

Recording EPR spectra using the Loop Gap Resonator (LGR)

This protocol gives step-by-step instructions for recording EPR spectra of spin labeled proteins (Nitroxide label like MTSSL) using the LGR **assuming the LGR is already in place and you have been trained on the instrument.**

- Reading this protocol **does not replace** the **training** provided by Dr.Erbse. (*Annette Erbse, Office C316, ext. 2-0528, erbse@colorado.edu*)
- Before you are allowed to use the instrument by yourself, **one-on-one training** with Dr.Erbse **and** an okay directly from Dr.Erbse is **required**.
- After training you will receive access to the EPR Google Calendar. All users should use the Google Calendar to reserve instrument time and should log into the logbook at the beginning of each use. **Please check which resonator was in use.** If a different resonator was or will be used before the time you want to use the LGR Contact Dr.Erbse (2-0528, erbse@colorado.edu). She will help you to change the resonator. Keep in mind that you must notify her at least a day in advance to allow time for the resonator change to be fit into her schedule. The earlier she knows the more likely she can make time!
- **Do not attempt to change the resonators without Dr.Erbse** or with the help of anyone else but her. You can **damage the instrument** severely and **you will lose the privilege** to use the instrument, regardless of damage.

Good Practice and Required Procedure:

Always take a spectrum of a clean, buffer filled capillary **before you start your samples and after you have measured the last sample for the day.** This is to check for possible contaminations of the LGR. Please save those spectra so that we have an up-to-date record in case some contamination should happen.

Important:

- **The LGR resonator can only be cleaned by the manufacturer in case of heavy contamination. It will take several weeks and will be very expensive. Please be extremely careful to avoid contaminations.**
- **If you notice/suspect any contamination, inform Dr.Erbse immediately. The faster we can start clean up measures, the higher the chance of success without sending the resonator in.**

Reserve Instrument Time

1. Make sure your PI has provided a speedtype for the EPR time.
2. We are charging \$5/hour. The money is used for upkeep and minor repairs.
3. After training you will receive access to the EPR Google calendar. This will give you access to the instrument signup. All users should use the Google calendar to reserve instrument time and should log into the logbook at the beginning of each use. It would be best if you could plan your experiments well in advance. But please try to reserve time at least 2 days in advance. Please make a note to the admin during signup stating the resonator you want to use.
4. In order to allow everybody to have a chance to get instrument time we have the following rules:
 - Up to the **Friday the week before** you want to use the EPR you can reserve up to two days in a block.
 - In the week you were planning to use it, if nobody else has reserved time, you can reserve additional days.
 - Users that were part of the SGI Grant that paid for the instrument can reserve 3 days in a block up to the **Friday the week before** they want to use the EPR and more days in the week if nobody else has signed up.
 - If your experimental plans change, please adjust your reservation accordingly.
 - If you reserve the EPR send me an email about your reservation it **at the same time.**

Power up the EPR

1. Sign the logbook.
2. Switch the water on by opening both the inlet and the outlet valve.

3. Turn on the heat exchanger



4. Switch on the console (green button)



5. Switch on the magnet

6. Watch the green LED on the bridge. It should change from off to blinking green to steady green. If it does not, something is wrong. Stop and inform me.



7. Open XEPR program (one click)



8. Go to acquisition menu → connect to spectrometer → Local host → OK
9. Load the calibration data file: Go to acquisition menu → spectrometer configuration → signal channel → calibration data set → Loop → apply → save → close
10. Open tuning panel by clicking  → option → AFC option:

AFC AC gain%: 100

DC gain: 1

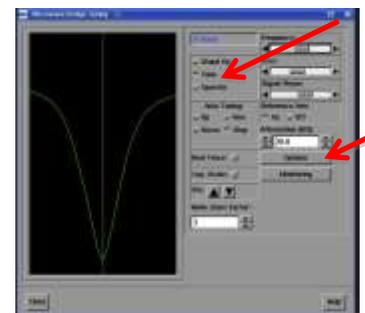
Time constant: medium high

Modulation%: 100

11. close AFC option window

12. In the tune window, click tune, wait for the Frequency, Bias and Phase slider to be activated, set the attenuation to 20 dB.

