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O2k-MultiSensor: Mitochondrial respiration media for HRR and simultaneous O2k-Fluorometry

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1. Abstract

Three different mitochondrial respiration media widely differing in chemical composition, MiR05, MiRK03, and Buffer z, were compared regarding their suitability for combined respirometry and fluorometry. Using the O2k in combination with the O2k-Fluo LED2-Module, a substrate-uncoupler-inhibitor-titration (SUIT) protocol was applied to HEK 293T cells and respiration was determined with simultaneous detection of H_2O_2 production by use of the Amplex Red (AmR) assay system. We observed that respiration was equally well supported by MiR05 and Buffer z, whereas oxygen fluxes detected with MiRK03 were consistently lower at all coupling and substrate states. Estimated H_2O_2 production rates were largely comparable with all three media, while assay sensitivity was comparatively low but very stable with MiR05, and higher but declining over time in the other two media. A combination of MiR05 and Buffer z, MiR07-T1, proved to support respiration just as well as MiR05, gave similar estimates for H_2O_2 production rates, and showed AmR assay sensitivity that was comparable to that of MiR05 but slightly less stable over time. Altogether, medium composition appears critical for valid multi-sensor respirometry and fluorometry and requires evaluation for different specific applications.

2. Introduction

Mitochondrial respiration media are designed to support and preserve optimum mitochondrial function. The most important variables to be considered are osmotic pressure, ionic strength, ion composition, binding of Ca^{2+} and free fatty acids, and antioxidant capacity. Multi-parameter measurements such as the simultaneous determination of respiration and H_2O_2 production using the O2k in combination with the O2k-Fluo LED2-Module may impose yet additional requirements on the medium, necessitating evaluation of mitochondrial respiration media suitability for each specific application.

3. Media compared for use in multi-sensor respirometry

Two experimental series were conducted examining respiration and simultaneous H_2O_2 production in HEK 293T cells. The first experiments compared measurements employing three respiration media widely differing in chemical composition as outlined in Table 1. MiR05 has been specifically designed for use with the O2k to optimally support mitochondrial respiration during prolonged and comprehensive substrate-uncoupler-inhibitor-titration (SUIT) protocols [1, 2]. MiRK03 has been modified after a medium described by [3], intended for use in H_2O_2 production measurements with Amplex Red (AmR), and Buffer z is a medium introduced by Neufer and colleagues [4], and has been primarily used with permeabilized muscle fibres.

Table 1.

	MiR05	MiRK03	Buffer z	MiR07-T1
		[mM]		
Sucrose	110	-	-	110
K-lactobionate	60	-	-	-
K-MES	-	-	105	105
HEPES free acid	-	20	-	-
K-HEPES	20	-	-	-
Taurine	20	-	-	-
KCl	-	130	30	-
KH_2PO_4	10	10	10	10
$MgCl_2$	3	3	3	3
EGTA	0.5	0.5	1	0.5
BSA	1 mg/ml	1 mg/ml	5 mg/ml	1 mg/ml
pH	7.1	7	7.1	7.1

4. Instrumental setup

Instrumental setup and connection of the O2k-Fluo LED2-Module followed standard procedures as described in detail elsewhere [5]. For the simultaneous measurement of H_2O_2 production using the AmR assay system, two Fluorescence Sensors Green were connected to the

Fluorescence-Control Unit and sensitivity of the sensor and intensity of the LEDs were adjusted by setting the Gain for sensor to 1000 and the LED Polarization voltage [mV] to 500.
For each experiment, 3 instruments with 2 chambers each were run in parallel, with chambers containing 2.2 ml of either MiR05, MiRK03, or Buffer z. The assignment of which O2k was used for which medium was changed for each experiment, so that there was neither any bias relating to POS usage nor to the LED2-Module or the fluorescence sensors applied. Media were equilibrated to 37°C at a stirring rate of 900 rpm with stoppers in the 'open O2k-Chamber' position before closing the stoppers and thereby adjusting the final chamber volume to 2 ml.

