1 Setup

The setup comprises two cameras A and B which sample the mouse brain with a frequency $f_0 = 100$ Hz at times t_i. In each sampling interval of duration $\Delta t = 1/f_0$, each camera recorded two frames, one at time t_i with laser illumination on, and one at time $\tilde{t}_i := t_i + \Delta t/2$ with laser illumination off. Camera A yielded the signals $D(t_i)$ (donor) and $V(\tilde{t}_i)$ (volume), while camera B yielded $A(t_i)$ (acceptor) and $O(\tilde{t}_i)$ (oxygenation).

During preprocessing, A and O will be spatially transformed to yield images which are in the same reference frame as D and V. In general, a transformation T is a combination of a rotation R and shift S, with the rotation performed first, $T = S \circ R$.

Notation: r denotes space (x, y), t time. Signals have a transient which is often excluded from processing. Here, t_0 is a starting time (currently bin 101), and t_e the end of the recorded interval. $X \longleftarrow X+Y$ means that variable X gets replaced by the result of $X + Y$. Typically for one trial, signal $X(r, t)$ depends on space and time dimensions. These dependencies are not mentioned if a mathematical operation is performed over all dimensions.

2 Preparations for Preprocessing

2.1 Stage 1: Get Reference Image

- First stage extracts 4 reference images D_{ref}, V_{ref}, A_{ref}, O_{ref} from the D, V, A, O signals:
- ...for all signals, reference image is the middle frame from the first trial of the first experiment.
- ...for all signals, heartbeat is extracted as variance of the bandpassed signal
- ...results are 2D arrays stored in 4 x 2 files

2.2 Stage 2: Make Heartbeat Mask

- Second stage serves to define a ROI mask $M(r)$ using heartbeat information:
- ...shows signal D reference image and overlays D heartbeat mask
- ...lets user select threshold level and saves initial mask.

2.3 Stage 3: Refine Mask

- Third stage loads mask from second stage and lets user add additional regions to the mask, saves final mask.

3 Preprocessing

Stage 4 performs preprocessing on the basis of the previously extracted reference images (Stage 1) and the defined mask (ROI, Stage 2 and Stage 3). The following steps are performed **separately** for every experiment and every trial in a data set, unless a specific experiment and/or specific trial are selected by command line. Naming of the steps same as in log files.

(A) Loading data, reference and mask

No important operations performed on data, except detecting pixels with limit values (signal out-of-bounds).

(B–optional) Binning

Binning/integration of the data in n x n regions by summing signals for reducing noise (n configurable). Reduces also memory footprint.

(C) Alignment of cameras

First, signals from camera B are aligned to camera A (**global** alignment). Then, movements between frames are compensated by alignment over time. Finally, the mask M is updated to only include common field of view after camera rotation.

Global camera alignment T_0

for GECI: this step probably not required since only D and V are used.

D and V remain fixed, A' and O' will be rotated. $||\ldots||$ means quadratic norm:

a) ...compute best matching transformation of A_{ref} onto D_{ref}:

$$T_0 \quad = \quad \text{argmin}_T||T \circ A_{ref}, D_{ref}|| \tag{1}$$

b) ...apply rot/trans to A_{ref} and to all A's, and to O_{ref} and to all O's:

$$\begin{aligned} A_{ref} &\longleftarrow T \circ A_{ref} && (2)\\ A &\longleftarrow T \circ A && (3)\\ O_{ref} &\longleftarrow T \circ O_{ref} && (4)\\ O &\longleftarrow T \circ O && (5) \end{aligned}$$

Local frame alignment $T(t)$

a) ...compute best matching rot's/trans's of all D onto D_{ref}:

$$T_D(t) \quad = \quad \text{argmin}_T||T \circ D(t), D_{ref}|| \tag{6}$$

b) ...compute best matching rot's/trans's of all V onto V_{ref}

$$T_V(t) \;=\; \text{argmin}_T||T \circ V(t), V_{ref}|| \tag{7}$$

c) ...average over best matching rot's/trans's for D and V, $T(t) = \langle T_x(t)\rangle_{x=\{D,V\}}$

d) ...apply average rot's/trans' to all D, V, A, O.

