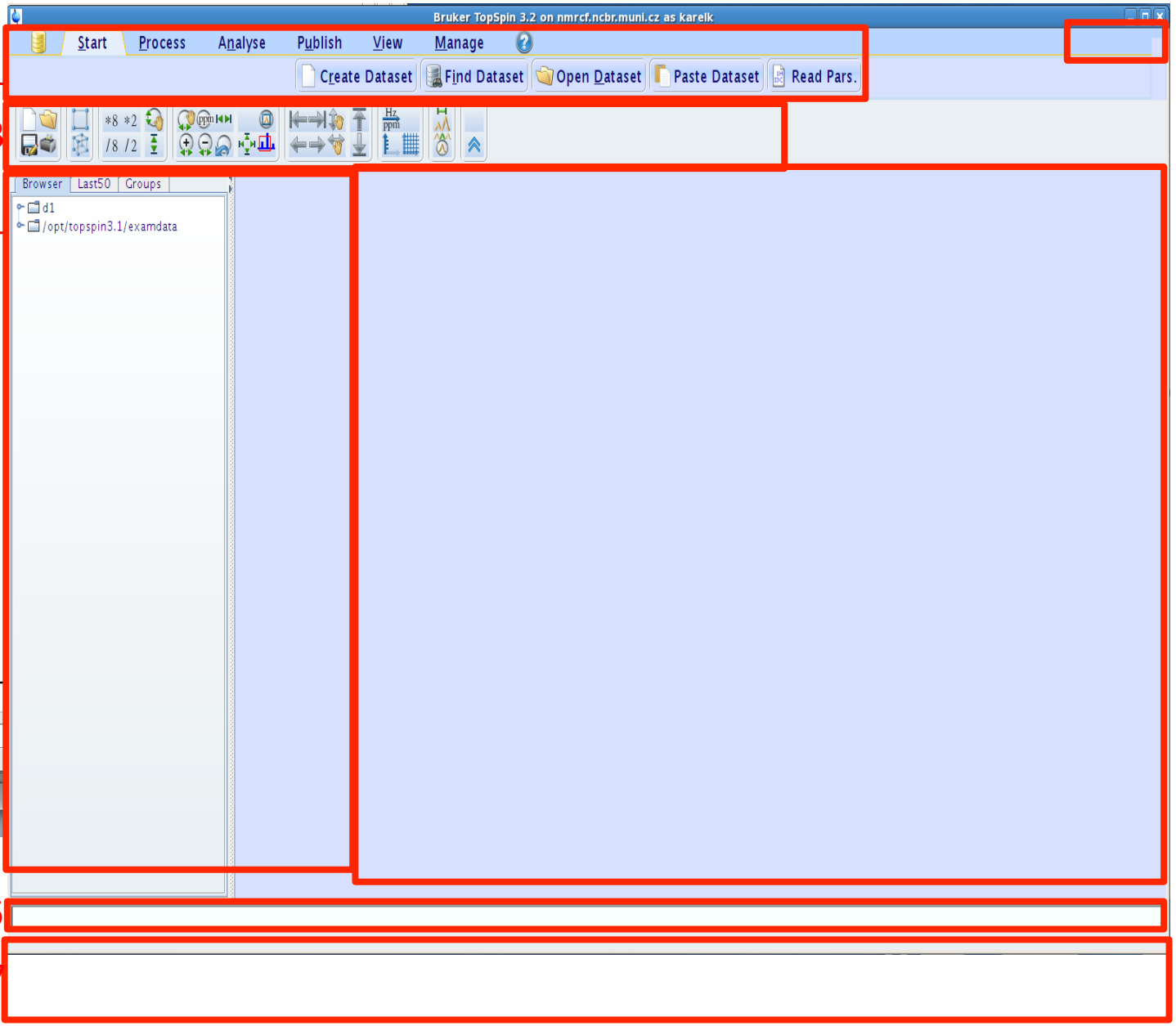
⇒get to know as much about the sample as possible

1) Optimal temperature
2) Salinity
3) Concentration
4) Buffer (phosphate, Tris, Hepes etc.)
5) Solvent for lock (D2O vs. H2O/D2O 90/10)
6) Isotopes that can be measured (what has been labeled
    i. ^{1}H
    ii. ^{15}N only
    iii. $^{13}C+^{15}N$
    iv. Specific labeling (ILV – Ile-Leu-Val)

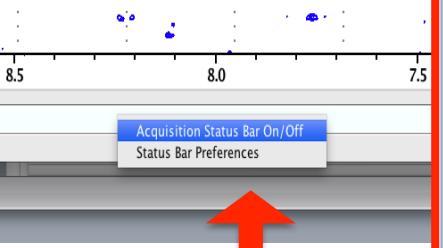
C) Preparing the sample – **10-15 minutes**

1) Measure the pH of the sample
2) Transfer the sample into the NMR tube
3) Spin the tube (optional) in the hand-driven centrifuge
4) Check the difference between the rotors
    i. White (5 mm / 3 mm) ~21 g
    ii. Blue ~ 14.5 g
    iii. Porcelain – for high temp.
    iv. 1.7 mm rotor (curiosity)
    v. Shaped tube rotor – for salty samples (see the positioning)
5) Adjust the rotor position with the tube in the gauge

Topspin 3.X – layout description



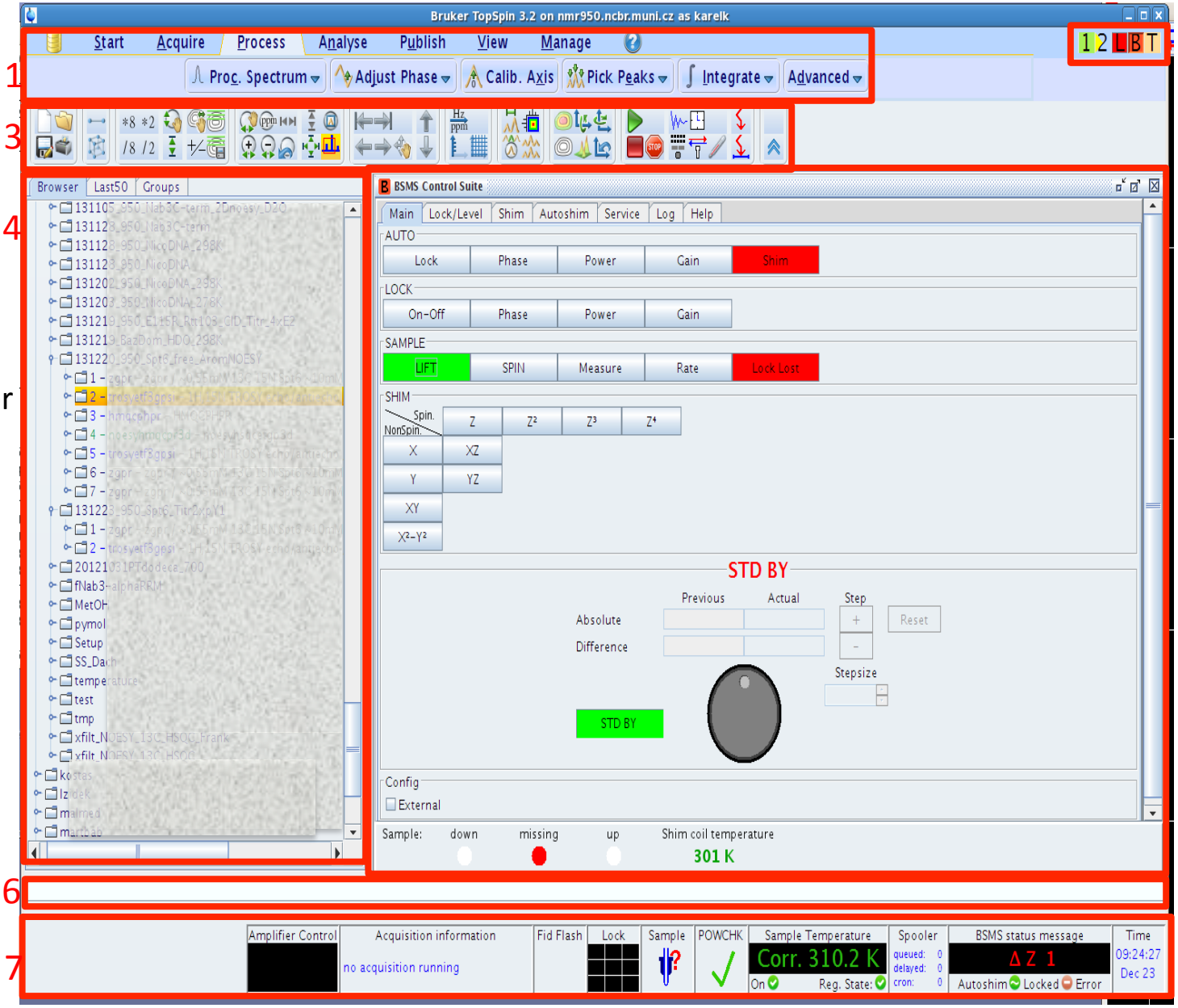
1 – Menu/submenu bar
2 – Windows navigator
3 – Toolbar
4 – Browser
5 – Data/window area
6 – Command line
7– Status panel



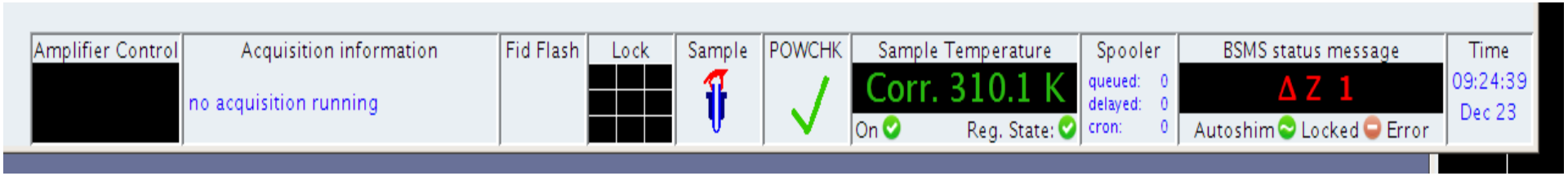
Right mouse click in the status bar area toggles it on/off

Topspin 3.X – layout description

1 – Menu bar
2 – Windows navigator
3 – Toolbar
4 – Browser
5 – Data/window area
6 – Command line
7– Status panel

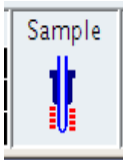


- Prior any manipulation with the spectrometer or TopSpin, check the status panel first – namely, what is the acquisition status, temperature and what is the lock signal status.

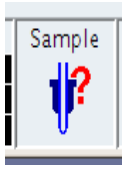


- In case there is a sample of your colleague still in the magnet, take it out from the magnet (*vide infra*) and take best care about the sample.

