Direct Thrombus Imaging as a Means to Control the Variability of Mouse Embolic Infarct Models

The Role of Optical Molecular Imaging

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Background and Purpose—High experimental variability in mouse embolic stroke models could mask the effects of experimental treatments. We hypothesized that imaging thrombus directly would allow this variability to be controlled.

Methods—We optically labeled thrombi with a near-infrared fluorescent (NIRF) probe C15 that is covalently linked to fibrin by factor-XIIIa. Labeled thrombus was injected into the left distal internal carotid artery (ICA) of C57BL6 mice (n=47), near its bifurcation, and laser-Doppler cerebral-blood-flow (CBF) was assessed for 30 minutes. NIRF thrombus imaging was done ex vivo at 24 hours.

Results—CBF variably decreased to 43.9±17.3% at 5 minutes (rCBF; 11.2~80.4%). NIRF thrombus imaging at 24 hours showed variability in distribution (ICA bifurcation, adjacent and/or remote areas) and burden (2279±1270 pixels; 0~5940 pixels). Final infarct size was also variable (21.0±10.3%; 4.7~60.3% of the bihemispheric volume). Despite this heterogeneity, a strong thrombus-infarct correlation was maintained. The left hemispheric target infarct size (% of the hemisphere) correlated with thrombus burden, as a stronger predictor of infarct volume (P<0.001, r=0.50) than rCBF (P=0.02, r=−0.34). The infarct size was best predicted by a combination of thrombus imaging and CBF: left-hemispheric big-thrombi (>1865 pixels)/low-rCBF (≤42%) had an infarct volume of 56.9±10.4% (n=12), big-thrombi/high-rCBF had 45.9±23.5% (n=11), small-thrombi/low-rCBF 35.7±17.3% (n=11) and small-thrombi/high-rCBF 27.3±16.4% (n=12).

Conclusions—This is the first study to demonstrate that the highly heterogeneous nature of the mouse embolic stroke model can be characterized and managed by using near-infrared fluorescent thrombus imaging combined with CBF monitoring to stratify animals into useful subgroups. (Stroke. 2011;42:3566-3573.)

Key Words: thrombus imaging ■ embolic cerebral infarction ■ molecular imaging ■ optical imaging

Ischemic stroke caused by cerebral thromboembolism is a leading cause of death and disability. Much effort has been put into developing effective neuroprotective treatments, but with limited success.1,2 A major difficulty in stroke research has been experimental variability in animal stroke models,3−5 leading to failures in identifying neuroprotective drugs. Embolic stroke models, in which preformed clots are injected into the middle cerebral artery–anterior cerebral artery (MCA–ACA) bifurcation area, mimic human stroke more closely than do other models of cerebral ischemia.6−9 However, they provide less control over the location and extent of the resulting cerebral infarction.3 Factors, such as spontaneous lysis6 or distal embolization of thrombi after injection into the MCA, as well as anatomic variations in the circle of Willis, could have major and variable impacts on the resulting infarct.10 This variability could mask and confound the potential therapeutic effects11 of neuroprotective drugs, and by adding to experimental noise, might mask or heighten the effects of experimental treatments, leading to hard-to-interpret data.

What is needed is an imaging methodology that would allow the visualization of thrombi11−14 and their characterization after injection into the cerebral vasculature; this would control for at least some of the experimental variability related to clot lysis, migration, and fragmentation.

To address this issue, we labeled thrombi optically with a molecular imaging thrombus marker—a 15-amino acid pep-
tide that is known to be recognized by activated coagulation factor XIII (FXIIIa)\textsuperscript{15} and labeled on the $\epsilon$ amino groups of lysine residues\textsuperscript{12,14} with Cy5.5 fluorescent dye (C15). This probe is covalently linked to the fibrin strands of the clot by the enzymatic action of FXIIIa, when it crosslinks fibrin strands during the process of clot maturation.\textsuperscript{15,16}

When inducing embolic strokes in mice, researchers monitor the decrease of cerebral blood flow (CBF) in the MCA with a laser-Doppler flowmeter. We hypothesized that not only CBF decrease,\textsuperscript{17} but also thrombus location and status, would affect the induced infarct territory or size, which is one of the most important outcomes in stroke research. To characterize the heterogeneity of embolic cerebral infarction and predict the final infarct size at 24 hours, we devised a technique of ex vivo near-infrared fluorescent (NIRF) imaging to measure the distribution and extent of the cerebral thromboemboli remaining at 24 hours; this was combined with in vivo laser-Doppler flowmetry (LDF) to monitor the CBF for the initial 30 minutes after embolic MCA occlusion. We also tried to demonstrate potential usefulness of quantitative visualization of thromboemboli in vascular research.