5. Sample preparation

As model for our investigations, we used HEK 293T cells, the application of which for combined respirometry and fluorometry has been previously described [6]. These cells have been shown to largely preserve respiratory features after cryopreservation at -80°C and we thus used freshly thawed cells for our experiments. For this purpose, a cryo-tube containing cells frozen in 250 µl standard freezing medium (Fetal Calf Serum with 10% DMSO) was removed from the -80°C freezer, 500 µl pre-warmed DPBS were immediately added, and the cells were quickly thawed by gentle pipetting to obtain a homogenous cell suspension. Finally, 100 µl of the suspension was injected into the O2k chambers of all three instruments containing the different media, yielding a final concentration of $1.5*10^6$ cells/ml.

6. The SUIT protocol for combined respirometry and fluorometry

Following the initial instrumental adjustments, the SUIT protocol was started by adding the components of the AmR detection system for H_2O_2 production. To this end, we injected AmR (final concentration, f.c., 10 µM), HRP (f.c. 1 U/ml) and SOD (f.c. 5 U/ml), and recorded a baseline. Then 0.1 µM H_2O_2 from a freshly prepared stock solution was added to enable conversion of the fluorescence signal into a concentration of the fluorescent product of the AmR assay system, resorufin [7]. In addition, from the response to the H_2O_2 addition the assay sensitivity could be calculated, expressed as change in fluorescence, detected as voltage, per µM of H_2O_2 added [V/µM]. Together with several further additions of 0.1 µM H_2O_2 at different respiratory states examined during the experiment, this enabled us to estimate and compare the change in sensitivity over time as detected in the different respiration media.
Subsequently, cells were added and a SUIT protocol for permeabilized cells was followed as outlined here:

Cells+Dig: NS_1PM 2D 3S 4AF 5DNCB 6U 7Rot 8Ama

E				6U	7Rot	8Ama
P	2D	3S	4AF	5DNCB		
L	1PM					
	N	NS	NS	NS	S	ROX
	CI	CI&II	CI&II	CI&II	CII	ROX

Complete SUIT:
AmR-HRP-SOD-H_2O_2-Cells-Dig-H_2O_2-PM-D-H_2O_2-S-AF10-AF20-DNCB2-DNCB4-H_2O_2-U-H_2O_2-Rot-Ama-H_2O_2

N, S, and ROX denote N-junction substrates, succinate and residual oxygen consumption, respectively.

P:	pyruvate, 5 mM
M:	malate, 2 mM
Dig:	digitonin, 10 µg/ml
D:	ADP, 2.5 mM
S:	succinate, 10 mM
AF:	auranofin, an inhibitor of thioredoxin reductase, 10 and 20 µM
DNCB:	dinitrochlorobenzene, an inhibitor of glutathion peroxidase, 2 and 4 µM
U:	uncoupler CCCP, added in steps of 1 µM, from 1-5 µM
Rot:	rotenone, 0.5 µM
Ama:	antimycin A, 2.5 µM

AF and DNCB are supposed to inhibit components of the mitochondrial antioxidative defense system and were thus added to evoke particularly strong responses in H_2O_2 production rates.

7. Respiratory rates, H2O2 production and changes in assay sensitivity

Figure 1 depicts an example of the simultaneous determination of respiration and H_2O_2 production with the AmR assay as applied with freshly thawed HEK 293T cells examined in MiR05. In Figure 2, the results of four independent experiments run with four separate cell stocks with each mitochondrial respiration medium are summarized. These experiments indicate that MiR05 and Buffer z support respiration of the HEK cells at comparable rates, while respiration was consistently lower in all coupling and substrate states when measured with MiRK03. In addition, in MiRK03 cells reproducibly required a lower concentration of uncoupler CCCP to elicit ETS capacity (not shown).