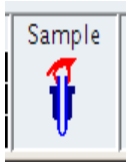
Sample status:



down

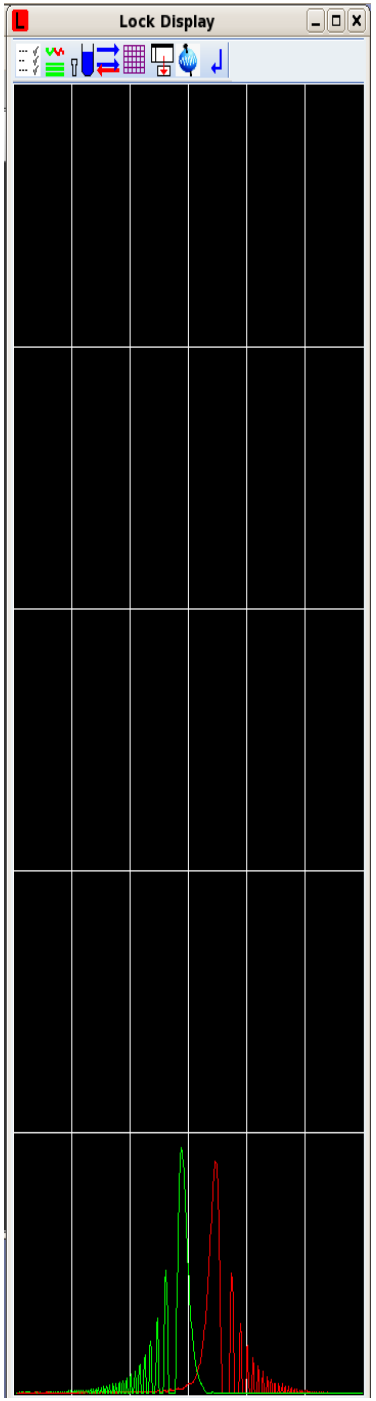


missing



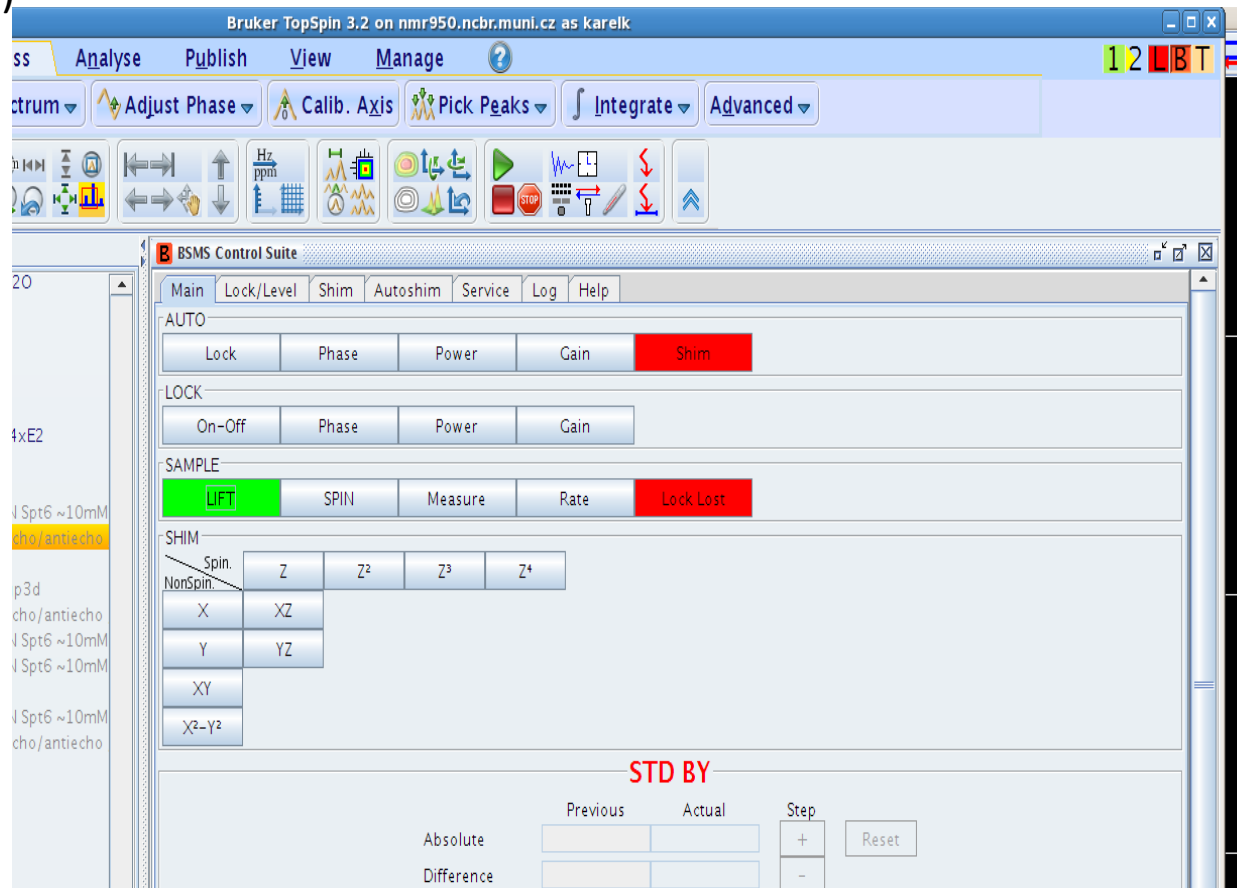
up

- To eject the sample from the magnet – assure yourself there is no acquisition running, the lock is off (no straight line is sweeping the lock window but dispersive sinusoidal curves can be seen in the lower area of the lock window – see the figure on the right) and there is no mechanical obstacle that could prevent sample ejection. Then either type **ej** on the cl or use the BSMS window to manipulate with the lift.



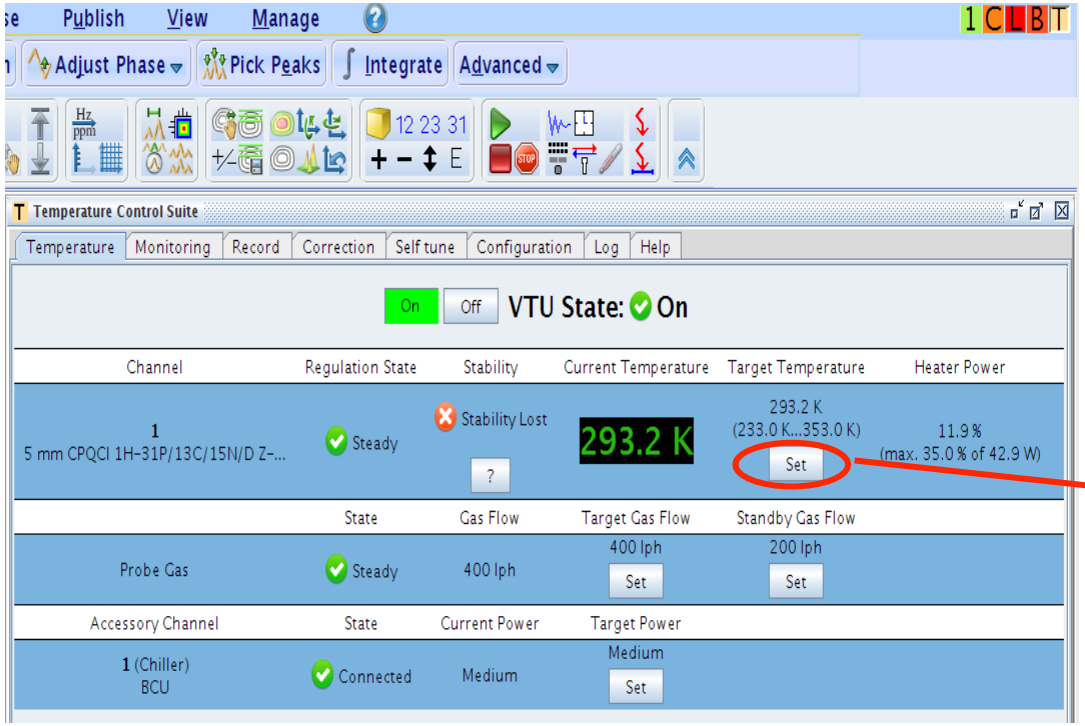
Ejecting the sample

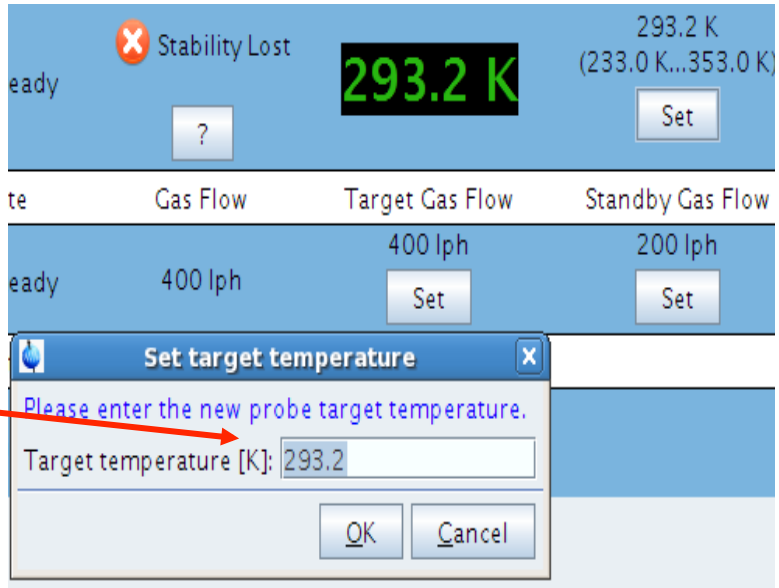
- In the Main panel of the window B (BSMS window – Bruker Smart Matching /Shimming system), click Sample - Lift for turning on the airflow (button turns green)
- Sample mail will be set in action and brings the sample down
- Spectrometers that have no sample mail will announce the sample ejection by increased airflow and sample „dancing“ on the top of the magnet. In this case, after removing the sample from the magnet, do not forget to switch off the airflow by clicking the Lift button once again (turns grey)

