Supplementary Material for 'Quantitative microplate-based respirometry with correction for oxygen diffusion'

Akos A. Gerencser[1*], Andy Neilson[2], Sung W. Choi[1], Ursula Edman[1], Nagendra Yadava[1], Richard J. Oh[1] , David A. Ferrick[2], David G. Nicholls[1], Martin D. Brand[1]

1. Buck Institute for Age Research, Novato, CA, USA
2. Seahorse Bioscience, North Billerica, MA, USA

*** correspondence:** Buck Institute for Age Research, Novato, CA, USA; e-mail: agerencser@buckinstitute.org; Tel: (415)-2092273

The aim here is to express the complete **OCR(*t*)** calculation (Eq. 13 and Eq. 14; see in the article) in a spreadsheet in a user-friendly and accurate manner.

Handling temporal differentiation and integration

Differentiation required for the calculation of Eq. 14 is noise sensitive. To diminish noise originating from the differentiation, Savitzky-Golai (SG) kernel filtering is used with a window with of 7: [0.107, 0.071, 0.036, 0, -0.036, -0.071, -0.107]. This enables substantial smoothing, but also discards 3 data points from the beginning and 3 from the end of each measurement cycle. In addition, when calculating **OCR(*t*)** and $1/\boldsymbol{k_P} > 0$ the data is differentiated twice. Unless the differentiation is implemented twice as a calculation of second derivatives with a single kernel filtering, there is a resulting loss of 6 ticks at the beginning and 6 at the end of each measurement cycle. Therefore $d^2[O_2]_M/dt^2$ in Eq. 14 is calculated by a second derivative SG kernel [0.0595,0,-0.0357,-0.0476,-0.0357,0,0.0595]: the $[O_2]$ values filtered with the above kernel are divided by the square of the time point values filtered with the first derivative order kernel. The kernel coefficients were generated in Mathematica 5.2. (Wolfram Research) according to Press and Teukolsky[1].

Eq. 13 must be integrated using polynomial interpolation, because of the nature of integration of the e^x function (error is accumulated by time when using the trapezoid method). This integration using interpolation can be implemented as a pre-calculated formula, which calculates the integral from (time, value) data pairs. Here the second order polynomial (Eq. 16; for independent variable *x*) exactly fitting on ((*ta*,*a*),(*tb*,*b*),(*tc*,*c*)) data points is integrated. The integral is shown in Eq. 18 as calculated for the second time point (between *x* = *ta* and *x* = *tb*).

$$a + (-ta + x)\left(\frac{a - b}{ta - tb} + \frac{\left(\frac{-a+b}{ta-tb} + \frac{b-c}{tb-tc}\right)(-tb + x)}{-ta + tc}\right) \tag{Eq. 17}$$

$$-\frac{(ta - tb)\left(-c\,(ta - tb)^2 + b\,(ta + 2\,tb - 3\,tc)\,(ta - tc) + a\,(2\,ta + tb - 3\,tc)\,(tb - tc)\right)}{6\,(ta - tc)\,(tb - tc)} \tag{Eq. 18}$$

Thus integrals are determined by a running calculation of Eq. 18 on each (time, value) data pair using time points one step before, current, and one step after for each time point. This is followed by summing the integrals of all earlier time points in each time point. These formulae were generated in Mathematica 5.2. (Wolfram Research) using the InterpolatingPolynomial and Integrate standard functions.

Eq. 13 integrates **$[O_2]_M(t)$** from the beginning of the experiment, thus the output depends on the complete history of the measurement. In addition this means that all data points have to have their correct time stamp compared to the beginning of the whole experiment. This includes the time spent in mix and wait positions. Because $[O_2]$ data in the mix position is inaccurate, and not available in the wait position, **$[O_2]_M(t)$** has to be interpolated (assumed) between the last point of a measurement phase and the first point of the next measurement phase. To this end a simple exponential rise is substituted into the mix and wait phase data (Eq. 19).

$$b - (b - a)\,e^{-\frac{t}{30}} \tag{Eq. 19}$$

In Eq. 19 *a* is the last **$[O_2]_M(t)$** value of the measurement phase before the mix and *b* is the first data point of the next measurement phase. *t* starts from zero at the first substitute tick. 30 is a time constant in seconds. This calculation precedes performing Eq. 13.

Performing smoothing differentiation and integration with interpolation in Excel

See “differentiation and integration.xls”.

- First order differentiation with Savitzky-Golay kernel (diff. order = 1, polynomial order = 2, width = 7): see formula in cell 'First order diff'!C5. First and last 3 points are discarded.
- Second order differentiation with Savitzky-Golay kernel (diff. order = 2, polynomial order = 2, width = 7): see formula in cell 'Second order diff'!C5. First and last 3 points are discarded.
- Smoothing with Savitzky-Golay kernel (diff. order = 0, polynomial order = 2, width = 7): see formula in cell 'smooth'!B5. First and last 3 points are discarded.
- Integration with 2^{nd}-order interpolation: the integral for each tick is calculated by fitting a second order polynomial on 3 data points, and integration of the polynomial (see formula in 'Integration with interpolation'!C3). The first integral (C2) is set to zero. To obtain the complete integral, tick integrals are summed in column D.