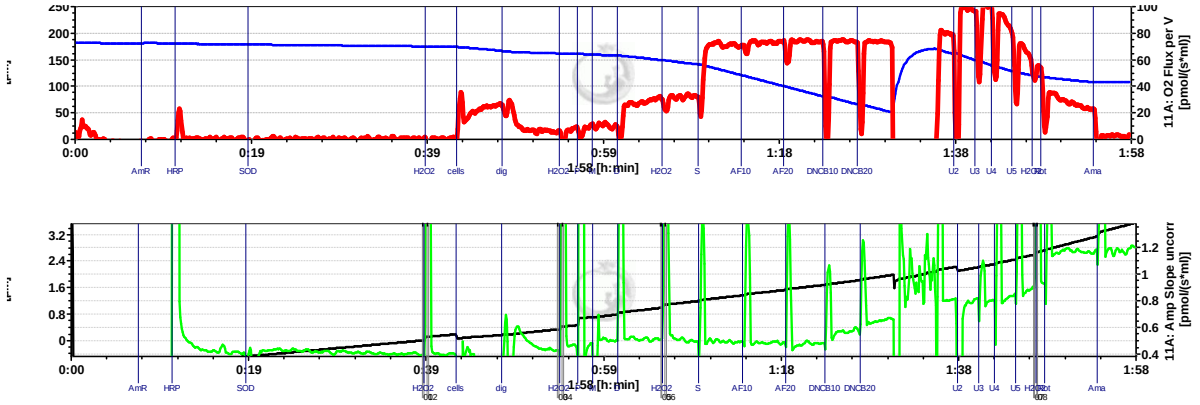

Fig. 1. Respiration (upper panel) and H_2O_2 production (lower panel) of HEK 293T cells determined with MiR05. Oxygen concentration is shown as the blue trace (left Y-axis [µM]), oxygen flux per chamber volume is displayed in red (right Y-axis [pmol·s-1·ml-1]). The black line in the lower panel represents the fluorescence signal of the AmR assay product resorufin (left Y-axis [µM]), the green line shows the volume-specific H_2O_2 flux (right *Y*-axis [pmol·s^{-1}·ml^{-1}]) derived from the change in fluorescence over time. Details on the additions are outlined above. Experiment: 2015-11-19 P11-02.DLD

Data depicted in Figure 3 summarize H_2O_2 production rates determined in the presence of the three different media under the various coupling and substrate states. A general observation was that the AmR chemical background was highest with MiR05 and was significantly lower in MiRK03 and Buffer z (not shown), in line with our previously published data [7]. As this chemical background flux was unrelated to H_2O_2 production by the cells, it was subtracted from all fluxes determined in the presence of cells. Net H_2O_2 fluxes were slightly negative in the ROUTINE state as determined with MiR05, and only slightly above zero with MiRK03 and Buffer z, presumably reflecting the radical scavenging capability of the intact cells. Upon permeabilization with digitonin, releasing cell components including unbound scavengers, H_2O_2 fluxes increased in all 3 media. Similar to the pattern seen with respiration, fluxes determined with MiRK03 were almost always somewhat lower than in the other media. In MiR05 and Buffer z, H_2O_2 fluxes were almost identical up to the point when the glutathion peroxidise inhibitor DNCB was added. While the thioredoxin reductase inhibitor auranofin consistently failed to elicit any increase in H_2O_2 fluxes in any of the 3 media, DNCB produced a dose-dependent increase which was larger in MiR05 and MiRK03 than in Buffer z (Figure 4).