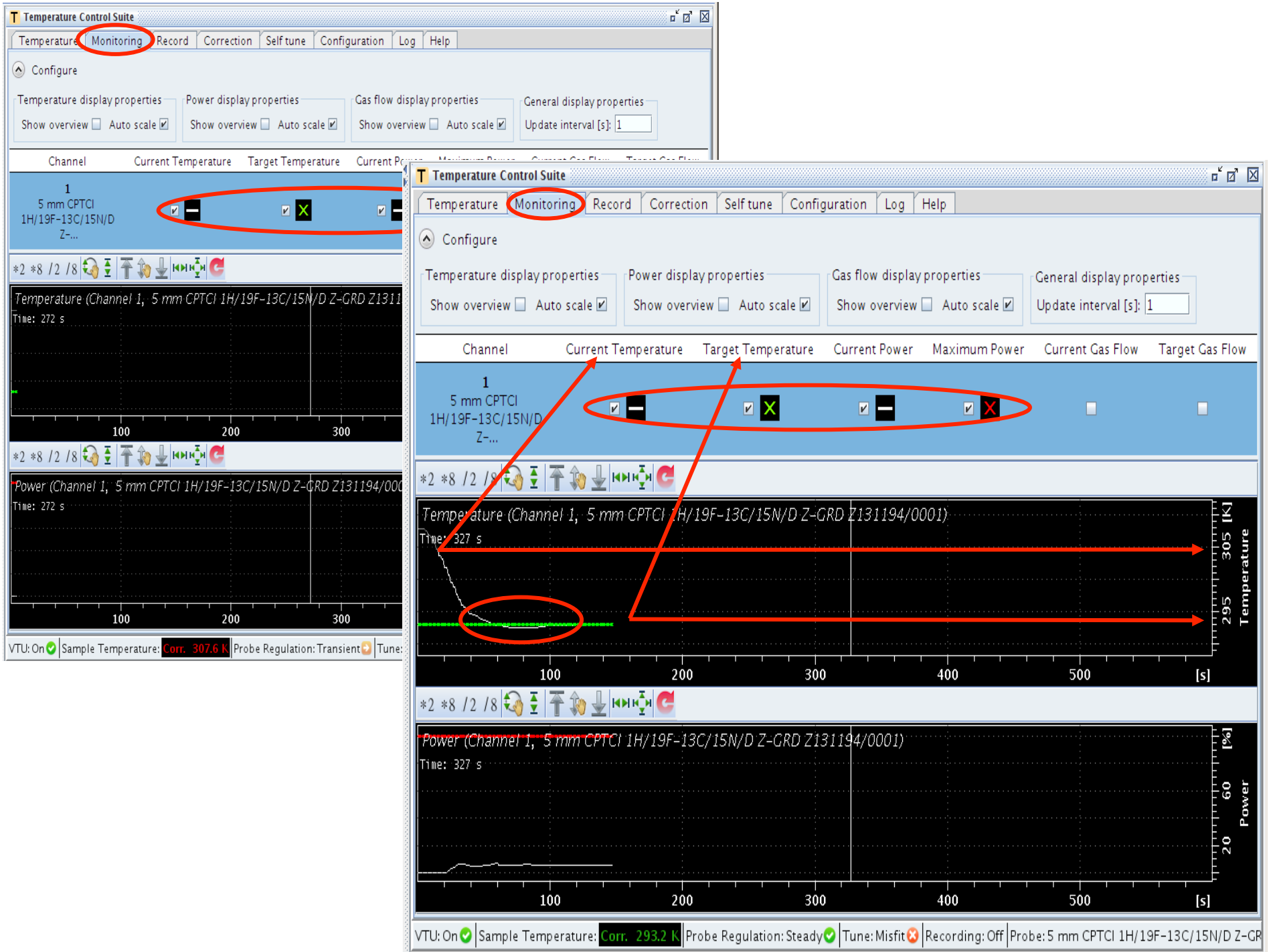


Temperature control

- Once there is no sample in the magnet, set the temperature you want for your sample/measurement – this is done by opening the Temperature control window. This can be done by ***i)*** double click the Sample Temperature window in the status panel, ***ii)*** type **edte** in the command line (cl). This will open new window called **T**. Set the temperature and in the monitoring tab check first four check-boxes and control the progress of temperature. As soon as the temperature is stabilized, you may insert your sample into the magnet.





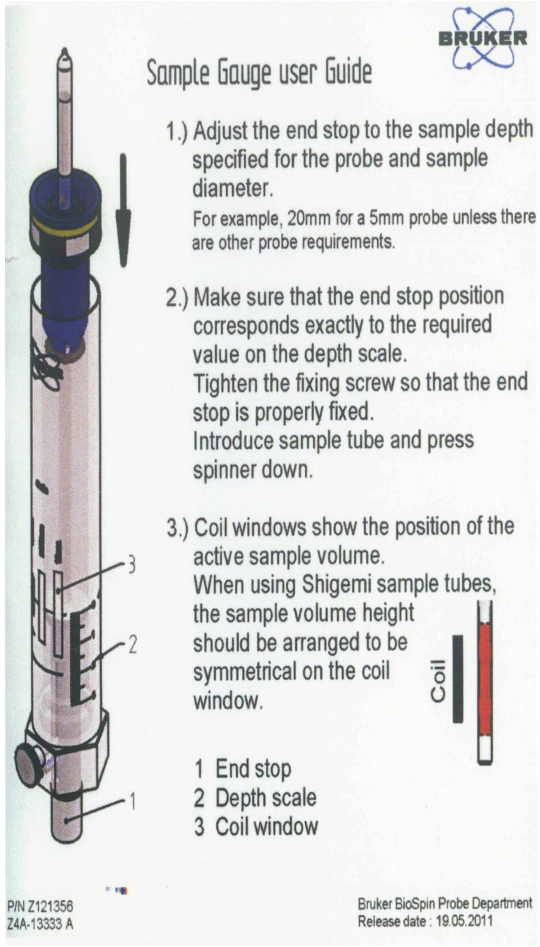

Temperature Control Suite
Temperature
Monitoring
Record
Correction
Self tune
Configuration
Log
Help
Configure
Temperature display properties
Show overview
Auto scale
Power display properties
Show overview
Auto scale
Gas flow display properties
Show overview
Auto scale
General display properties
Update interval [s]: 1
Channel
Current Temperature
Target Temperature
Current Power
Maximum Power
Current Gas Flow
Target Gas Flow
1
5 mm CPTCI
1H/19F-13C/15N/D
Z-...
Temperature (Channel 1, 5 mm CPTCI 1H/19F-13C/15N/D Z-GRD Z131194/0001)
Time: 327 s
Temperature [K]
305
295
100
200
300
400
500
[s]
Power (Channel 1, 5 mm CPTCI 1H/19F-13C/15N/D Z-GRD Z131194/0001)
Time: 327 s
Power [%]
60
20
VTU: On
Sample Temperature: Corr. 293.2 K
Probe Regulation: Steady
Tune: Misfit
Recording: Off
Probe: 5 mm CPTCI 1H/19F-13C/15N/D Z-GR

Sample/tube handling

- Inserting the tube into the spinner
  - Do not push the tube straight down the spinner, the tube may break down
  - Screw the tube slowly into the spinner and at the same time gently push the spinner upward the tube

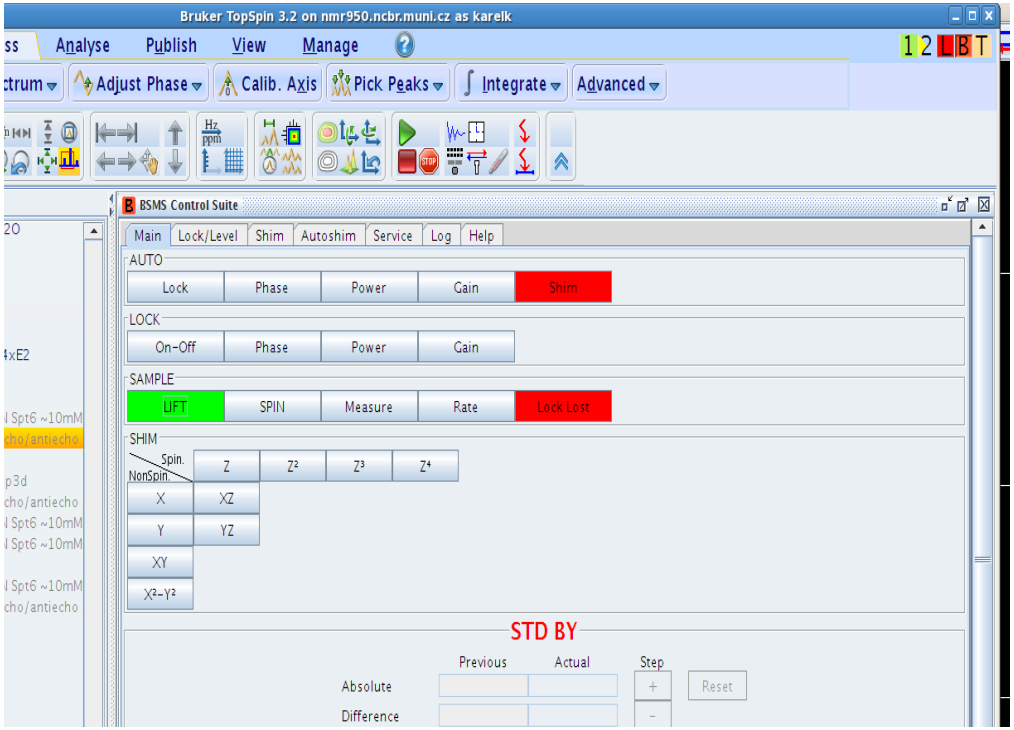
Adjusting/centering the tube in the spinner

- Put the tube with the spinner into a depth gauge and screw the tube until the length of the sample is symmetrical around the middle line crossing the coil-representing boxes



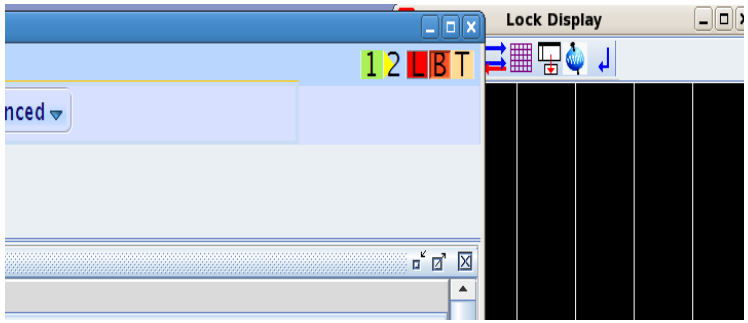
Inserting the sample

- After the desired temperature is reached, in the Main panel of the window B (BSMS window – Bruker Smart Magnet System), click (skip this in case of sample mail) Sample - Lift for turning on the airflow (button turns green)
- Place the sample either into the sample mail or on the top of the magnet bore when „maximum“ airflow is reached
- Turn off the airflow by clicking the Lift button again (goes gray again)
- For the magnets equipped with sample mail, place the spinner with the tube into the sample rack and close it
- Wait until the sample is positioned in the probe



