Supplementary Data for “Fluorescence Lifetime Imaging of Physiological Free Cu(II) Levels in Live Cells with a Cu(II)-Selective Carbonic Anhydrase-Based Biosensor”

The Supplementary Data section contains a detailed description of the phasor plot for the convenience of readers unfamiliar with this form of lifetime imaging presentation, and data fit to two components from in situ calibration measurements for the sensor.

1. Principle of the Phasor Plot

Fluorescence microscopy is a powerful approach for elucidating cell biology by relating molecular species and their amounts to their positions in the cell. Recently, fluorescent indicators that transduce the presence or level of chemical analytes as changes in fluorescence lifetime have proven useful in the study of a number of cellular processes by fluorescence lifetime imaging microscopy (FLIM) (1-4). A central issue in studies of this type is the depiction of fluorescence lifetime data for individual pixels of an image captured in a microscope. Some lifetime-based fluorescent indicators exhibit an essentially monoexponential decay which changes monotonically with analyte concentration; well-known examples include sulfopropyl acridinium for halide ions (5, 6) and ruthenium tris (polypryridyl) complexes for oxygen determination (7) Somewhat more common are lifetime-based indicators which exhibit a change in lifetime upon binding the analyte: in this case, the indicator exhibits differing proportions of two different lifetimes, with the proportions of the lifetimes reflecting the fractional occupancy of the binding site with the analyte and thus its concentration (8) (1, 9, 10). The difficulty is displaying the information for a pixel whose fluorescence comprises multiple decays: for instance, emission arising from multiple lifetimes minimally requires the lifetimes (τi) and their proportions (described as fractions of emission fi or pre-exponential factors αi) to describe the decay. Recognizing this, Redford and Clegg (11) adapted a formalism used for displaying dielectric relaxation to displaying phase fluorimetric data that is admirably suited for lifetime imaging.

In frequency domain fluorometry (also known as phase modulation fluorometry), the sample is excited with light that is amplitude-modulated at a high circular modulation frequency ω (= 2π x the frequency f, typically 1-1000 MHz). Because of the finite amount of time the fluorophore spends in the excited state, the fluorescence emission is phase-delayed by an angle φ and demodulated with respect to the excitation by a factor m to extents which for a single exponential decay) are simple functions of the lifetime and frequency:

\[
\tan(\phi) = \frac{\omega \tau}{S-1}
\]

\[
m = \frac{1}{(1 + \omega^2 \tau^2)^{1/2}} \quad S-2
\]

In principle one can know the entire time-dependent decay by measuring φ and m over all ω; in practice, one measures these parameters at multiple frequencies, and for non-imaging studies fits the data to some assumed decay law (12). If the decay is a single exponential the apparent lifetime calculated from S-1 or S-2 is the same, and invariant with frequency; also, m = cos φ at any frequency. If there are multiple decays present, m < cos φ and the apparent lifetime calculated from S-1 is less than that calculated from S-2, at any frequency. If the φ's and m's are plotted vs. frequency on a log scale for
a single exponential decay, one obtains a sigmoidal curve for each that is nearly a mirror image of the other Figure S-1. In Figure S-2 the curves are shown for monoexponential decays of 0.8 and 4.0 nsec, and a 50:50 (f_1=f_2) mixture of the two.

Figure S-1 Frequency-dependent phases (solid line) and modulations (dashed lines) for monoexponential decays of 4 (green) and 0.8 nsec (purple), and a 50:50 (f_1=f_2) mixture of the two (orange).

If two exponentials are present, the frequency-dependent phase angles and modulations describe a distorted sigmoid, whereas the curves for a monoexponential are the same, merely shifted in frequency.

The phasor plot of Redford and Clegg plots (for each individual pixel) the measured phase and modulation at some suitable frequency; for the example in Figure S-1, a range between 20 - 200 MHz would be satisfactory. The phasor plot uses polar coordinates, such that the phase angle $\phi$ is the angle a vector anchored at the origin makes with the x-axis, and the vector's length equals $m$. For monoexponential decays $m = \cos \phi$, and the points fall on the semicircular arc in the figure: a lifetime of zero would map to 1,0, and an infinite lifetime would map to 0,0. For a multiexponential decay the points map to the interior of the arc, with the exact values calculable from the sine and cosine transforms of the components. Thus an indicator that exhibited a monoexponential decay that changed monotonically with analyte concentration (e.g., due to simple collisional quenching) would map to points on the arc, whereas an indicator that exhibited different lifetimes when free or bound with analyte, would exhibit points in the interior of the arc as the fractional saturation varied from zero to 100%. An example of this is shown in Figure S-3, which depicts the mapping of pixels at 100 MHz having lifetimes corresponding to those in Figure S-2; e.g., 0.8 and 4 nsec, and a 50:50 (f_1=f_2) mixture of the two:
**Figure S-2** Phasor plot of φ (28.7 degrees) and m (0.89) at 100 MHz for 0.8 nsec monoexponential decay. The solid black circle is at G = m cos φ = 0.78 and S = m sin φ = 0.41. Redrawn from Redford and Clegg.