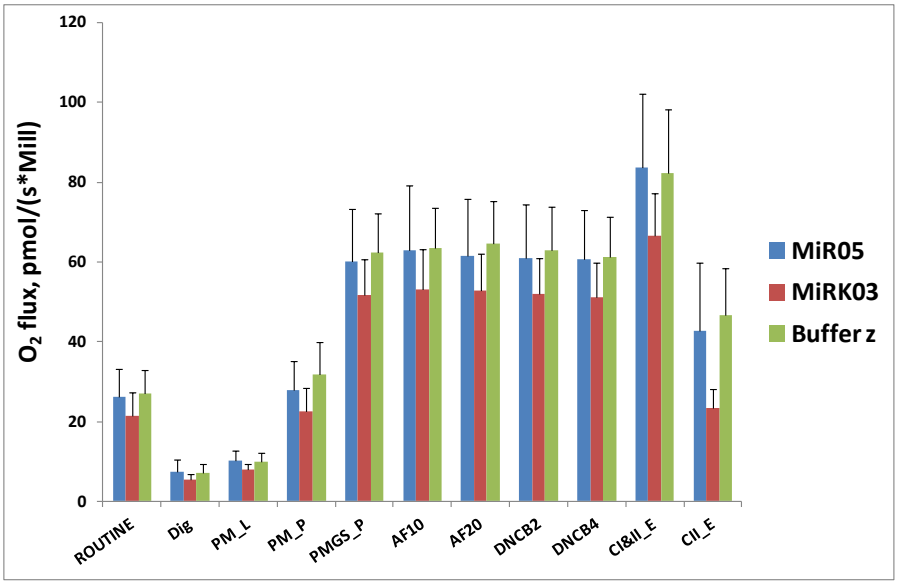

Fig. 2. Respiration rates of HEK 293T cells in different coupling and substrate states, as examined with 3 different mitochondrial respiration media. Data shown are means ± SD of 4 experiments, each measured in duplicate. Cell stocks used had been previously cryopreserved at -80°C for between 6 and 263 days.

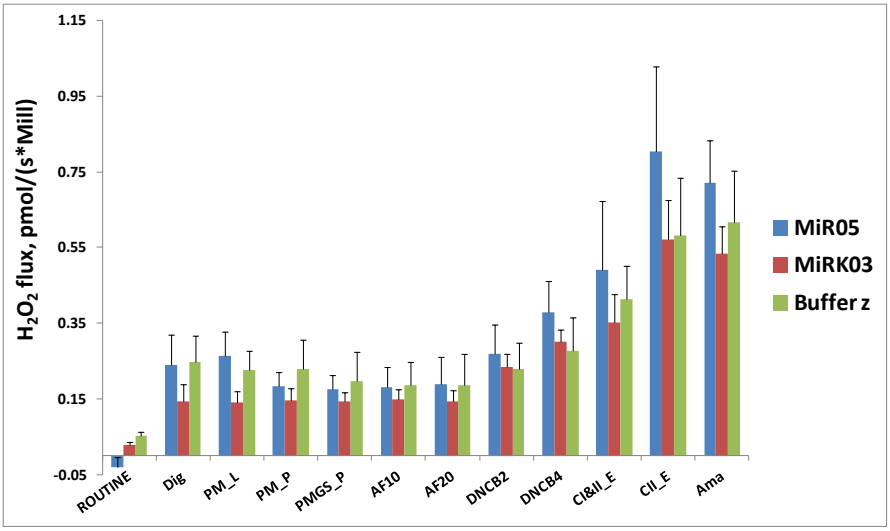

Fig. 3. H_2O_2 production rates of HEK 293T cells in different coupling and substrate states, as examined with 3 different mitochondrial respiration media. Data shown are means ± SD of 4 experiments, each measured in duplicate. H_2O_2 fluxes were corrected for the slope determined in the absence of cells.

Absolute and relative AmR assay sensitivities and their change over the time course of the SUIT protocol are shown in Figure 5. Initial sensitivity determined before adding cells was highest in MiRK03, followed by Buffer z and a much lower value in MiR05. Following addition of cells sensitivity was considerably reduced in all three media, and this was particularly

pronounced in MiRK03 and Buffer z. During subsequent measurements at different coupling and substrate states sensitivity continued to decline in these media, whereas it remained fairly constant in MiR05.