Windows navigator

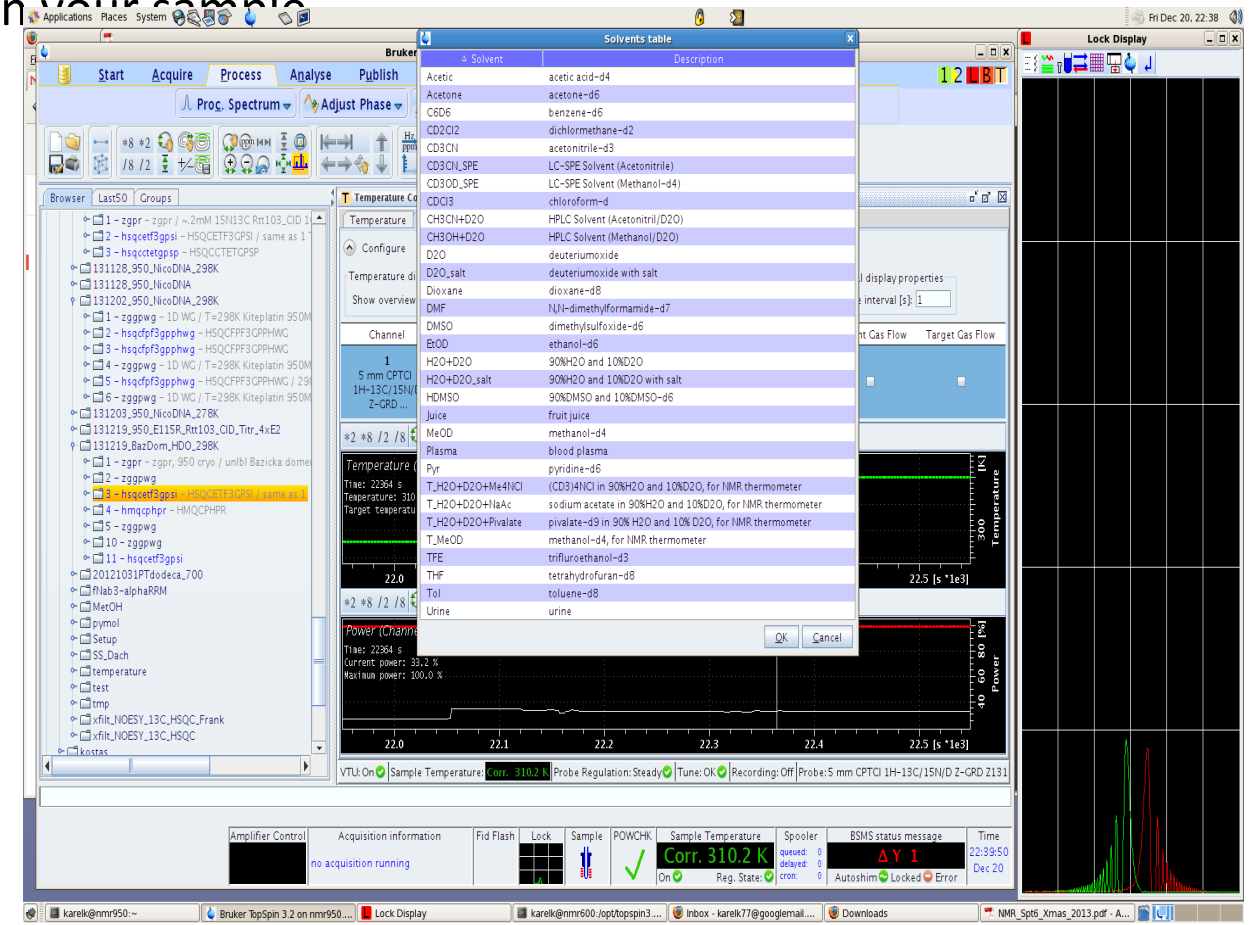
- Window B
  - represents BSMS display, called by command **bsmsdisp**; provides info about lock, field, shim-control, etc.
- Window T
  - temperature control window; called by **edte**
- Window L
  - lock-level window, called by **lockdisp**
- Numbered windows
  - Spectra, left pointing triangle indicates window with acquisition in progress or in no acquisition running, where was is running.



As most of the above windows are needed every time one is measuring and typing the commands to open them up is boring and timewasting, one may write a macro to open these with on command (e.g. **kk**, ‚**edmac kk**‘ to see details)

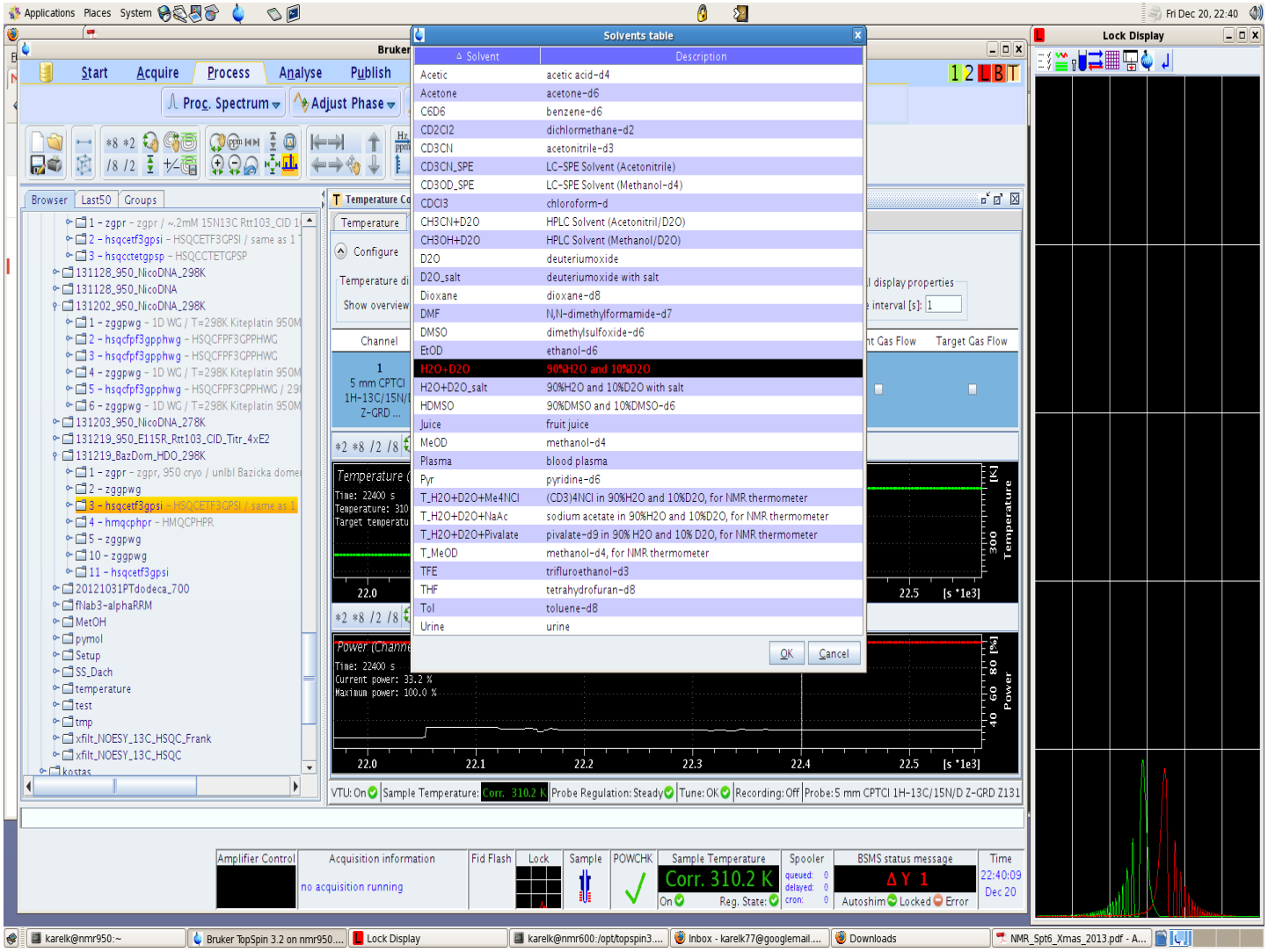
Locking the magnetic field

- Locking on the reference nuclei (deuterium)
- Once the sample is in the magnet (check Sample status icon on status bar, status LED of the sample mail etc.), usually lock signal appears as red/green signal in dispersion mode. In case you do not see that line – it may indicate that previous user was using different deuterated solvent or there is no ^{2}H in your sample
- Type **lock** on the cl



Locking the magnetic field

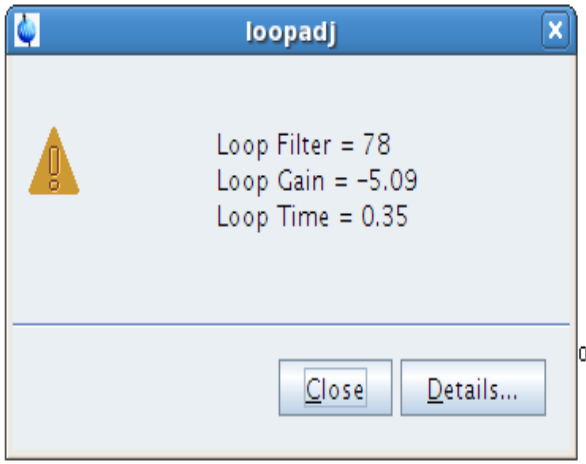
- Table with solvents will pop up
- Select the right solvent from the table and click the OK-button or double click the solvent of choice



Homogeneity of the field

Adjusting homogeneity of the magnetic field – as the more homogeneous magnetic field results in a narrower lock signal which results in a higher d.c. voltage, one aims for an optimum lock signal by adjusting various shim currents

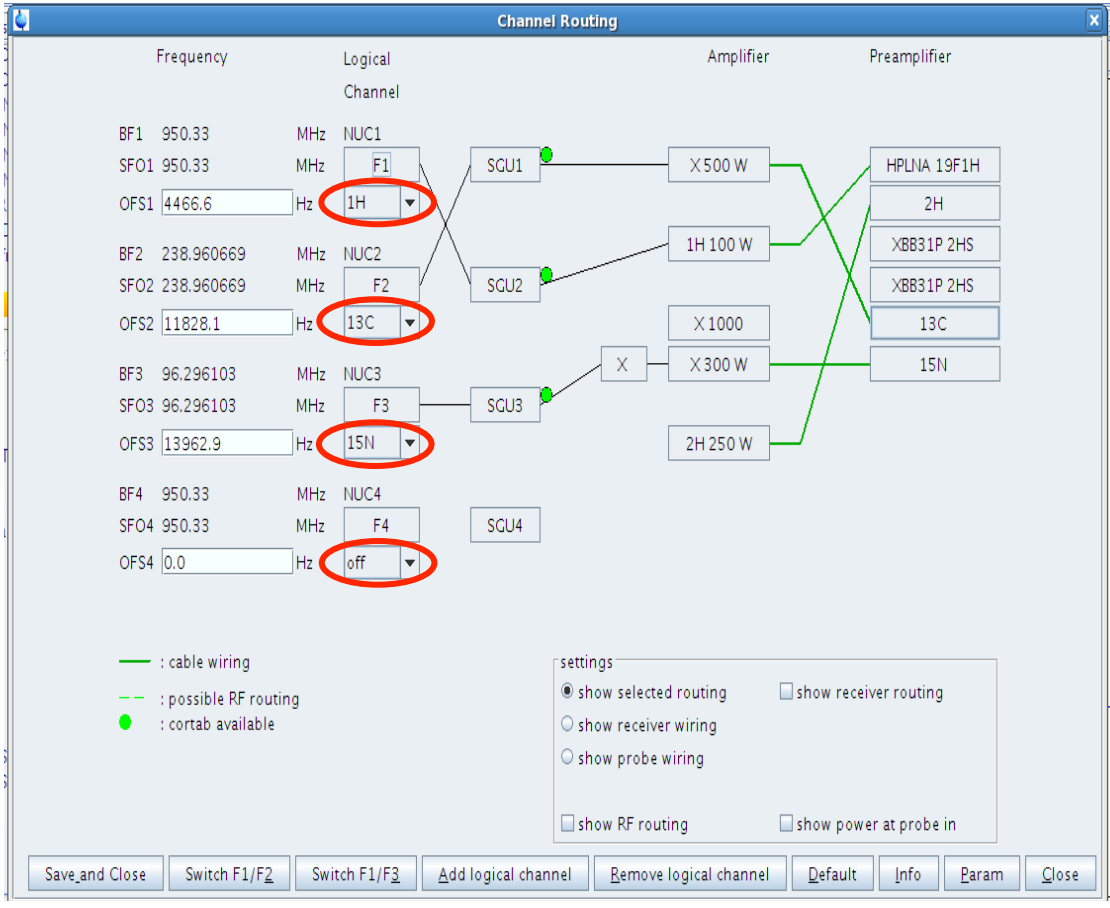
- Should you be brave and experienced enough, do it manually in the BSMS display via the Shim tab or (recommended) use the topshim option
- Type **topshim** on the cl – runs automatic 1D shimming
- Once the topshim finished switch on the the Autoshim in the Autoshim tab
- As there may be needed to adjust the lock phase, lock gain and other parameters, type **loopadj** on the cl (optimizes lock phase, lock gain, loop phase, ...) which will take care about all of it. In case there is sufficient lock signal, menu with three lines will appear to confirm everything went smoothly and was set up. Should there be low lock signal, error message will appear.



