Time-Resolved Fluorescence Imaging of Blood-Brain Barrier Disruption in Living Mice

Rationale and Study
In this application, stroke regions were identified and blood-brain barrier (BBB) leakage of the left unilateral middle cerebral artery occlusion (MCAO) was detected in a living mouse model. This study demonstrates the ability to assess the extent of ischemic injury and thereby to determine tissue viability through the use of a time domain fluorescence imaging technique. The proposed application provides critical insight on stroke pathophysiology and is easily applied in the assessment of therapeutic strategies.

Unilateral MCAO Mouse Model
CD-1 mice were anesthetized for induction with 1.5% isoflurane and maintained in 1.0% isoflurane in 69% N₂O and 30% O₂ using a vaporizer. The animals were subjected to occlusion of the left middle cerebral artery (MCA) for one hour using an intraluminal filament. Consistency of stroke procedure can be verified by measuring blood pressure, blood gases, and pH.

Imaging Protocol
A group of 6 mice subjected to MCAO/reperfusion was injected with 100 nM of Cy5.5 near-infrared fluorescent probe via the tail vein. Mid-body regions of mice were imaged with the ART Optix® optical imaging system according to the timeline depicted in Figure 1. A 670 nm pulsed laser diode was used to excite the Cy5.5 probe and fluorescence signal was collected at 700 nm via predetermined appropriate filters. Afterwards, mice brains were removed, sliced, stained with triphenyltetrazolium chloride (TTC) solution and optically scanned using similar acquisition characteristics.

Figure 1. Imaging timeline overview

Background scan

Start of ischemic injury induction

End of ischemic injury induction and Cy5.5 injection via tail vein

1 hour ischemic injury induction

24 hours reperfusion

Fluorescence scan

Time (hour)

0 1 25
Histochemistry Procedure

After imaging, animals were perfused with heparinized saline and 4% paraformaldehyde. Brain sections were collected using a vibrotome and visualized in the near-infrared mode using a fluorescent microscope. Tomato Lectin-FITC was used to stain the cerebral vessels while DAPI was used to stain the nucleus.

Data Analysis Methodology

For each animal under study, the inherent auto-fluorescence signal was subtracted using the background scan and the manual option available in the OptiView™ analysis software for background removal. This procedure led to net fluorescence intensity images. Concentration and depth information maps were generated using the OptiView Depth and Concentration tool. Finally, fluorescence lifetime maps were derived by fitting the time-resolved acquired data with a double-exponential Levenberg-Marquardt algorithm. The fitting range was selected so as to minimize fitting errors.

In vivo Imaging Results

The average fluorescence intensity signal over the group of injured species was 6 times higher in the ischemic left hemisphere compared to the contra-lateral hemisphere (P<0.05, with a Student's 2-tailed t-test) (Figure 2b). Comparatively, the baseline average fluorescence intensities for left and right hemispheres before ischemia were similar (Figure 2a).

Additionally, the group of 6 mice subjected to MCAO ischemia/reperfusion injury exhibited a higher Cy5.5 concentration in the left damaged hemisphere compared to the contra-lateral uninjured section.

<table>
<thead>
<tr>
<th></th>
<th>Fluorescence intensity</th>
<th>$\tau_{av}$</th>
<th>$\tau_1$</th>
<th>$A_1$ (%)</th>
<th>$\tau_2$</th>
<th>$A_2$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-ischemia</td>
<td>Left</td>
<td>(1.15 ± 0.09)</td>
<td>(1.0 ± 0.05)</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Right</td>
<td>(1.15 ± 0.09)</td>
<td>(1.0 ± 0.05)</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Post-ischemia</td>
<td>Left</td>
<td>(6.97 ± 0.22)</td>
<td>(1.60 ± 0.10)</td>
<td>(2.1 ± 0.2)</td>
<td>20</td>
<td>(1.0 ± 0.05)</td>
</tr>
<tr>
<td></td>
<td>Right</td>
<td>(1.21 ± 0.18)</td>
<td>(1.93 ± 0.10)</td>
<td>(4.2 ± 0.2)</td>
<td>9</td>
<td>(1.7 ± 0.1)</td>
</tr>
</tbody>
</table>

Table 1. Average fluorescence intensity and lifetime values obtained non-invasively in the head, for a group of 6 mice. $\tau_{av}$ represents the average weighted fluorescence lifetime in ns, while $\tau_1$ and $\tau_2$ correspond respectively to the long-term and short-term fluorescence lifetimes (units in ns). $A_1$ and $A_2$ are the amplitude of the first and second exponential lifetimes, respectively.
These results are indicative of Cy5.5 leakage across the BBB. In particular, the highest concentrations were found within a depth of 2-7 mm, corresponding mainly to the brain cortex and some striatum region (Figure 3).

Fluorescence lifetime analysis (Table 1) demonstrated that ischemic hemisphere is mainly contributed by Cy5.5 probe lifetime ($\tau_2 = 1.0 \pm 0.05$ ns and $A_2 = 80\%$). Conversely, the contra-lateral hemisphere showed no Cy5.5, and fluorescence lifetime components likely originate from endogenous fluorophore ($\tau_2 = 1.7 \pm 0.1$ with $A_2 = 91\%$).

**Ex vivo Imaging Results**

The fluorescence intensity map of *ex vivo* brain slices showed higher Cy5.5 signal in the left hemisphere in all slices compared to the contra-lateral hemisphere (Figure 4a) and dual lifetime fitting analysis demonstrated that main fluorescence lifetime signal in slices #1, #2 and #3 comes from the Cy5.5 probe (in average, $\tau_2 = 1.0$ and $A_2 = 87\%$) (Figure 4b). These results indicate that the left ischemic hemisphere in brain slices #1, 2 and 3 has the highest BBB leakage and Cy5.5 extravasations.

Focal ischemia was confirmed for each mouse through histologic analysis (Figure 4c). The brain slices were TTC stained and the ischemic lesions were made visible. Normal brain sections were stained brick red while cerebral infarct remained pale. This can be observed in slices #1, #2 and #3.

**Histopathology Analysis**

Histopathologic sections of the damaged region and the contra-lateral uninjured hemisphere were viewed in fluorescence microscopy. Figure 5 shows Cy5.5 leakage (red) in left ischemic hemisphere, with slice #1, #2 and 3 exhibiting the highest parenchymal staining intensity. These results are in agreement with the histology analysis and with the *in vivo* and *ex vivo* fluorescence imaging findings.
Discussion and Conclusion

The Cy5.5 probe with a molecular weight of 1 kDa does not traverse the BBB under physiological conditions. Therefore, its leakage from the intravascular compartment reflects a disrupted BBB caused by focal ischemia. This disruption was revealed through fluorescence imaging of mice subjected to MCAO/reperfusion injury. Both fluorescence intensity and lifetime analysis were able to differentiate between ischemic and normal contra-lateral hemisphere in living mice. Parallel histopathological analysis of brain slices extracted from injured species confirmed the location of the ischemic regions that were identified through in vivo time-resolved fluorescence imaging.