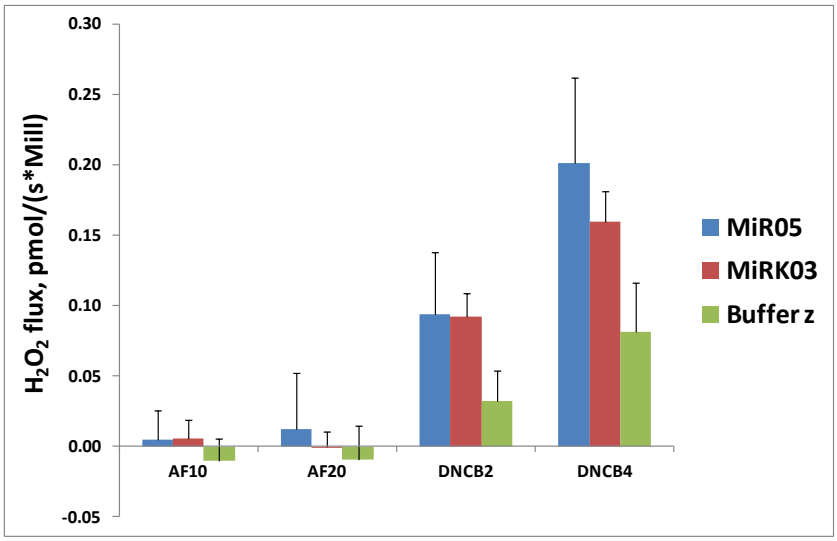

Fig. 4. Changes in H_2O_2 production rates of HEK 293T cells determined with three different media after adding thioredoxin reductase inhibitor auranofin at 10 and 20 µM and of the glutathione peroxidase inhibitor DNCB at 2 and 4 µM. Data are means ± SD of 4 experiments, each measured in duplicate. H_2O_2 fluxes were corrected for the slope determined in the absence of cells.

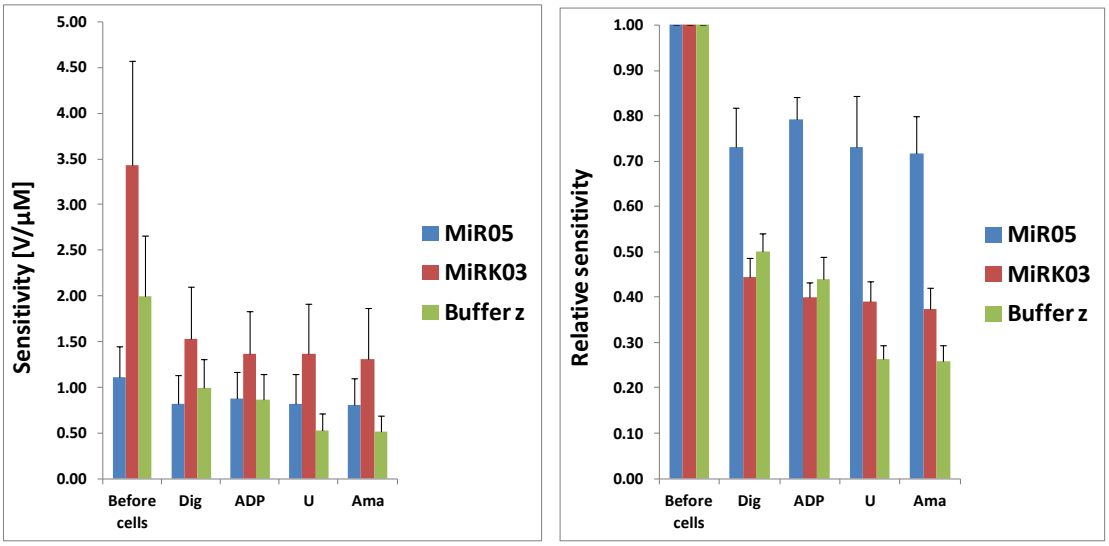

Fig. 5. Changes in AmR assay sensitivity over the time course of the SUIT protocol as observed in three different mitochonrial respiration media. The left panel shows sensitivty expressed as V/µM, the right panel displays these data as normalized to the first determination of sensitivity before adding cells. Data are means ± SD of 8 chambers used in 4 independent experiments, holding that chambers used for duplicate measurements of respiration can be regarded as independent entities in this instance.

