

## SUPPLEMENTAL MATERIALS

### 1. Derivation of Neutral Red Absorbance from the intensity of backscattered light.

Mathematically, the reduction in the amount of backscattered light (I) in the presence of Neutral Red can be treated in a way similar to the more familiar, absorption-mediated decrease in transmitted light (T). In a very thin slice of absorbing medium, the reduction in transmitted light – dT is proportional to light intensity, slice thickness (dx), and the concentration (c) and specific extinction coefficient ( $\epsilon$ ) of the absorbing agent, or

$$-dT = T * c * \epsilon * dx, \quad \text{or} \quad -dt/T = c * \epsilon * dx \quad (\text{formula 1})$$

Integrating this over total depth (x) of absorbing medium gives rise to:

$$-\int_{T_0}^{T_x} T^{-1} dT = \int_0^x c * \epsilon * dx, \quad \text{or} \quad \ln(T_x / T_0) = -c * \epsilon * x \quad (\text{formulas 2,3})$$

or in reverse form to:

$$T_x = T_0 * e^{-c * \epsilon * x} \quad (\text{formula 4})$$

Here, specific absorption ( $c * \epsilon$ ) is proportional to the natural logarithm of transmitted ( $T_x$ ) to initial light intensity ( $T_0$ ). As transmitted light passes through many thin optical layers, a reverse process takes place, causing reflection of a fraction of transmitted light. Neglecting surface reflection and assuming a simple proportional factor ( $r_p$ ) between backscattered (I) and transmitted (T) light, TV camera recorded light backscattering becomes a function of T(x), integrated over medium depth of x:

$$I = r_p * \int_0^x T(x) dx = r_p * T_0 \int_0^x e^{-c * \epsilon * x} dx \quad (\text{formula 5})$$

In the final form, this becomes:

$$I = r_p / (c * \epsilon) * T_0 * \left| e^{-c * \epsilon * x} \right|_0^x = T_0 * r_p / (c * \epsilon) * \left| 1 - e^{-c * \epsilon * x} \right| \quad (\text{formula 6})$$

Assuming that almost all light is absorbed within the tissue by the time it reaches the deep end and does not bounce from the back-end of the tissue back to the front (i.e.  $\lim e^{-c * \epsilon * x} \rightarrow 0$ ), the formula for backscattered light obtains the form of:

$$I = T_0 * r_p / (c * \epsilon), \quad \text{or simply} \quad I = T_0 * r_p / A \quad (\text{formula 7})$$

with  $A$  representing the previous double absorption term  $c \cdot \epsilon$ . In this context, light reflected within the tissue is inversely proportional to tissue absorbance ( $A_T$ ). Assuming that the absorbance due to Neutral Red ( $A_{NR}$ ) is simply additive, i.e. does not interfere or synergise with tissue absorbance, backscattered light from tissue containing Neutral Red ( $R_{NR}$ ) would be defined by the formula:

$$INR = T_{0^*} r_p / (A_T + A_{NR}) \quad (\text{formula 8})$$

and reflected light ( $R_T$ ) from naïve tissue not containing Neutral Red as:

$$I_T = T_{0^*} r_p / A_T \quad (\text{formula 9})$$

Under these conditions, the ratio of backscattered light from tissue not containing ( $I_T$ ) to tissue containing Neutral Red ( $I_{NR}$ ) will simply represent the ratio of  $A_T + A_{NR}$  to  $A_T$ :

$$I_T / I_{NR} = (A_T + A_{NR}) / A_T \quad \text{or} \quad I_T / I_{NR} = 1 + A_{NR} / A_T \quad (\text{formulas 10, 11})$$

and subtracting 1 on both sides, this resolves into:

$$(I_T - I_{NR}) / I_{NR} = A_{NR} / A_T \quad (\text{formula 12})$$

## 2. Colour Balance and Green & Blue Absorbance Calculations

Recorded images from tissues containing or without Neutral Red were used to determine the colour balance (Green-Red, Blue-Red, Blue-Green), and then employed to calculate the absorbance in green, blue and green/blue ratio, needed for the pseudo-colour pH maps.