$$D(t) \;\longleftarrow\; T(t) \circ D(t) \tag{8}$$
$$V(t) \;\longleftarrow\; T(t) \circ V(t) \tag{9}$$
$$A(t) \;\longleftarrow\; T(t) \circ A(t) \tag{10}$$
$$O(t) \;\longleftarrow\; T(t) \circ O(t) \tag{11}$$

(D) Inter-frame interpolation

Time alignment of frames recorded with laser off to times of frames recorded with laser on. Performed for volume and oxygenation:

$$V(t_i) \;:=\; 0.5(V(\tilde{t}_i) + V(\tilde{t}_i - \Delta t))$$
$$O(t_i) \;:=\; 0.5(O(\tilde{t}_i) + O(\tilde{t}_i - \Delta t))$$

(E–optional) Heartbeat removal

Goal is to clean the signals from potential heartbeat artefacts. Heartbeat is extracted from volume signal V using a band-pass filter and subsequent singular value decomposition to identify the strongest principal component $h_t(t)$. The assumption is that the 'clean' signals X_{clean} are contaminated by heartbeat $h_t(t)$ with unknown spatial distribution $x_r(r)$ via $X(r,t) = X_{\text{clean}}(r,t) + x_r(r)h_t(t)$. Here, $X_{\text{clean}}(r,t)$ is assumed to no longer contain any overlap with h_t, thus $\sum_t X_{\text{clean}}(r,t)h_t(t) = 0$:

a) Bandpass-filtering by F_{band} and removing of temporal mean over whole time:

$$H_{\text{tmp}} \;:=\; F_{\text{band}} \circ V \tag{12}$$
$$H(r,t) \;=\; H_{\text{tmp}}(r,t) - \langle H_{\text{tmp}}(r,t)\rangle_t \tag{13}$$

b) Singular-value decomposition to identify strongest principal component:

$$\text{SVD}\,[H(r,t)] \;\longrightarrow\; h_r(r) \text{ and } h_t(t) \tag{14}$$

c) Removal from all recorded signals $X \in \{D, V, A, O\}$ with coefficient $x_r(r)$ derived by computing scalar product followed by normalization:

$$x_r(r) \;:=\; \frac{\sum_{t\in[t_0,t_m]} \tilde{X}(r,t)\tilde{h}_t(t)}{\sum_{t\in[t_0,t_m]} \tilde{h}_t(t)^2} \tag{15}$$
$$X(r,t) \longleftarrow X_{\text{clean}}(r,t) \;=\; X(r,t) - x_r(r)\tilde{h}_t(t) \tag{16}$$

d) Side remarks: Heartbeat removal uses the processing mask $M(r)$, cleanup only performed for r with $M(r) = 1$. Tilde means removal of temporal mean (for h, temporal mean computed within $[t_0, t_m]$, for X, temporal mean computed over whole time).

(F–optional) Acceptor/donor scaling

for GECI: definitely not applicable.

From previous step **E–OPTIONAL** use heartbeat amplitudes $a_r(r)$ and $d_r(r)$ in donor and acceptor, respectively, and compute scaling factors f_a and f_d:

$$f_a = \sqrt{\frac{d_r\,\overline{A}}{a_r\,\overline{D}}} \tag{17}$$

$$f_d = 1/f_a \tag{18}$$

with $\overline{X} = \langle X(t)\rangle_{[t_0,t_m]}$ for $X \in \{A, D\}$ denoting temporal mean without the transient at the beginning:

$$A \longleftarrow f_a(A - \overline{A}) + \overline{A} \tag{19}$$

$$D \longleftarrow f_d(D - \overline{D}) + \overline{D} \tag{20}$$

(G) Conversion to relative signal changes

Performed for all signals $X \in \{D, V, A, O\}$

$$X(t) \longleftarrow \begin{cases} X(t) \Big/ \langle X(t)\rangle_{[t_0,t_m]} & \text{if denom is nonzero} \\ 0 \quad \text{else.} \end{cases} \tag{21}$$

(H) Cleaning by regression

Signal smoothing for noise reduction

The goal of smoothing is to remove noise in estimating regression coefficients. Specific care is taken at mask borders.