13. If you need publication quality data, warm the instrument up for at least 15 min before you start tuning and recording spectra. If you just want to work out conditions and parameters you can start right away. Keep an eye on the diode current and frequency lock since they might drift more if the EPR is not warmed up yet.



Loading a Sample:

Important:

- **Never move a sample into or out of the resonator without first switching into “tune mode” and setting the attenuation to 40dB or higher.**
 - **Avoid contaminating the resonator. The LGR cavity can only be cleaned by the manufacturer in case of heavy contamination. It will take several weeks and will be very expensive. Please be extremely careful to avoid contamination.**
 - **Make sure the capillary is sealed properly. If you are using a TPX capillary make sure it is intact. Wipe everything carefully from the outside before you insert samples. Be gentle. Never use force. If you break the capillary, you will contaminate the resonator!!!**
 - **Accidents do happen. If you break a capillary inside the resonator inform Dr.Erbse immediately!**
1. The active volume of the LGR is 4 μl . 4 μl sample is sufficient to measure a spectrum if the capillary is positioned right. If you are working with a glass capillary, positioning is not that accurate, if you have enough material use 6 μl to be on the safe side.

For Glass Capillaries:

- Pipette 6 μl (see point 1) in a fresh 500 μl Eppendorf tube and avoiding forming bubbles.
- Fill capillary using capillary action (sometimes it helps to tap the capillary slightly at the bottom of the eppendorf tube or to tilt it slightly. It depends on how viscous your sample is). Avoid bubbles.
- Do not fill the capillary from a large stock solution. It is too easy to dip it in all the way and contaminate more of the outside wall than necessary.
- Wipe the capillary with a Kim Wipe carefully. Always use a fresh Kim Wipe for a new sample!
- Seal the capillary by carefully pressing it in the clay.
- Wipe the capillary again several times using a fresh section of a fresh Kim Wipe each time.



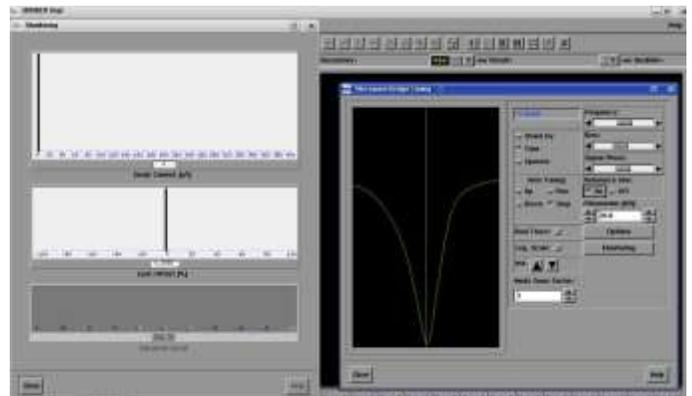
- Make sure the EPR is in tune mode and at attenuation of 40 dB or higher.
- Insert the capillary carefully into the holder on the LGR. Leave about 2 cm out. **Be careful. Never use force. If you break the capillary you will contaminate the resonator!!!**
- Tighten the holder to secure the capillary in position. Again **be careful. Never use force. If you break the capillary you will contaminate the resonator!!!**

For TPX Capillaries:

- Pipette 4.5 μ l in a fresh 500 μ l Eppendorf tube avoiding formation of bubbles.
- Put a fresh 10 μ l tip on the pipette, gently press the TPX capillary onto the pipette tip. Expel air from the pipette before you dip the TPX into the solution, and pipette the 4 μ l sample into the TPX capillary. Try to avoid bubbles.
- Seal the TPX by gently pressing it into the clay.
- Wipe the TPX several times from the outside using a fresh section of a Kim Wipe every time.
- **Be careful! If the long capillary part of the TPX seems to be more flexible than usual, you might have partially broken it. Never insert a TPX into the resonator that is not completely intact**
- Screw the TPX capillary onto the holder. Leave about three turns of the thread of the TPX visible.
- Insert the capillary into the LGR and screw the holder in place.
- **Be careful. Never use force. If you break the capillary inside the LGR you will contaminate the resonator!!**

