Q1 – In the paper from Krumschnabel et al (2014) (attached), they say that changes in the medium composition affect the sensitivity of the reaction between AmR and H2O2, with sensitivity being much lower in the MiR05 medium (although it may increase by replacing lactobionate with KCl). The question is: which medium should we use? Would it be better to use an alternative medium such as MiRK03Cr or is it worse for estimating mitochondrial respiration parameters? If it depends on the tissue we are analysing, how can we know which medium is better in our samples?

A1. It seems you have done a great job synthesizing all the references you need for your measurements. *Are you planning to assess both ATP production and H2O2 release? I think H2O2 assay is relatively flexible as for which product you use (you can use a basic KOH to pH your solution for H2O2 assay, but you have got to use a special one for ATP assay). I guess you will prepare your buffer/substrates and inhibitors just once, and this would be with the right KOH for ATP assay*. **ATP assay, you need to be precise to use Calcium free chemicals.** I added the supplier for the references you can’t find in Sigma. Just be aware that in a Oroboros chamber, **you will have to run ATP assay OR H2O2 assay**. Both assays have to be run separately because fluorescence probes are not compatible. **Note that H2O2 assay might not be feasible from homogenate tissue, and you might have to isolate your mitochondria by differential centrifugations.**

Q2 – When should we measure ROS? I understand that we should obtain H2O2 concentrations only while measuring ROUTINE respiration, am I wrong? In that case, should we calibrate ROS sensors only before measuring it? Also, can calibration with H2O2 impact mitochondrial function and, therefore, subsequent estimates? Finally, according to Krumschnabel et al (2014), the amount of H2O2 needed for calibrations might depend on the expected ROS production, how can we know that?

A2. *I do measure and calibrate H2O2 at each respiratory stage*. What I call state 2 – you might call it routine, OXPHOS, LEAK. Enclosed my protocol. *Many research groups also look at OXPHOS respiration fuelled with Complex I substrates (as in Arias-Reyes paper, NADH-linked..) and then OXPHOS respiration fuelled with OXPHOS I and II substrate. This is when you want to know the contribution of complex 1 to the ROS production.* Most ROS are produced by Complexes 1 & 3. There are many way to measure H2O2 production, I guess it will depend on your project general aim. *As for the buffer, I would just follow Krumschnabel paper and its recommendation, unless you need to do something also for your research question*. *Krumschnabel’s paper are very far from perfect, but this is what people do. If you also want to determine ATP production (I keep saying that because it is what I initially thought you would do…) I would suggest you work with a single buffer. But if you want to know what is the best buffer in your experimental conditions/tissue/…, you can compare fluxes in both buffers. I think these fine-tuned points will be very quickly fixed once you run a few assays in the Oroboros. Same for the amount of H2O2 needed for calibration, this will be a 30min things to fix in front of the Oroboros. Best is you give a go for some runs, share the datlab file with me, and I feedback.*

Q3 – Again, in Krumschnabel et al (2014), they say that the amount of AmR to be added may vary depending on the type of tissue and, specifically, mitochondrial density. In lizard brains I expect big mitochondrial densities, but I do not know in other tissues. How do we know how much AmR to add in our sample? Something similar may occur with the volume of medium needed for isolating mitochondria, how can we know how much we need?

A3: **Consider a week of preliminary experiment to tune as many parameters as you want** – the concentration in probe is an obvious one. Compare assays following Krumschnabel recommendation for [Amp], run the same assay with half this concentration, and run the same assay with double this concentration. Calculate the fluxes in H2O2 and oxygen, and then compare the fluxes. **Too much Amp might slow down the respiration. Not enough might give a signal to noise ration too small.**