Colour balances were calculated from images from naïve mice without Neutral Red. The Blue-Red (Bb-r) balance for each individual pixel of the image was determined by subtracting the Optical Luminosity Values (OLV, 0-255, 8bit) for Red (OLV<sub>red</sub>) from that for blue (OLV<sub>blue</sub>). Similar formulas were also used for the Green-Red and Green-Blue balance. In the case of Green-Red (Bg-r), 30 points were added, as the 8bit Optimas calculations only support operations in the 0-255 range and luminosity in green was lower than in red. For the Green-Blue balance, additional 20 points were added, for the same reason:

$$Bb - r = OLV_{blue} - OLV_{red} \quad (\text{formula 13})$$

$$Bg - r = OLV_{green} + 30 - OLV_{red} \quad (\text{formula 14})$$

$$Bg - b = B_{g-r} + 20 - B_{b-r} \quad (\text{formula 15})$$

For processing RGB images from Neutral Red injected mice for pH map data, red-derived Blue (rdB) value was calculated by starting with  $OLV_{red/nr}$  and adding the Blue-Red balance, and, in case of red-derived Green (rdG), the Green-Red balance

$$rdB = OLV_{red/nr} + Bg - r \quad (\text{formula 16})$$

$$rdG = OLV_{red/nr} - 30 + Bg - r \quad (\text{formula 17})$$

The relative absorption in the blue bandwidth (Abl) was calculated according to formula 7 by subtracting  $OLV_{blue/nr}$  from rdB and dividing it by  $OLV_{blue/nr}$ ; that for absorption in green (Agr) by subtracting and dividing it with  $OLV_{green/nr}$ . Relative absorption in green to blue (Agr/bl) was obtained by dividing the two:

$$Abl = (rdB - OLV_{blue/nr}) / OLV_{blue/nr} \quad (\text{formula 18})$$

$$Agr = (rdG - OLV_{green/nr}) / OLV_{green/nr} \quad (\text{formula 19})$$

$$Agr/bl = Agr / Abl \quad (\text{formula 20})$$

### 3. pHi Calibration

We used the pH-mediated changes in the absorption spectrum of brain-permeable pH indicator Neutral Red (La Manna, 1984), via colour photometry of backscattered light ( $I_{NR}$ ). The apparent pHi was deduced from Neutral Red absorption in the green and blue wavebands using 3CCD RGB video camera and Optimas 6.5 image processing software. This change in absorption was first calibrated using 10mm tall, fluid well columns containing 10% brain homogenate in 0.1M phosphate/boric acid buffer with defined pH values ranging from 5.0 to 8.0 (fig 1A) (La Manna 1984). Homogenates of fresh brain tissue in 0.9% saline were prepared by passing the tissue sequentially through 20, 23 and 26-gauge needles. In a microtitre plate (Nunc) 200 $\mu$ l standard brain homogenate solutions were made containing 10% brain homogenate, in 0.1M phosphate/boric acid buffer corrected to known pH (5.0-8.0). To explore whether intravital binding of the indicator would change its pH or spectroscopic properties, in one set of experiments, brain homogenates were mixed with neutral red *in vitro*; in the second, brain homogenates were prepared from animals injected with Neutral Red 2 hours previously. Calibration curves were constructed using brain homogenates from mouse pups injected *in vivo*, 2h previously, with neutral

red (as above), and with unstained brain homogenate to which 15µl of 0.1% Neutral Red was added. Phosphate/borate buffered brain homogenates without Neutral Red were used as controls for light backscattering ( $I_0$ ) in the absence of Neutral Red absorption. Absorption in blue and green (and red), were calculated from  $I_0/I_{NR}$  for blue and green wavebands, respectively (see formulas 1-12, supplemental material 1), and the resulting Green/Blue ratio (G/B) fitted using a formula derived from the law of mass action:

$$NR_{acidic} / a \leftrightarrow H^+ + NR_{basic} / b; pH - pK_{nr} = \log_{10}([NR_b]/[NR_a])$$

with the relative absorptions  $c_1$  and  $d_1$  of acidic Neutral Red in the green and blue wavebands, and  $c_2$  and  $d_2$  for the basic Neutral Red, respectively.