Tuning:

1. Make sure you are in tune mode and attenuation is set to 40 dB or higher.
2. Insert your sample into the LGR as described above.
3. **Never move a sample in or out of the resonator without first switching into "tune mode" and setting the attenuation to 40dB.**
4. **Be careful. Never use force. If you break the capillary inside the LGR you will contaminate the resonator!!!**
5. Adjust the attenuation to 20 dB, click "monitor".
6. Enlarge the monitor window and arrange it so that you can still click the sliders in the tune window.
7. Note to working with the sliders:

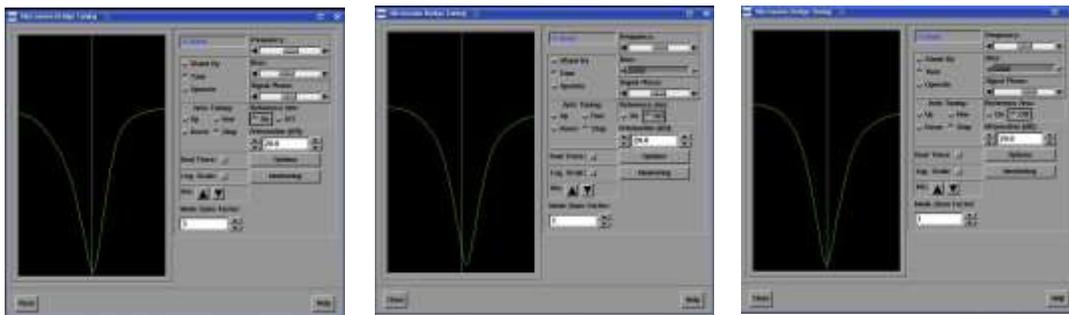


Dragging the slider – changes in big steps

Clicking in the white to the left and right of the slider – medium steps

Clicking the black arrows – smallest steps

8. Adjust the frequency slider bar to find and center the dip.
9. Adjust signal phase slider to make the dip close to symmetric
10. Adjust the iris to maximize the dip. **Be gentle!!!!** If you break or bend the Iris we will have to replace the resonator (\$15000.00)
11. Click the reference arm on and off a few times. The signaling phase is correct if the dip moves up and down but not to the side. If it moves to the side when the reference arm is tuned off, leave the reference arm off and re-centre the dip using the frequency slider. Turn the reference arm back on and adjust the signal phase slider to move the dip back to the centre. Try and switch the reference arm off and on again. Repeat the adjustment process until the dip only moves up and down.



12. With the reference arm switched off, click operate.
13. Center the lock offset with the frequency slider so that it is close to 0 (in the middle). Be careful when you click the arrows, shifting the slider directly or clicking in the white to the left or right is too big a shift in one step.
14. Adjust the iris to keep the diode current as low as possible (3-9 is ok) while adjusting the frequency to keep the lock offset close to 0
15. Switch attenuation to 19 dB and adjust the iris again to keep the diode current as low as possible (3-9 is ok) while adjust frequency to keep the lock offset close to 0
16. Repeat step 15) going down one step with the attenuation at a time.
17. If you are not doing Power Saturation going to 10 dB is enough.
18. Go back to 20 dB again and put the reference arm back to “on”
19. Centre the lock offset.
20. Adjust the bias so that the diode current is close to 200 μ A.
21. Centre the lock offset with the frequency slider so that it is close to 0 (in the middle) by clicking the arrows.
22. Adjust the signal phase. Observe the diode current while shifting the phase slider to the left or right using the arrows. The diode current will change. If it goes down try the other direction for the phase slider. If it goes up keep going until it goes down again. You are

trying to find the maximum. Once the bias current goes down again, back up a few clicks with the phase. The maximum is broad, therefore it will take a few clicks before you see the diode current go down once you are in the plateau at the maximum.