Selecting the nuclei

- Prior starting any set up, pulse checks or measurements, make sure the channel routing is correct and all the channels that are going to be measured are active.
- To do so, type **edasp** on the cl
- set the desired nuclei from the pop-up menus: typically channel 1 – ^{1}H, channel 2 - ^{13}C, channel 3 – ^{15}N
- Click on Default to set proper routing

for the channels and Save and Close the settings.

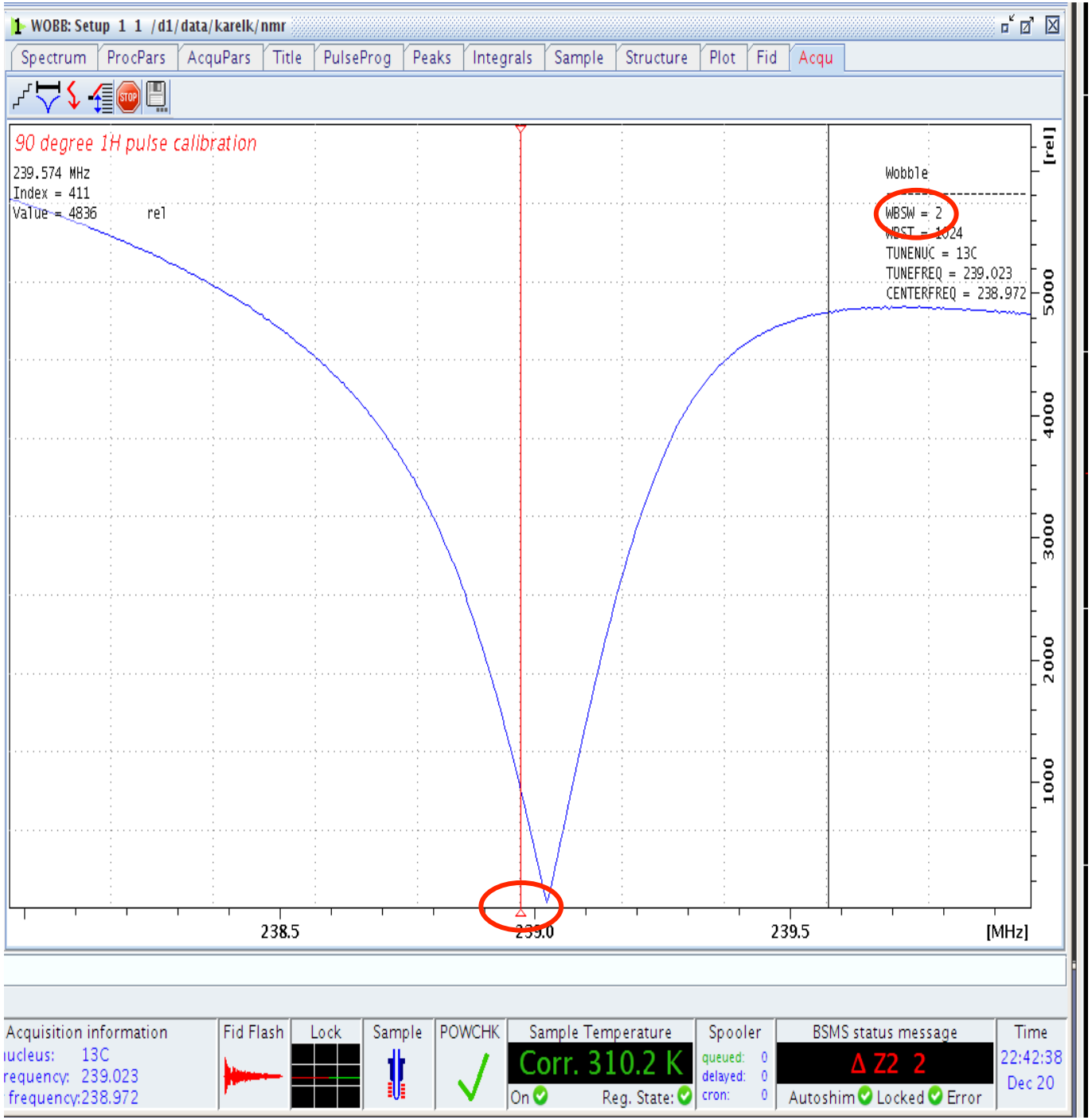


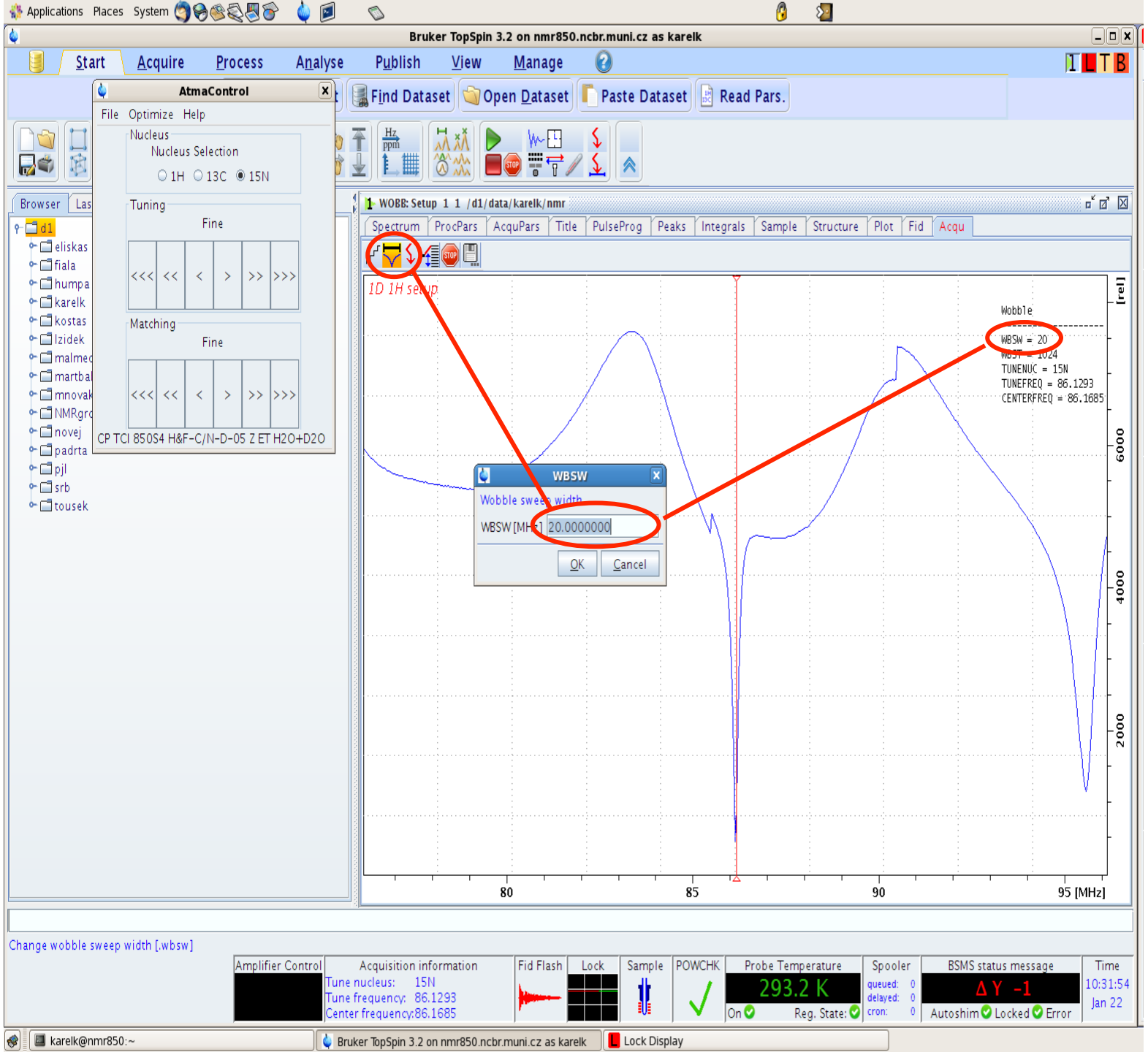
Tunning and matching the probe

- For obtaining optimal signal-to-noise ratio, one needs to tune the probe with the sample inserted. It is done by adjusting two mutually interactive capacitors. One tunes the circuit to the desired resonance frequency (**tuning**) and the other matches the impedance (**matching**). Tuning/Matching can be achieved either manually directly on the probehead that is NOT equipped with ATM unit or automatically from a PC in case the probehead IS equipped with the ATM unit. Automatic Tuning/Matching Automatically (atma)
- Start with **atma** (AutoTuneMatchAuto)on the cl – typically, this will tune the probehead automatically without any human intervention
- Once atma is done, it is recommended to check the tuning manually
- Type **atmm (**Automatic Tuning/Matching Manual**)** on the cl – window with a black wobbling curve that needs to be ftuned to the minimum indicated by vertical red line is opened
- The precision of the displayed curve is driven by wobble sweep width. Set it prior wobbling by command **wbsw** on the cl to 2 MHz or click wbsw icon and set it there.
- N.B. Sometimes the atmm gets stuck when trying to change the sweep width. Using of following command and its option **atmm manwbsw** has proven to improve the wobble behavior. Again, create own macro or use **wkk**.