**Figure S-3** Phasor plot of phase and modulation at 100 MHz of monoexponential 0.8 (purple) and 4.0 (green)nsec decays, and a 1:1 mixture of the two (red).

The commercial software plots points on the phasor plot using the phases and modulations of individual pixels in an image, and one can highlight areas in the image based on their lifetime properties by selecting a subset of points with a small circle to indicate the region of interest (in ∆φ and m space). This is illustrated in **Figure S-4**, which is a cartoon of phasor plots for an hypothetical dye which exhibits a short lifetime in the nucleus and a long lifetime in the cytoplasm. Highlighting the points on the short lifetime portion of the arc (purple) provides pixels mainly in the nucleus, whereas the longer lifetime pixels on the phasor plot (green) map to the cytoplasms of the cells.
2. *In situ* Calibration of Biosensor: Two Component Fits

We also fit the phase and modulation data from the *in situ* calibration to two discrete lifetime components, rather than a distribution of average lifetimes. While determination of analyte concentrations from multicomponent decays is routine (reviewed in (8) (1, 9, 10)) in a test tube, the precision of FLIM data is regularly not as good as in non-imaging fluorometers.

As expected, most pixels fall inside the semicircular arc *(Figure 5)* indicating lifetime heterogeneity since the free copper concentration is near $K_D$ for binding to the protein and substantial proportions of both free and bound forms can be expected. Regions of interest comprising about 500 pixels within the images of twenty cells in multiple fields of each copper concentration at seven frequencies were fit to a two component decay law *(Equation S-4)*; those results are summarized in *Supplemental Table I*:

$$I(t) = I_0 \sum (\alpha_i e^{t/\tau_i}) \quad (S-4)$$

where $I(t)$ is the fluorescence intensity as a function of time, and $\alpha_i$ and $\tau_i$ are the preexponential factor and lifetime, respectively, of the $i$th component.
Supplemental Table I. Fitted lifetimes and preexponential factors for sensors in cells for each copper buffer.

<table>
<thead>
<tr>
<th>[Cu(II)]</th>
<th>$\tau_1$ (nsec)</th>
<th>$\alpha_1$</th>
<th>$\tau_2$ (nsec)</th>
<th>$\alpha_2$</th>
<th>$\chi^2_R$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apo 5.11 fM</td>
<td>5.11±0.79</td>
<td>0.27±0.04</td>
<td>1.32±0.29</td>
<td>0.72±0.06</td>
<td>17</td>
</tr>
<tr>
<td>Holo 4.17 fM</td>
<td>4.17±0.40</td>
<td>0.31±0.01</td>
<td>0.94±0.27</td>
<td>0.68±0.02</td>
<td>13</td>
</tr>
<tr>
<td>0.9 fM</td>
<td>4.41±0.16</td>
<td>0.33±0.02</td>
<td>0.99±0.12</td>
<td>0.68±0.02</td>
<td>5</td>
</tr>
<tr>
<td>0.9 pM</td>
<td>4.23±0.41</td>
<td>0.28±0.02</td>
<td>0.76±0.18</td>
<td>0.72±0.02</td>
<td>7</td>
</tr>
<tr>
<td>10 pM</td>
<td>3.71±0.16</td>
<td>0.25±0.02</td>
<td>0.57±0.10</td>
<td>0.75±0.02</td>
<td>14</td>
</tr>
<tr>
<td>70 nM</td>
<td>3.06±0.11</td>
<td>0.14±0.02</td>
<td>0.50±0.06</td>
<td>0.86±0.02</td>
<td>20</td>
</tr>
</tbody>
</table>

Values are reported as means ± standard deviation; goodness of fit is expressed as the reduced chi-squared $\chi^2_R$. The lifetimes were permitted to “float” in this analysis since the lifetime values for free and bound forms may differ in the cell from the buffer solution values obtained in Figure 4. It can be seen that the “floating” values for the lifetimes in the various Cu(II) concentrations in most cases are close to the expected values (4 and 0.8 nsec); moreover, the preexponential factors show the expected trend of decreasing for the longer lifetime component and increasing for the shorter lifetime component, respectively, as the copper concentration is increased. These fits were made to rectangular regions of interest within the cellular images. These results are near those obtained for the indicator alone in the metal ion buffers (Figure 4) and the average lifetime values reflect the trend of the data.

References