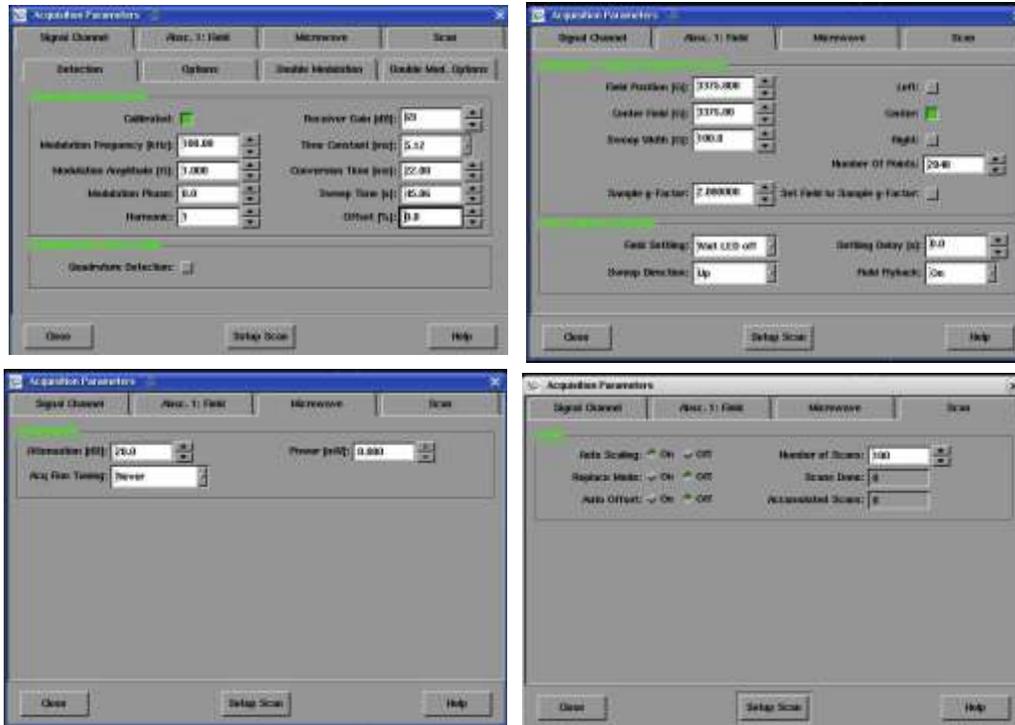
23. Adjust the bias so that the diode current is close to 200 μA and adjust the frequency so that the offset is close to zero.
24. Lower the attenuation one step at a time from 20 dB to 17 dB. If the EPR is tuned well the diode current and the frequency lock offset should not shift much.
25. If it does shift too much, take a deep breath and start the tuning process from the beginning. If it does not go on to 26)
26. Go back to 20 dB, fine tune by adjusting the bias to 200 μA and the lock offset to 0.
27. You are now ready to measure.

EPR Measurement:

1. First create an experiment: Click Exp 
2. The Build Experiment window opens. If you are doing a 1D experiment, simply scanning the field, the default is ok. Just click create. If you are doing a 2D Experiment, enable the second abscissa.
3. Parameter setting: The easiest way to set parameters is to load them from a previous experiment and adjust them as needed. Since we will have measured spectra together you will have older spectra you can use.
4. Load a spectrum.
5. Click "load acquisition parameter from spectrum" 
6. Type the appropriate handle number in the box and hit enter. The old parameters are now loaded.
7. Click gearbox.  The wheels should go from not touching to touching and the receiver level dial should start working. The parameters are now sent to the instrument.
8. Click the parameter box  and check the receiver gain. If you anticipate a normal signal strength (measurable with a receiver gain under 70 dB), set the receiver gain to 68 dB or lower. Click setup scan and activate the setup scan by clicking setup scan in the new window. You should see the centre field signal in the window (if not, shift centre field in steps of 10 G until you see it). Optimize the receiver gain so that the signal is a little smaller in amplitude when half of the screen. Switch the setup to scan off and close the window.