$$G / B = ((c_1 / d_1 - c_2 / d_1) / (1 + 10^{(pK_{nr} - pH)}) + c_2 / d_1) / ((1 - d_2 / d_1) / (1 + 10^{(pK_{nr} - pH)}) + d_2 / d_1)$$

with the  $c_1/d_1$  building the upper (acidic) asymptote and  $c_2/d_2$  ( $= c_2/d_1 / d_2/d_1$ ) the lower (basic) asymptote of the calibration curve shown in Supplemental fig.1B. Spectroscopic differences between intravital and invitro admixture of Neutral Red were minimal. Best fit minimum squares algorithm for intravital Neutral Red revealed an apparent  $pK_{nr}$  of 6.35,  $c_1/d_1$  of 3.84,  $c_2/d_1$  of 1.05, and  $d_2/d_1$  of 0.87. The calculated curve fitted the data points with an  $r^2$  value of 0.977. In the following part, the pH was calculated using the reverse formula:

$$pH = pK_{nr} + \log_{10}((d_2 / d_1 * G / B - C_2 / d_1) / ((1 - 2 * d_2 / d_1) * G / B + c_1 / d_1))$$

#### 4. Single image processing technique

Since both basic and acidic forms of Neutral Red absorb only weakly in the red part of the visible spectrum compared with their maxima in blue and green, respectively, we next explored the potential of using the red waveband recording from the same section as a surrogate marker for the “would-be” intensity of backscattered green and blue light in the absence of Neutral Red. This would then allow calculation of absorbance from the actual (with Neutral Red) and the “would-be” intensity (without Neutral Red) of backscattered light in the green and blue waveband, allowing pixel by pixel determination of pH from directly recorded RGB images of the cut brain surface.

As shown in fig.1, the colour balance analysis of backscattered light following unilateral carotid occlusion alone (fig.1C), showed very similar mean values for the green to red differential (i.e.

difference in the optical luminosity values/OLV in between blue and red) averaged across the brain for the occluded (left) and non-occluded (right side) in the absence of Neutral Red. Similar absence of significant ipsi- to contralateral difference was also observed for the average blue to red, and blue to green balance. Further analysis also showed very similar levels of intra-image, pixel to pixel standard deviations on the occluded, as well as the non-occluded side (fig.1D). Similar absence of ipsi- to contralateral difference for mean and SD values was also observed after 90 min exposure to 8% oxygen (fig.1E,F). Moreover, the inter-pixel standard deviations were also relatively moderate, i.e. at most 4 OLV points or just 10-20% of the absolute colour balance values.

To determine whether these balances stay the same or change before, during or after hypoxic/ischemic insult, we next examined the changes in the green to red, blue to red and green to blue colour balance in un-operated control animals, after carotid occlusion alone, after 30-90 min exposure to 8% oxygen and at 2-24h of re-oxygenation (fig.1G-I). With the exception of the moderate ipsi- to contralateral difference of 3-4 points in green to red, and blue to green balances at 30 and 60 minutes of 8% oxygen ( $p < 0.05$ , paired t-test), all other time points did not reveal a significant difference between occluded and non-occluded side. As shown in fig.1J, inclusion of the 3.5 point green/red & green/blue balance correction for the occluded and non-occluded side at 30min hypoxia produced a slight decrease in acidosis on the ipsilateral, and slight increase on the contralateral side but the effect was moderate and did not significantly alter the resulting image maps.