Performing the compartment model correction in Excel:

See “compartment model correction of OCR example.xls”

- **Columns A-D:** data from the ‘Level Data’ sheet corresponding to the mean fluorescence values of the O_2 probes in 20 measurement wells. Data correspond to Fig. 4. Ticks are shown **from the first measurement point** (initial mix is discarded) **to the end** of the experiment.
- **Column E:** mean fluorescence data of four temperature wells.
- **Column F-H:** a time scale in seconds has to be established. For the XF24, the time scale is calculated **from the Time stamps, and not from the Ticks**, because ticking stops during wait phases. The calculation of $\mathbf{[O_2]_W(\mathit{t})}$ requires accurate absolute timing for the whole experiment including mix and wait periods to calculate integrals below. The ‘Time’ column (**B**) also has rounding errors in the seconds; this is fixed in column (**G**). Column **(H)** is the corrected time scale of the experiment. **Ticks and corresponding time points are inserted manually for the wait periods.**

- **Column I:** $[O_2]$ is calculated with the temperature-dependent **gain correction** (Eq. 5). This is the measured $[O_2]$: $\mathbf{[O_2]_M(\mathit{t})}$. Then **mix and wait periods** (shown in green) are filled with interpolated values using Eq. 19.
- **Columns J-L:** first and second temporal derivatives and smoothed $[O_2]_M$ signal calculated using kernel filtering. This provides $\mathbf{d[O_2]_M(\mathit{t})/dt}$, $\mathbf{d^2[O_2]_M(\mathit{t})/dt^2}$ and $\mathbf{[O_2]_M(\mathit{t})}$ for Eq. 13. $\mathbf{[O_2]_M(\mathit{t})}$ is smoothed (in L) to achieve similar temporal smearing of the data that happens during differentiation with kernel filtering (i.e. each value is calculated from the values three time points before and three time points after the actual time point).
- **Column M:** non-corrected rates calculated in pmol/min/7 μl for comparison only (7 μl is the geometric volume of the entrapped volume). Three ticks at the beginning and at the end of each measurement cycle are discarded, because of the border effect of the kernel filtering.
- **Column N:** The $e^{(kAW + kW)t}$ tag of Eq. 13.
- **Column O:** The time scale is copied form (**H**) to make the application of the precalculated integrator formula easy (see “differentiation and integration.xls”).
- **Columns P-R:** calculation of the integral of Eq. 13 for each time point. The expression within the integral (**P**) is integrated first between ticks using polynomial interpolation (**Q**), then these tick integrals are summed (**R**).
- **Column S:** calculation of the result of Eq. 13, this yields $[O_2]$ in the walls of the chamber.
- **Column T:** Calculation of the real biological **OCR(*t*)** by Eq. 14 in mmHg/s.
- **Column U:** Conversion of **OCR(*t*)** into pmol/min/22.7 μl (22.7 μl is the value of V_C, thus these values reflect the **OCR(*t*)** per well. **Rates at 3 ticks before and after mix and wait periods have to be removed**, because of the border effect of the kernel filtering.
- **Column AB-AD:** Definition of $\mathbf{[O_2]_0}$ defines the units of $[O_2]$ throughout the spreadsheet. The K_{SV} is obtained from F_0 by Eq. 2. The apparent time and rate constants and the probe response constant are given in s and 1/s, respectively. V_C, the apparent chamber volume, is in μl.

- **Optimization:** A simplified version of the optimization procedure is given here. Optimization in Excel can be done by the ‘Goal Seek’ function. Set ‘Set cell’ to AD15 (this is the calculated variance); ‘To value’ to 0; ‘By changing cell’: give here one cell corresponding to the ‘Tau’ values in AC9-AC13.

To analyze new data with the spreadsheet, replace the contents of the columns ‘Time’ (C), ‘S2 O2’ (D), ‘S2 O2 temp well’ (E) with new time stamps, fluorescence intensities and control fluorescence intensities, respectively. If a proper time scale, or measured [O_2] data is available, columns (H) and (I) can be replaced by the new data. If the new [O_2] data has different units than mmHg, set the ambient [O_2] in cells AD4 accordingly.

Next, set the length of calculation columns (F-U) according to the length of the newly inserted data, by selecting cells F7-U7 and copying/pulling down contents to the bottom.

If data contains mix and wait periods or pauses, insertions of time points and filling in missing [O_2] values is required.

To compare calculations performed with $\boldsymbol{k_{AW}} = 0$ or $\boldsymbol{k_{AC}} = 0$, enter time constants from Table 1 into **columns AB-AD**.

Reference List

1. Press, WH.; Teukolsky, S. *Computers in Physics* **1990,** *4*, 669.