a) The mask M is filtered with a 2D-Gaussian $G^{2D}_{\sigma_r}$ of width $\sigma_r = 2$ yielding a smoothed mask

$$\overline{M}(r) = G^{2D}_{\sigma_r}(M(r)) \quad .$$

$\overline{M}$ contains normalization factors for correcting the values of recorded signals in the vicinity of 'holes' implied by the original mask.

b) All signals $X \in \{D, V, A, O\}$, masked by the unsmoothed M, are spatio-temporally filtered with a 3D-Gaussian with spatial extent σ_r and temporal extent $\sigma_t = 0.1$, and normalized:

$$X_G(t) := \frac{1}{\overline{M}} G^{3D}_{\sigma_r,\sigma_t}(M\,X)$$

c) In a final step, all values X_G *outside* the mask M are set to one (we are now already computing with ratios).

Signal cleaning by regression

The goal of regression is to remove oxygenation and volume signals from the acceptor and donor signals, as well as offsets and linear trends. Notation X^- means $X-1$ for $X \in \{D, V, A, O, D_G, V_G, A_G, O_G\}$. The following mixture model with unknown coefficients $V_d(r)$, $V_a(r)$, $O_d(r)$, $O_a(r)$, $t_d(r)$, $t_a(r)$, $c_d(r)$, $c_a(r)$ is assumed:

$$\begin{aligned} D_G^-(t) &= V_d V_G^-(t) + O_d O_G^-(t) + d_G^-(t) + t_d\left(\frac{t}{T} - \frac{1}{2}\right) + c_d + \eta_D(t) \\ A_G^-(t) &= V_a V_G^-(t) + O_a O_G^-(t) + a_G^-(t) + t_a\left(\frac{t}{T} - \frac{1}{2}\right) + c_a + \eta_A(t) \end{aligned}$$

The following operations are only performed for 'valid' locations r with $M(r) = 1$:

a) Perform linear regression on the *smoothed* signals in $[t_0, t_m]$ such that energy functions $E_2(d_G^-)$ and $E_2(a_G^-)$ are minimized.

b) Use the computed coefficients to clean up the *unfiltered* signals $D(t)$ and $A(t)$:

$$\begin{aligned} d(t) &= D(t) - V_d V^-(t) - O_d O^-(t) - t_d\left(\frac{t}{t_m} - \frac{1}{2}\right) - c_d \\ a(t) &= A(t) - V_a V^-(t) - O_a O^-(t) - t_a\left(\frac{t}{t_m} - \frac{1}{2}\right) - c_a \end{aligned}$$

for GECI: regression only performed for D (first equation), without regressor O.

(I–OPTIONAL) Save oxygenation, volume, and mask

No important operations performed on data.

(J) Final steps

OPTIONAL post-processing binning/integration of the data in n x n regions by summing signals for reducing noise (n configurable).

J1–for GECI

Saves acceptor, donor, and mask.

J2–for GEVI/dual signal mode

Computes acceptor/donor ratio and again divides by mean
NOT REALLY CLEAR WHY DIVISION BY MEAN IS AGAIN PERFORMED HERE

$$r = a/d \tag{22}$$

$$r \longleftarrow r/\langle r \rangle_{[0,T]} \tag{23}$$

...or, as an approximation for $a \ll 1, d \ll 1$:

$$r \approx 1 + a - d \tag{24}$$

Some remarks on signal scaling

Fluorescence signal variations $\Delta F(t)$ are very small with a large offset F_0[1], $|\Delta F| \ll F_0$:

$$\Delta F(t) = F(t) - F_0 \tag{25}$$

$$\text{with: } F_0 = \sum_t F(t) \tag{26}$$

It is thus common to work with ratios $\Delta F(t)/F_0$ which are centered around zero, typically around 0.01 ... 0.001.

Acceptor signal a and donor signal d variations are different in their amplitudes, even if these variations originate from the same physical sources (and they can also have very different offsets). Let us denote these different scaling amplitudes with p_a and p_d. When acceptor and donor signal are offset against each other, these amplitudes have to be equilibrated first.

For determining p_a and p_d, one method is to compute how strongly a specific known signal is expressed in a and d. For this purpose one takes the heartbeat $h(t)$ which was previously derived from a different source.