8. Evaluating MiR07-T1, a combination of MiR05 and Buffer z

Based on the above described results, we reasoned that a combination of MiR05 and Buffer z could be a worthwhile approach towards an optimized medium for simultaneous determination of respiration and H_2O_2 production. While both media were found to support respiration equally well, Buffer z appeared to reduce chemical background of the AmR assay at elevated sensitivity, while MiR05 diplayed the desired stability of sensitivity. Thus, in a second experimental series, we compared MiR05 with the newly designed MiR07-T1, replacing taurine, K-lactobionate and HEPES from MiR05 with an equimolar amount of K-MES as present in Buffer z (Table 1). Again, we used freshly thawed HEK 293T cells, but in this case applied a somewhat simplified SUIT protocol by omitting the use of auranofin and DNCB. The inclusion of creatin in both media had no impact on respiration, but served to extend the applicability of the results for future use with other mitochondrial preparations such as permeabilized muscle fibres.
An example for an experimental run is depicted in Fig. 6, a summary of 4 such experiments is shown in Fig. 7.

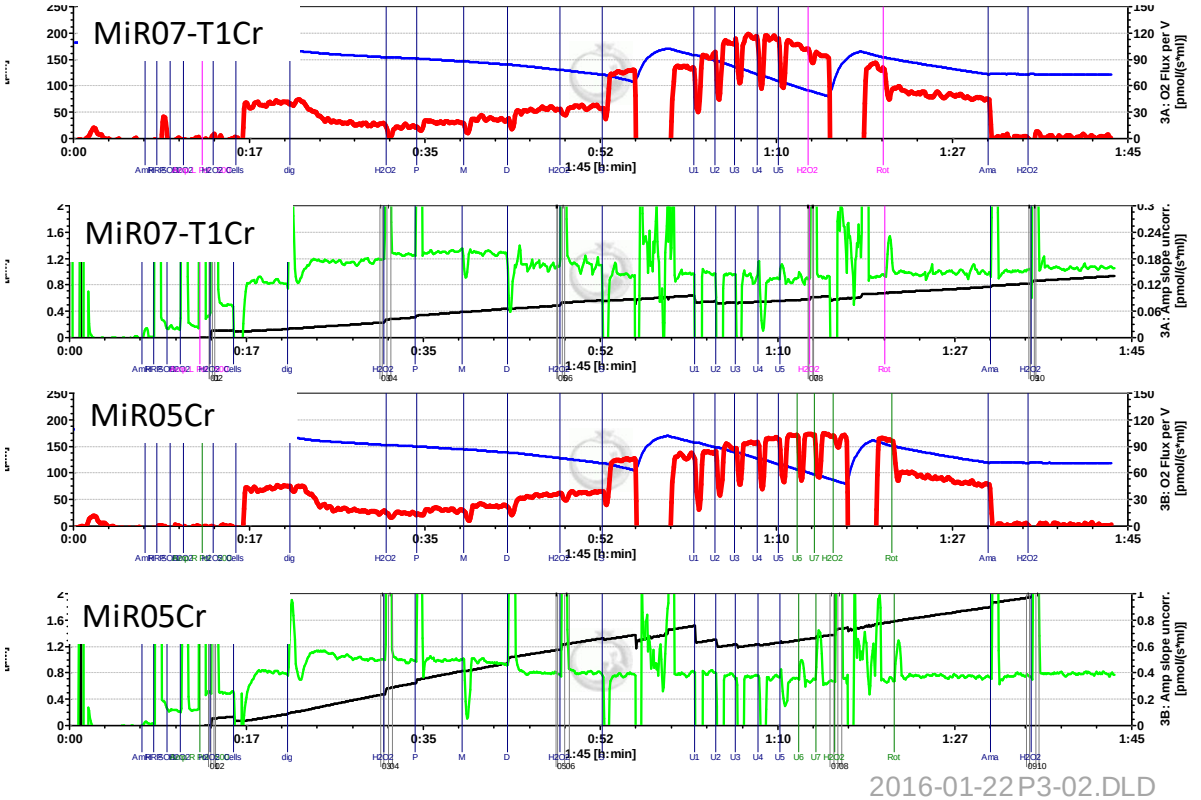

Fig. 6. Respiration (first and third panel) and H_2O_2 production (second and forth panel) of HEK 293T cells determined with MiR07T1Cr and MiR05Cr. Details as explained in the legend to Fig. 1.

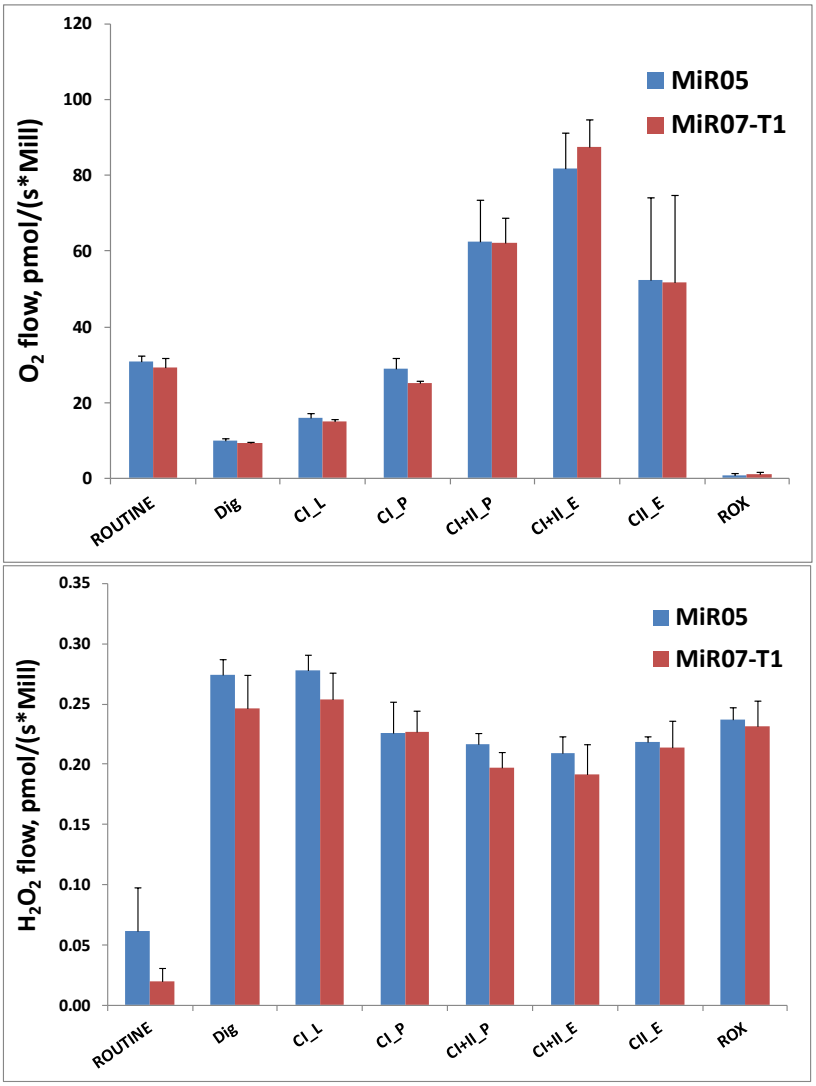

Fig. 7. Respiration rates (upper panel) and H_2O_2 production rates (lower panel) of HEK 293T cells in different coupling and substrate states, as examined with MiR07-T1Cr and MiR05Cr. Data shown are means ± SD of 4 experiments, 3 of which were measured in duplicate. Cell stocks used had been previously cryopreserved at -80°C for 164 days.