Tunning and matching the probe

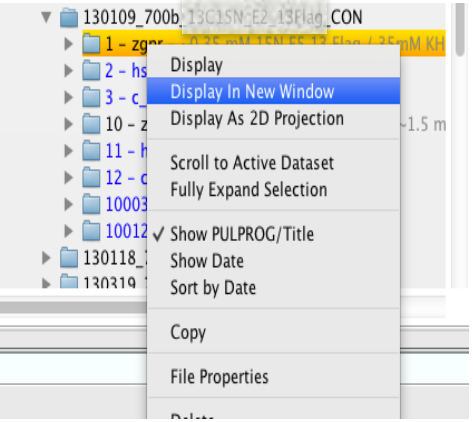
- While tuning the probehead „manually “ with atmm, keep an eye on the lock-level signal. It may drop significantly during the tuning so once finished, run topshim once again. Typically one is tuning the channels starting at lowest frequency (typically, ^{15}N followed by ^{13}C and then ^{1}H) but in case of (cryo)-probeheads with a new design (lock signal is dropping while tuning/matching) are supposed to be tuned from highest frequencies down (i.e. ^{1}H->^{13}C->^{15}N). In any case, check the tuning curve of a given nuclei iteratively.
- Tune/match each channel starting from the nuclei with the lowest frequency (on the 850/950MHz spectrometers in reversed order)
- Adjust the displayed sweep width (parameter WBSW (wobble sweep width) in the upper right corner, *vide infra*)
- Get the minimum of the curve to required position by clicking the arrows that represent expected step of the tune/match
- Start with matching (get the minimum to the bottom), then tune (center the minimum on the reference line). As tune/match are interconnected, the procedure becomes iterative but optimum has to be always reached.
- For samples with high salt [c(NaCl)>=250~300mM], use either shaped NMR-tube or 3mm tube. Both tubes require special rotors and extremely gentle handling!

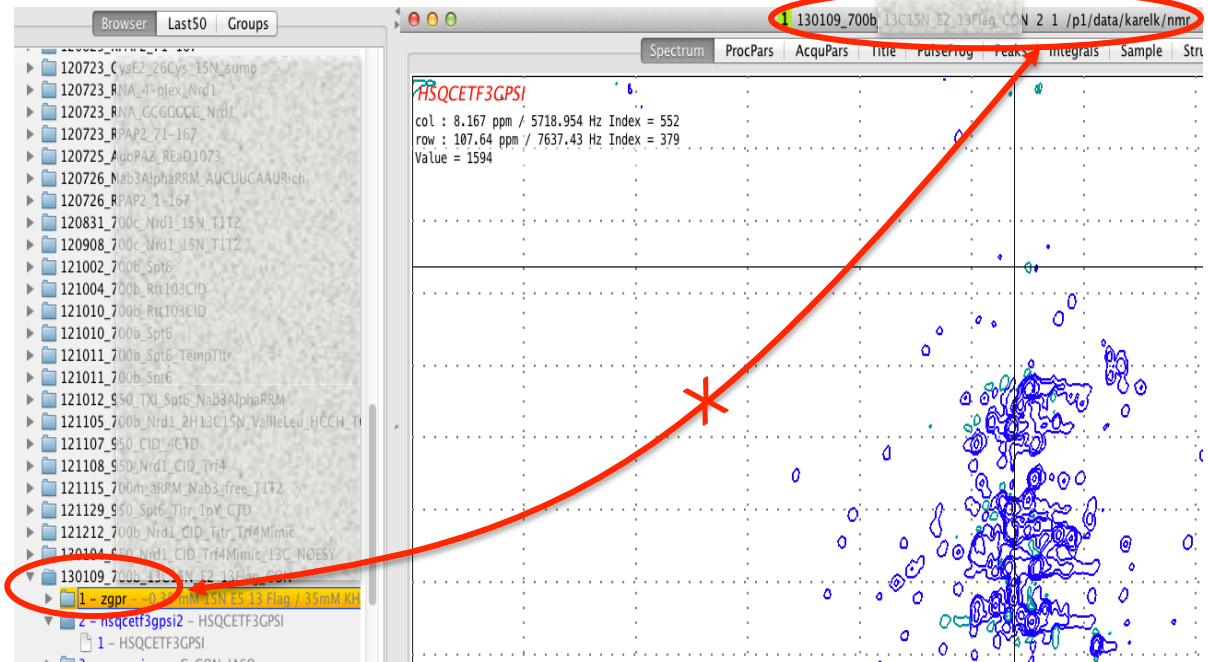

1 WOBB: Setup 1 1 /d1/data/karelk/nmr
Spectrum
ProcPars
AcquPars
Title
PulseProg
Peaks
Integrals
Sample
Structure
Plot
Fid
Acqu
90 degree 1H pulse calibration
239.574 MHz
Index = 411
Value = 4836 rel
Wobble
WBSW = 2
WBST = 1024
TUNENUC = 13C
TUNEFREQ = 239.023
CENTERFREQ = 238.972
[rel]
5000
4000
3000
2000
1000
238.5
239.0
239.5
[MHz]
Acquisition information
nucleus: 13C
frequency: 239.023
frequency:238.972
Fid Flash
Lock
Sample
POWCHK
Sample Temperature
Corr. 310.2 K
On
Reg. State:
Spooler
queued: 0
delayed: 0
cron: 0
BSMS status message
Δ Z2 2
Autoshim
Locked
Error
Time
22:42:38
Dec 20


Applications Places System
Bruker TopSpin 3.2 on nmr850.ncbr.muni.cz as karelk
Start Acquire Process Analyse Publish View Manage
Find Dataset Open Dataset Paste Dataset Read Pars.
AtmaControl
File Optimize Help
Nucleus
Nucleus Selection
1H 13C 15N
Tuning
Fine
<<< << < > >> >>>
Matching
Fine
<<< << < > >> >>>
CP TCI 850S4 H&F-C/N-D-05 Z ET H2O+D2O
Browser
d1
eliskas
fiala
humpa
karelk
kostas
lzidek
malmec
martbal
mnovak
NMRgrc
novej
padrta
pjl
srb
tousek
WOBB: Setup 1 1 /d1/data/karelk/nmr
Spectrum ProcPars AcquPars Title PulseProg Peaks Integrals Sample Structure Plot Fid Acqu
1D 1H setup
Wobble
WBSW = 20
TUNENUC = 15N
TUNEFREQ = 86.1293
CENTERFREQ = 86.1685
WBSW
Wobble sweep width
WBSW [MHz] 20.0000000
OK Cancel
[rel]
6000
4000
2000
80
85
90
95 [MHz]
Change wobble sweep width [.wbsw]
Amplifier Control
Acquisition information
Tune nucleus: 15N
Tune frequency: 86.1293
Center frequency:86.1685
Fid Flash
Lock
Sample
POWCHK
Probe Temperature
293.2 K
On
Reg. State:
Spooler
queued: 0
delayed: 0
cron: 0
BSMS status message
Δ Y -1
Autoshim Locked Error
Time
10:31:54
Jan 22
karelk@nmr850:~
Bruker TopSpin 3.2 on nmr850.ncbr.muni.cz as karelk
Lock Display

Creating a dataset

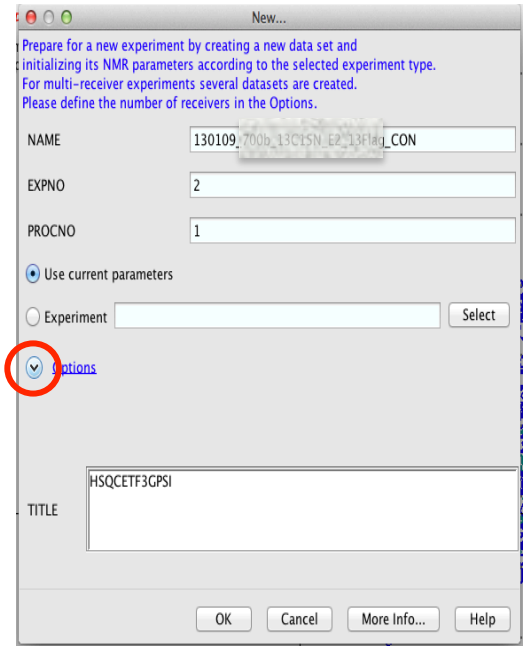
- Steps involving edasp and atma/atmm require an existing dataset. Each user typically keeps in his data-tree directory Setup/Calibration or something similar. In those dataset one usually doesn‘t change more than power-levels, (de)activates nuclei but not much more ...
- If one wants to create a dataset, there are several ways to do it
  - Copy an existing dataset
    - Select a dataset that was working previously ideally by clicking right button in the browser and open a s a new window. Often, different set is selected in browser and different in the window. Always check which spectrum you are working with!!!

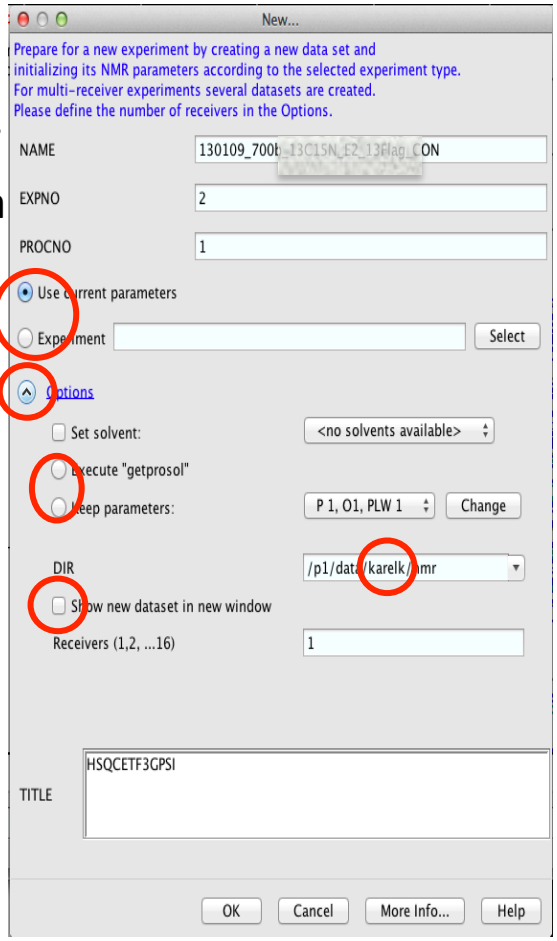




Creating a dataset

- Once being in the right dataset, type **edc** on cl and set the new destination of the experiment, experimental number, title, user
- In case dataset should be copied to the next ExpNo, use comma **iexpno** which will copy the dataset to next ExpNo





- If creating a new dataset, type **rpar** on cl to read standard dataset parameters of a required pulse sequence
- Type **getprosol** on cl to set actual pulse calibration. Should calibration of any nuclei vary from the standard ones (typically ^{1}H), type **getprosol 1H 13.55 13W** which means that 90° ^{1}H pulse is 13.55μs@13W taken as reference.
- Modify the rest of the parameters in AcquPars in Data area (TD – number of points collected during FID, DS – number of dummy scans, NS – number of scans, SW – spectral width, O1P – carrier frequency, ...)