In contrast to the green to red balance, that for blue to red increased, from approx 17.5 points for the right as well as the left side in control animals, peaking at approx 25 points after 90min of hypoxia ( $p < 0.05$  in one way ANOVA and posthoc Tukey, vs. the naïve, 30min hypoxia, and 2&6h of reperfusion). It then decreased to 13 points at 6h of re-oxygenation ( $p < 0.05$  vs. CROC, 60&90min hypoxia and 2&24h reperfusion) and returned to approx 23 points at 24hours. As expected, reverse changes were observed for the green to blue balance, with a peak in control animals and 6h re-oxygenation, and a trough following 90 min of hypoxia. To avoid systemic errors and for reasons of consistency, all pH map calculations were therefore based on settings derived from naïve mice not injected with Neutral Red but that experienced the same HI insult and re-oxygenation conditions as the animals injected with Neutral Red.

## SUPPLEMENTAL FIGURES

### Supplemental Figure 1. Image analysis: pH Calibration (A, B) and Colour Balance Measurements (C-H).

**A:** Photographic images of 10% brain homogenate in 0.1M phosphate/borate buffer corrected to known pH 5.0-8.0, with 15 $\mu$ l of 0.1% Neutral Red. **B:** Standard titration curve (dots) and best fit algorithm (lines) for absorbance green / blue for 10% brain homogenates at known pH. Half-maximal change was observed at pH 6.2. Note the minimal spectroscopic differences between *intravital* (red dots and line) and *invitro* (blue dots and line) admixture of Neutral Red. Mean +/- standard error of mean/SEM, n = 3 samples. **C, D:** Colour balance analysis of backscattered light following unilateral carotid occlusion (CROC) alone in the absence of Neutral Red, on the occluded (oc), and non-occluded (noc) side. The balance for Green minus Red (G-R), Blue minus Red (B-R) and Green minus Blue (G-B) were calculated as described in supplement 2. The average intrahemispheric mean is shown in C, the average intrahemispheric standard deviation (SD) in D (n =3). **E, F:** Pixel to pixel intrahemispheric mean (E) and SD (F) following unilateral carotid occlusion and 90 minutes hypoxia (n = 2 mice). **G-I:** Changes in backscattered light balance following carotid occlusion, hypoxia (H) and reoxygenation (reox) for Green minus Red (G), Blue minus Red (H) and Green minus Blue (I). n = 2-3 mice per time point. Note the difference between occluded and non-occluded side for Green-Red (G) at 30 and 60 minutes of hypoxia (\*p<0.05 in paired Student t-test). The Blue-Red balance (H) shows bilateral, significant difference at 90 min of hypoxia versus naïve, 30min hypoxia, and 2&6h of reoxygenation, and at 6h of reoxygenation versus occlusion alone, 60&90min hypoxia and 2&24h reperfusion (\*p<0.05, one way ANOVA followed by posthoc Tukey). Slightly less extensive changes were also observed at both time points for the blue Blue-Green balance. **J:** Pseudocolour pH map of coronal brain section following carotid occlusion and 30 minutes hypoxia with (left) and without (right) adjustment for inter-hemispheric difference in Green-Red colour balance.

**Supplemental Figure 2. A-G:** Real colour image (A) and pseudocolour pH maps (B-G) of all 15 coronal brain levels taken at 400 $\mu$ m intervals throughout the forebrain (top – rostral, bottom – caudal), starting with the olfactory bulb. **B:** naïve animals, **C:** 60 minutes of exposure to 8%O<sub>2</sub> (hypoxia alone), **D:** carotid occlusion alone, **E-G:** carotid occlusion with additional 15-60 minutes

hypoxia, showing the rapid onset of brain acidosis in the main part of the ipsilateral middle cerebral artery in the 15°-90° segment of the cerebral cortex.

**Supplemental Figure 3. A-G:** Pseudocolour pH maps of all 15 coronal brain levels, continuation of the time course from supplemental figure 1. **A:** carotid occlusion with additional 90 minutes hypoxia, **B-G:** re-exposure to room air for 1(B), 2 (C), 4 (D), 6 (E), 12 (F) and 24 (G) hours.