It was seen that both respiratory and H_2O_2 fluxes were highly similar in both media, an exception being a somewhat higher H_2O_2 production rate determined in the ROUTINE state with MiR05Cr (Fig. 7). Furthermore, MiR07-T1Cr showed a lower chemical background as related to resorufin fluorescence and a much higher sensitivity in the absence of cells. However, following the injection of cells sensitivity declined to a value close to that seen with MiR05Cr. Moreover, sensitivity was then fairly stable over tme with MiR05Cr, but showed a further 20% reduction with MiR07-T1Cr.

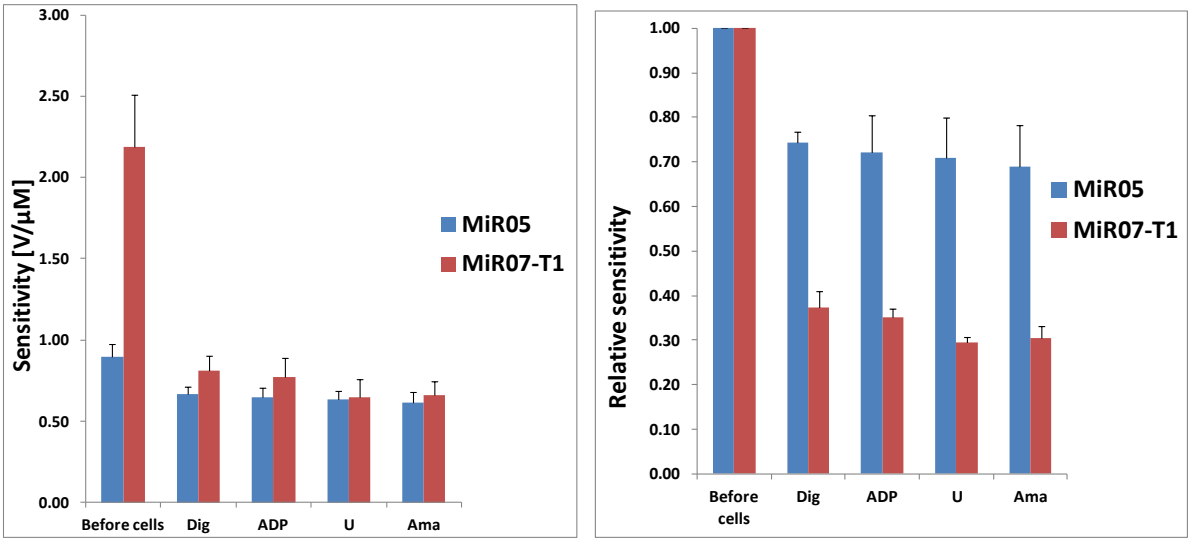

Fig. 8. Changes in AmR assay sensitivity over the time course of the SUIT protocol as observed in MiR05Cr and MiR07-T1Cr. Left panel: sensitivity expressed as V/µM, right panel: sensitivity normalized to the first determination before adding cells. Data are means ± SD of 7 chambers used in four independent experiments.

9. Conclusions

Mitochondrial respiration media can have considerable impact on the results obtained in both simple respirometric experiments and on Multi-Sensor studies. Specifically, media may support respiration to a variable extent and may display huge differences regarding their suitability for fluorometric assays. Thus, as shown in this study, media have to be optimized to allow assessment of well-supported mitochondrial energetics while at the same time showing no interference with fluorometric assays but ideally even stably supporting these.

10. References

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Acknowledgements

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