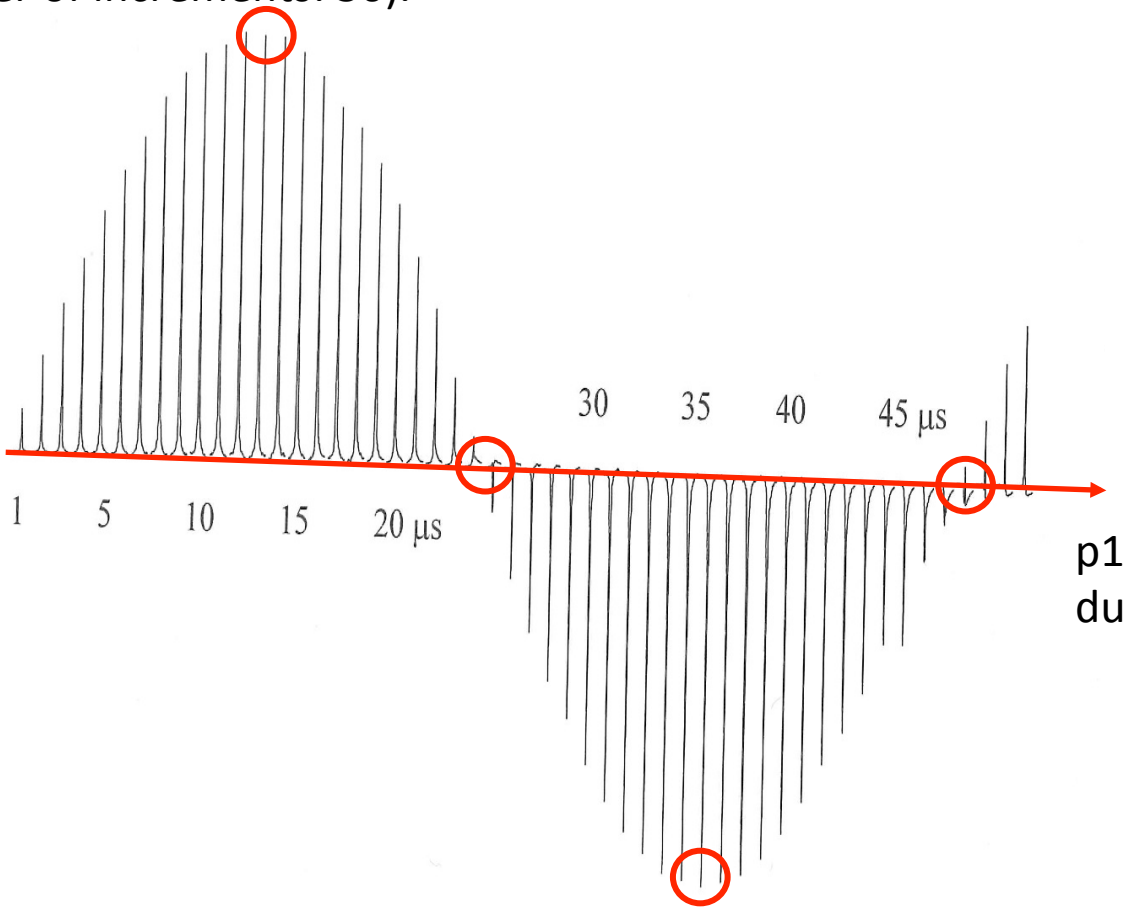
Calibration of 90° ^{1}H pulse

- Automatic calibration
  - Type **pulsecal** on cl – info-panel will pop-up with the length and power of 90° pulse. The parameters will be also automatically updated within the dataset
  - Pulsecal gives results reasonable enough to use them for nD experiments or to use them as a starting point for manual calibration

- Manual calibration
  - Type **p1** on cl, set the pulse length 1 μs and type **zgqfp**. This will run a macro that is composed of **zg** (zero go - start an experiment), **qsin** (multiply resulting FID with QSINE window function) and **fp** (perform Fourier transformation with phase correction). Alternatively zgefp does the same but instead of qsine exponential window function is used for FID apodization.
  - Water-line signal will appear at about ~4.7 ppm. Phase the line to pure absorptive line either manually through Process menu and manual phase adjusting. Phasing window can also invoked by typing **.ph**. If there are few signals in the spectrum automatic phase correction – **apk** command will work well. Check also symmetry of the signal and/or any abnormalities of the line-shape. Also measure the LWHH to get an estimate about the quality of the shimming.
  - Proton hard pulse (90°or $\pi/2$) is strongly dependent on the tube diameter, salt concentration, temperature and solvent used. The pulse-length can be as short as 8μs but can reach ~17μs for high salt samples. As the 90°pulse is usually determined by measuring the 360° since the pulse gives a minimum signal, type **p1** on cl and set it to four times the expected length of 90° ^{1}H hard pulse (i.e. 32 to 68μs). From the knowledge of sine-function one can easily guess whether the signal is longer 360°or shorter.
  - Once minimum in 360° is achieved, take one fourth the length of the pulse that gave a zero signal to obtain length for the 90° pulse.

Calibration of 90^{o} ^{1}H pulse

Another option how to calibrate pulse in to use command **paropt**, which will pop-up a window where one sets up initial value [of the pulse], increment, and number of increments. Result of such paropt will provide similar output (initial value: 1μs, increment:1μs, number of increments: 50).



Optimizing acquisition parameters

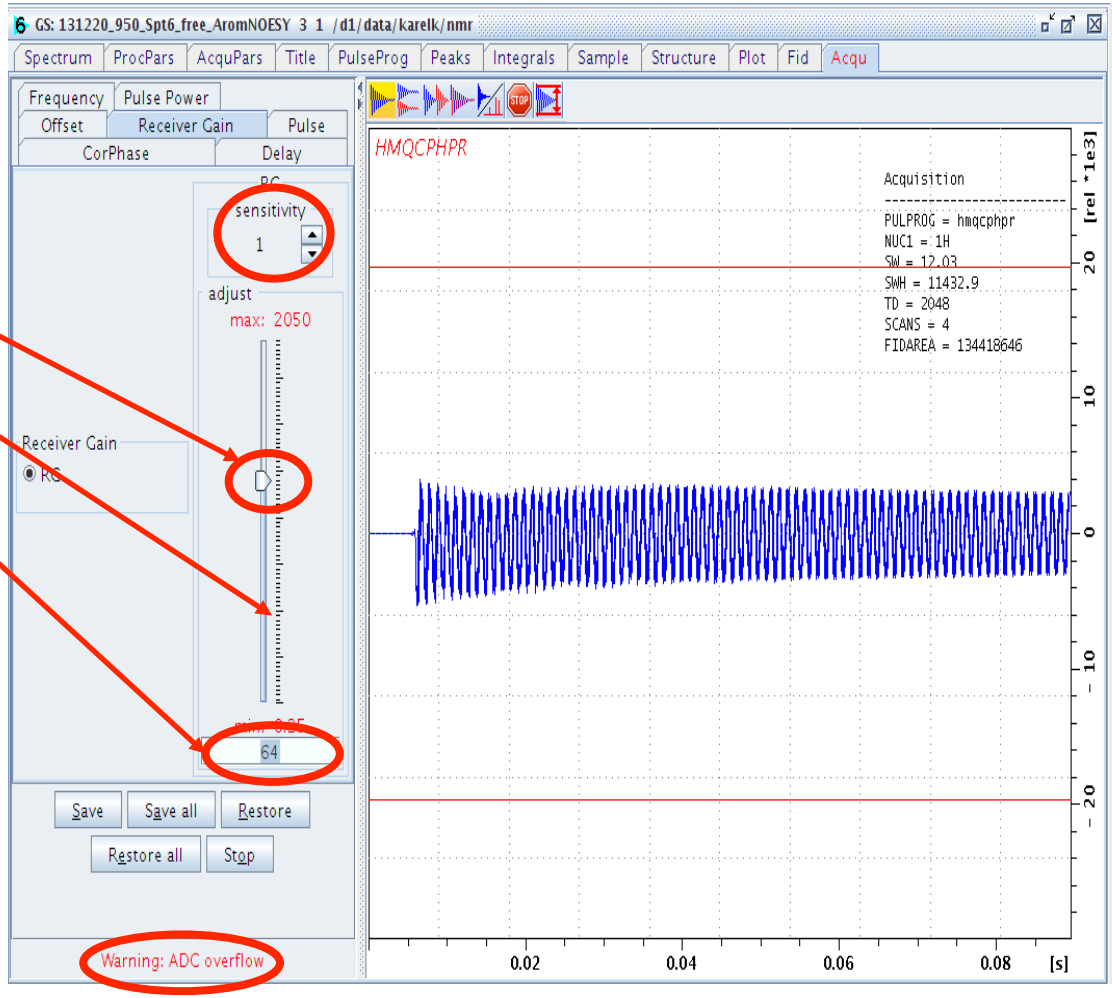
– Before starting any acquisition, it is wise to check whether all parameters are at their optimum or can be further improved. For the sake of optimization there is command **gs** (go scan) that will run an “infinite” loop enabling real-time manipulation with pulse-lengths, delays, receiver gain etc. Immediate impact on the FID is observed.

– Start with optimizing receiver gain – either by typing **rga** prior running gs or manually till ADC overflow disappears

– The parameters can be adjusted by:

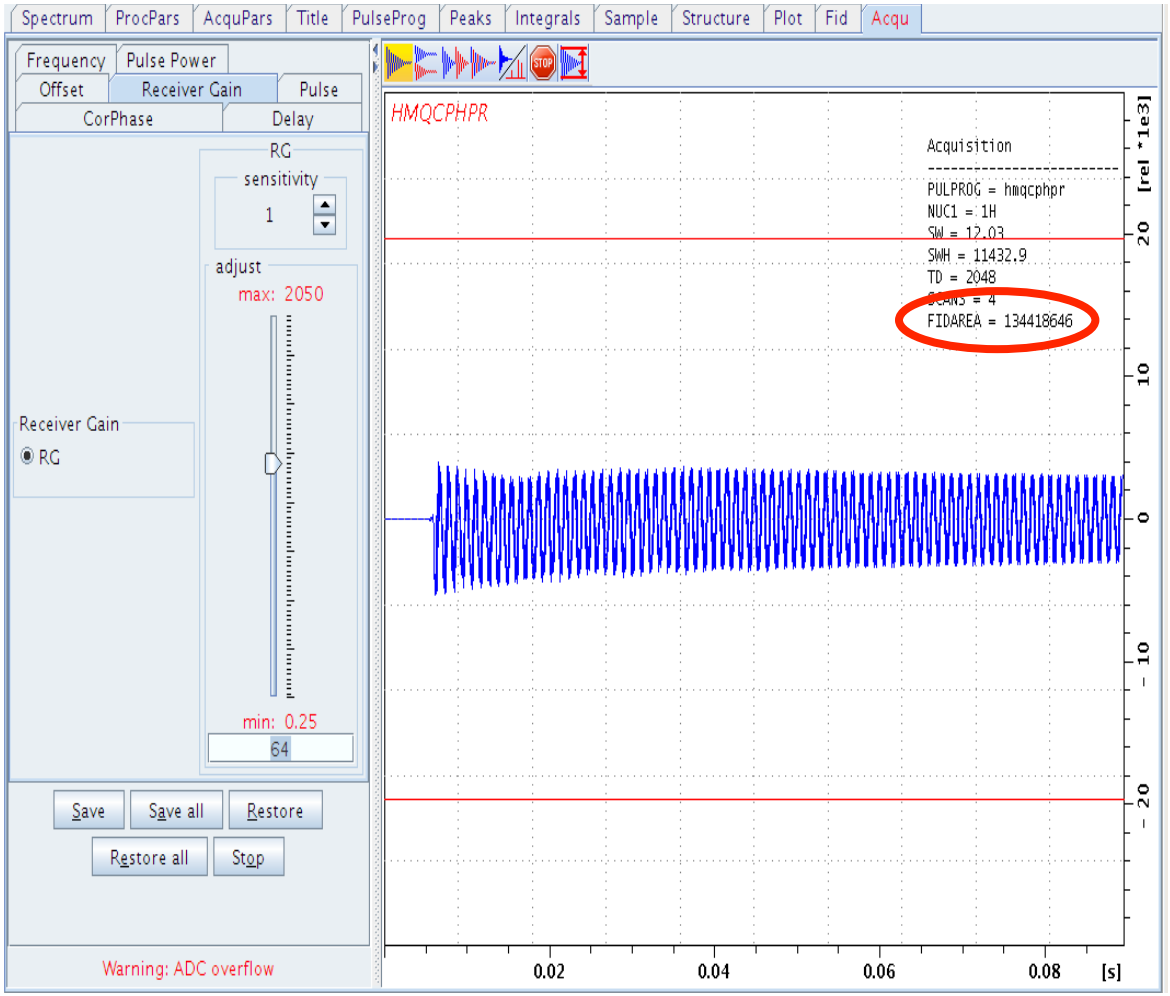
- dragging the slider (not recommended)
- directly typing desired value
- clicking on the slider-scale