9. If you anticipate a weak signal and have to measure with a receiver gain >70 dB DO NOT USE THE SET UP SCAN; it will confuse the instrument.
10. Good starting values for parameters are:
11. Do not change anything in Options, Double Modulation or Double Mod. Options.



12. If you have set the parameters, close the parameter window and check that the bias and lock offset meters are still centered. If they are not, fine tune again. Start a test scan by clicking the run button.
13. Stop the scan at the end of the first scan if you have a good signal or no signal at all. Do a few scans if you have a weak signal before you stop.
14. If you have no signal at all open up the Sweep Width to 200 or 300 G. You might be off with the centre field and start the scan again.
15. Check that your centre field peak is really in the centre of your field. Use the  button and drag the centre cross of the blue line to the centre field position.
16. Adjust your parameters according to the intensity of your signal.
17. Start scanning again and average as many scans as you would like to achieve a good signal to noise ratio.
18. If you have to average for a long time, keep an eye on the bias and lock offset. Make sure they are not drifting strongly. You can pause the scanning at the end of a scan and fine tune if necessary. If they drift out to far your spectra might show lineshape artifacts.

19. If you have to average 200 scans or more you might want to set up a 2D experiment and bin 50 scans at a time in a “slice”. You can average them later but this way you do not lose all the scans should your lock or diode current drift off completely without you noticing.
20. If the data acquisition is ended, save your spectrum. If you have done any manipulations or have a second spectrum loaded as secondary make sure that you really save the primary (which is your newly acquired spectrum). If you want to open your spectrum in any other application (Excel, WinSIM, etc.) you have to export it as ASCII file as well.
21. To remove the TPX from the resonator, change to tune mode and set attenuation to 40 dB or higher.

Never move a sample in or out of the resonator without first switching into “tune mode” and setting the attenuation to 40dB.

22. For a new sample start from Tuning the EPR.

Cleaning the TPX:

1. Blow air through the TPX using a designated empty syringe fitted with a pipette tip to blow out the clay and sample.
2. Flush the TPX with 5 ml miliQ-water using a designated clean syringe fitted with a pipette tip, followed by 5 ml absolute EtOH (using another designated syringe).
3. Dry the TPX by blowing again air through the capillary with the empty syringe while at the same time moving the tip very carefully over a Kim wipe to soak up droplets.
4. Be GENTLE and DO NOT RUSH. It is easy **to break or damage the TPX during cleaning and drying!**
5. **Note: Some proteins stick to the TPX very tightly. In rare cases you might have to soak the TPX in 10% SDS overnight. To estimate if that is necessary run a test spectrum of the cleaned, buffer filled TPX between samples.**
6. **Use a receiver gain of 74 dB and average at least 20 scans. You should only see noise.**
7. If you have used a TPX from another group, save a spectrum of the cleaned, water filled TPX at the end of your experiment to prove that you left the TPX clean

Turning the EPR Off

1. Turn the instrument off in the reverse order to turning it on:
2. Using Xepr set the instrument into “standby” mode.
3. Go to “acquisition menu” → “disconnect from spectrometer”. The instrument and parameter control panels should vanish.
4. Close the program.

Important:

Click no when you are asked if you want to save changes. **Never click yes.**
Yes would save changes for everyone!

5. Switch off the magnet power supply.
6. Switch off the console.
7. Switch off the heat exchanger.
8. Switch off the water.
9. Make sure the door is locked when you leave.
10. Better safe than sorry – **If you are the last to use the EPR on a day double check that everything is switched off on your way out of the building.**