We will discuss two scaling scenarios:

Multiplicative variation

Basic assumption is that fluorescence is *modulated* by physiological processes $p(t)$ with a certain amplitude, i.e.:

$$a(t) = a_0(1 + p_a\, p(t)) \tag{27}$$

$$d(t) = d_0(1 + p_d\, p(t)) \tag{28}$$

This leads to

$$\frac{\Delta a}{a_0} = p_a\, p(t) \tag{29}$$

$$\frac{\Delta d}{d_0} = p_d\, p(t) \tag{30}$$

Scaling with a factor $f_a = f$ and $f_d = 1/f$ brings both signals to the same amplitude, which is defined as the geometrical mean of p_a and p_d

$$\frac{\Delta a}{a_0} f^2 = \frac{\Delta d}{d_0} \Longrightarrow f = \sqrt{p_d/p_a} \tag{31}$$

$$\frac{\Delta a}{a_0} f = \sqrt{p_a p_d}\, p(t) \tag{32}$$

$$\frac{\Delta d}{d_0} / f = \sqrt{p_a p_d}\, p(t) \tag{33}$$

How to perform scaling with heartbeat measurements? If the overlap of the heartbeat $h(t)$ with signals a and d is computed and yields the coefficients h_a and h_d, then

[1] For signal analysis it is often assumed that $\Delta F(t)$ has zero mean which allows to extract F_0 by averaging over a recording.

$h_a = a_0\, p_a$ and $h_d = d_0\, p_d$, thus

$$p_a = \frac{h_a}{a_0} \tag{34}$$

$$p_d = \frac{h_d}{d_0} \tag{35}$$

Linear superposition

Basic assumption is that a physiological process $p(t)$ *adds* to an offset with a certain amplitude, i.e.:

$$a(t) = a_0 + p_a\, p(t) \tag{36}$$

$$d(t) = d_0 + p_d\, p(t) \tag{37}$$

This leads to

$$\Delta a = p_a\, p(t) \tag{38}$$

$$\Delta d = p_d\, p(t) \tag{39}$$

Scaling with a factor $f_a = (p_a + p_d)/(2p_a)$ and $f_b = (p_a + p_d)/(2p_d)$ brings both signals to the same amplitude, which is defined as the arithmetic mean of p_a and p_d

$$\Delta a\, f_a = \frac{p_a + p_d}{2}\, p(t) \tag{40}$$

$$\Delta d\, f_b = \frac{p_a + p_d}{2}\, p(t) \tag{41}$$

Acceptor-Donor Ratio

Final step of GEVI analysis is computing the acceptor-donor ratio according to the following equation:

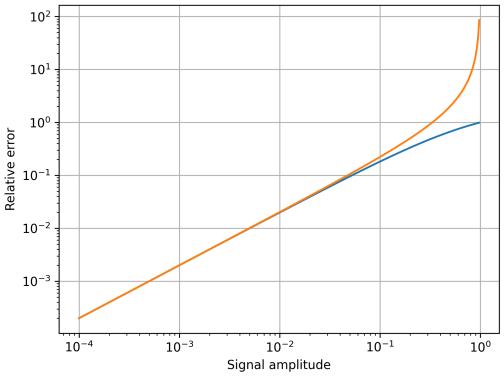
$$r(t) = \frac{1 + \frac{\Delta a}{a_0}}{1 + \frac{\Delta d}{d_0}} \tag{42}$$

Since $\frac{\Delta a}{a_0}$, $\frac{\Delta d}{d_0}$ are small against 1, we can use Taylor expansion to approximate:

$$r(t) \approx \left(1 + \frac{\Delta a}{a_0}\right)\left(1 - \frac{\Delta d}{d_0}\right) \tag{43}$$

$$\approx 1 + \frac{\Delta a}{a_0} - \frac{\Delta d}{d_0} \tag{44}$$

The following graph shows how the error relative to the donor ratio (acceptor ratio) scales with amplitude of donor ratio (acceptor ratio):


Relative error
Signal amplitude
10^{2}
10^{1}
10^{0}
10^{-1}
10^{-2}
10^{-3}
10^{-4}
10^{-3}
10^{-2}
10^{-1}
10^{0}