(clicking will be affected by the sensitivity)



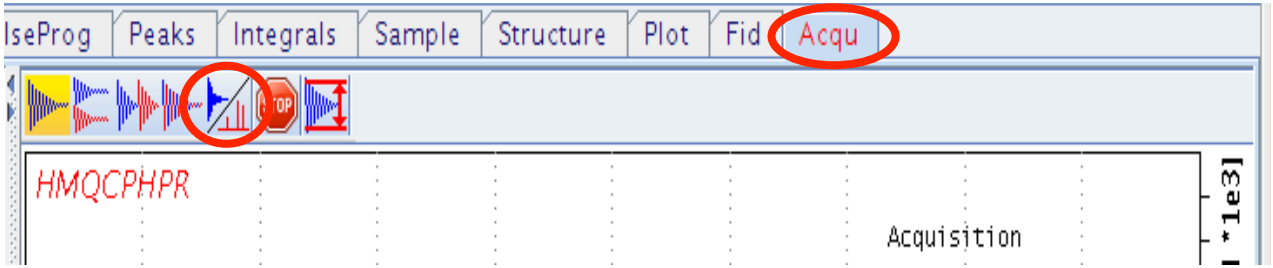
Optimizing acquisition parameters

- Other parameters that could be set are the exact position of receiver (Offset-tab), phase correction (particularly in case of WATERGATE water suppression; CorPhase) and duration of calculated shaped pulses (Pulse-tab).
- Always check the Warning message and FIDAREA which one wants to reach as low as possible as the signal in biomolecular samples is dominated by water which is supposed to be efficiently suppressed.



Start of the experiment

- Duration of the experiment
  - Type **expt** (experimental time) – gives the experimental time, required space on disc for the current dataset
  - Type **multiexpt** – allows to estimate duration of multiple experiments that are in row (e.g. ExpNos 1, 2, 3, 4 ...). Returns the time and date, when the experiments are finished.
- Running the experiment
  - Type **zgqfp** – starts and processes 1D experiment (composite macro of zg+qsin+fp) – in case of low concentrated samples, one set number of scans as high as 128 or even 1024. As such experiment takes already a significant amount of time, it is wise to check whether something is being acquired. Either check the on-line Fourier transform in the acquisition window or type **tr** to transfer the so far acquired data from spectrometer to computer and process them with **efp** or **qsin**+**fp**.



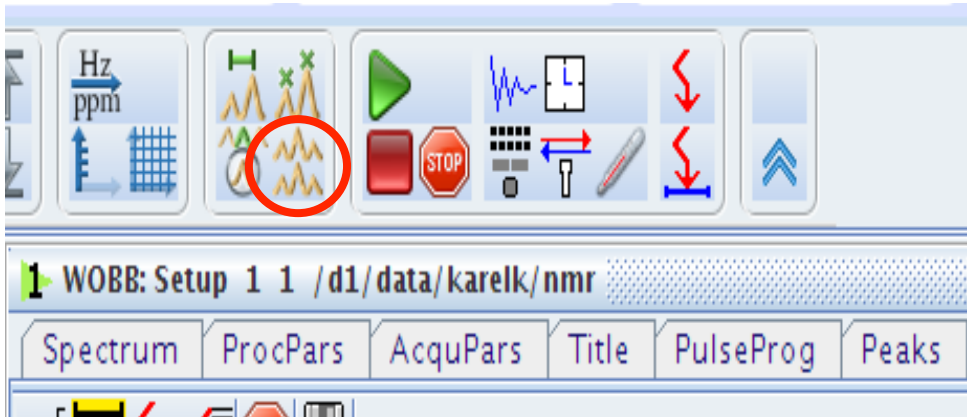
  - Type **zg** – starts the experiment without processing it
  - Type **stop** – immediately stops the experiment
  - Type **halt** – stops the experiment after the current scan

Start of the experiment

- In case of an nD experiment follow the same steps as in case of 1D, i.e. first set all parameters properly and check with gs-command that everything is correct and is not hurting sample/probe. Before typing zg, check the duration with expt or multiexpt to see when the experiment is going to finish.
- When the experiment is started with the zg-command, wait till the first FID is acquired. In the mean-time check that the temperature and lock are stable and not affected by the running experiment.
- Once first FID measured and stored, type **rser 1**, which will transfer the first FID to ~TEMP directory ExpNo 1, ProcNo 1. Process the FID with qsin+fp and phase it. This will give you a rough information about signal/noise ratio of your experiment – if there is no signal in the firs row, there will be not much signal at the end of the experiment (exceptions are, e.g. HNCACB or DQF COSY, but these are not experiments for beginners:-).

Start of the experiment

- Should it be possible to compare the running experiment with previous dataset, store the current ProcNo to ProcNo 2 by typing **wrp 2 y** – **wr**ite **P**rocNo to **2** and if there is already ProcNo 2, **y**es, overwrite it. Then go to the previous/reference experiment, type again rser 1; qsin; fp, phase the spectrum. Type **.md** or click the multiple icon and then type **rep 2** – **re**ad **P**rocNo **2**. Of course one can do all the overlay with mouse only but the clicking may be more time consuming as the directory gets filled with experiments and ~TEMP remains at the top (scrolling up, double-clicks …, keyboard is keyboard:-).



Quick reference:

1) bsmsdisp – open control panel
2) ej – eject sample
3) edte – open temperature control panel
4) ij – inject sample
5) lockdisp – display lock window
6) lock – select solvent for locking the magnet
7) topshim – automatic shimming
8) loopadj – adjust lock parameters
9) edasp – set up spectrometer routing
10) wbsw – set up wobble sweep width
11) atma – automatic tuning/matching
12) atmm – manual -----”-----
13) edc – copy dataset
14) iexpno – copy dataset to next ExpNo
15) rpar – read parameter set
16) getprosol – set up pulses according to prosol
17) pulsecal - calibrate ^{1}H 90° pulse
18) paropt – optimize parameter (e.g., p1 length)
19) rga – automatically set up receiver gain
20) gs – go scan – optimize acquisition parameters
21) expt – estimate duration of the experiment
22) multiexpt - ----”---- for multiple expts in row
23) zg – start acquisition
24) zgqfp – acquire 1D and apply qsin apodization
25) qsin – multiply FID with qsin window function
26) xfb – process 2D
27) xfb n – process 2D and remove 2ri, 2ir, 2ii
28) tr – transfer data 1D from spectrometer to PC
29) edmac *name* – create or edit macro *name*
30) wrp 2 y – write processed data to ProcNo 2 and if exists, overwrite
31) rser 1 – extract first FID of an nD experiment
32) .ph – start phase menu
33) .md – spectra overlay window
34) apk – automatic phase correction
35) show – show active processes
36) curplot – set up printer
37) print – print spectrum
38) acqu – switch to acquisition window
39) ii – if spectrometer doesn’t communicate
40) ii restart – if ii doesn’t help
41) stop – stop immediately acquisition, rough
42) halt – stop it smoothly, recommended
43) pulse – calculate pulse length based on 90°hard pulse parameters
44) calcpowlev – similar to pulse

Measurements:

1) zg – 1D ^{1}H check shimming => water line shape - **15 minutes**
2) zgpr – 1D ^{1}H with water presaturation **10 minutes**
   a. `pulsecal`
   b. `gs` – optimize
   c. `rg` – receiver gain
   d. `o1` – carrier position
   e. PLdB9 – water presaturation pulse
3) `z` or `zg` or `zgqfp`
4) `rpar zggpwg` – read parameters for 1D 1H with water presaturation using WATERGATE pulse scheme **20 minutes**
   a. `pulsecal`
   b. `gs` – optimize
   c. sp1 – decrease the FID area
   d. Corphase 2 – using the real time FT try to get water signal in antiphase
   e. `rga`
5) Measure 1D ^{1}H

2D NMR Measurements:

1) ^{15}N HSQC
   a. 100-140ppm in ^{15}N **25 minutes**
   b. 80-140 ppm in ^{15}N **30 minutes**
2) ^{13}C HSQC
   a. 0-80ppm in ^{13}C - aliphatic region **20 minutes**
   b. 100-140ppm in ^{13}C - aromatic region **10 minutes**
   c. ctHSQC – aliphatic with d23 13.3 ms **15 minutes**

Time Table overview		
Time [minutes]	Task	Note
20-30	NMR Hardware	
15-20	Sample Introduction	
10-15	Sample Preparation	
15-45	TopSpin	
15	1D ^{1}H zg	
10	1D ^{1}H zgpr	Presaturation
20	1D ^{1}H zggpwg	WATERGATE
10-20	Break	
25	^{15}N HSQC	Amides+NH2 sc
30	^{15}N HSQC	Amides+NH2+Arg NHs
20	^{13}C HSQC	Aliphatic
10	^{13}C HSQC	Aromatic
15	^{13}C ctHSQC	
205-255		Without break

Recommended literature:

1) John Cavanagh, Wayne J. Fairbrother, Arthur G. Palmer III, Mark Rance, Nicholas J. Skelton: *Protein NMR Spectroscopy, 2nd Edition: Principles and Practice*, **2005**, Academic Press
2) Stefan Berger, Siegmar Braun: *200 and More NMR Experiments: A Practical Course* **2004**, Wiley-VCH
3) Kurt Wüthrich: *NMR of Proteins and Nucleic Acids*, **1986**, Wiley – Interscience
4) Gordon C. K. Roberts: *NMR of Macromolecules: A Practical Approach (Practical Approach Series)*, **1993**