**Methods**

**Synthesis of the C15 Near-Infrared Fluorescent Thrombus Marker**

C15 NIRF imaging probes were synthesized as previously reported\textsuperscript{6,7,9} using C15 NIRF imaging agent or control Cy5.5 fluorochromes as optical markers. Briefly, 1000 $\mu$L of blood was drawn from C57/BL6 mice. Based on the results of pilot experiments, whole blood (70 $\mu$L) was mixed with the C15 probe (20 $\mu$mol/L, 30 $\mu$L) or equal concentration of control fluorochromes, and drawn up into a 30 cm-long polyethylene tubes using a 3 mL syringe. The tubes were stored at room temperature for 2 hours, then at 4°C for 22 hours. Then thrombi were gently removed from the tubes and washed 3 times with phosphate-buffered saline.

**Animal Experiments**

Embolic stroke was induced as previously reported\textsuperscript{6,7,9} with some modifications by injecting the C15-labeled clot (diameter, 0.15 mm; length, 15 mm) into the MCA–ACA bifurcation area of 10-week-old C57/BL6 mice ($n$ = 56; Supplemental Methods). Instead of 10 mm thrombus,\textsuperscript{9} we injected 15 mm thrombus after considering that the thrombus was diluted 7:3 during the labeling process.

Twenty-four hours later, the animals were euthanized, and the brains were removed and imaged ex vivo using a NIRF imaging machine.\textsuperscript{11,12,13,18} (Supplemental Methods). Fresh frozen sections of the brains were used for NIRF microscopic imaging (10-$\mu$m-thick sections) or immediate triphenyl tetrazolium chloride staining (2-$\mu$m-thick sections) to delineate the infarct area, whereas some specimens were paraffin-embedded for histology. Nine mice were excluded because of gross intracerebral or subarachnoid hemorrhage ($n$ = 6), imaging failure ($n$ = 2), or poor data quality ($n$ = 1).

**Quantitative Lesional Topography Analyses**

To assess quantitatively the extent and distribution of thromboemboli and ischemic brain injury, Cy5.5 signals on the NIRF images and whitish infarct areas on the triphenyl tetrazolium chloride-stained sections were mapped on templates using a custom-built software package\textsuperscript{11} (Supplemental Methods and Supplemental Figure S1). Based on the values of rCBF (% CBF relative to the baseline value) and/or thrombus extent (pixel numbers), various subgroup–mapsc were prepared.

**Computer-Simulated Virtual Neuroprotection Research**

To quantify the potential benefits of NIRF thrombus imaging and rCBF monitoring, we performed computer simulations (Supplemental Methods). Five sets of selected animals from the study cohort were randomly assigned to either a treatment group or a control group, and statistical comparisons were performed between the groups after either decreasing the measured infarct size according to the indicated neuroprotection rate (0–100% at 21 steps) or not.

**Statistical Methods**

Data are presented as mean±SD. Comparisons of continuous variables between groups were performed using the Student $t$ test or Kruskal-Wallis ANOVA test. In addition, Pearson correlation and multivariable regression analysis were performed. All statistical analyses were conducted using a software package (SPSS 18.0). A probability value $<0.05$ was considered statistically significant.

**Results**

**NIRF Imaging Allows an Assessment of Cerebral Thrombus Burden**

NIRF macroscopic (Figure 1A) and microscopic (Figure 1B) imaging confirmed specificity of the C15 probe for the thrombi, with C15 remaining tightly clot-associated; whereas free Cy5.5 dye washed away, consistent with covalent linkage of C15 to clot fibrin strands. As shown in Figure 1C, visual inspection with regular white light did not allow either cerebral infarction or cerebral thrombi to be shown. However, Cy5.5 NIRF tissue imaging showed scattered bright signal foci bilaterally over the expected locations of the cerebral arteries of the anterior part of the circle of Willis, mainly in the left MCA–ACA bifurcation area, but also contralaterally. Fluorescein isothiocyanate (FITC) channel tissue imaging showed infarct-related, auto-fluorescent signal in both hemispheres, more on the left ipsilateral to the clot, but clearly also contralaterally. Cy5.5 and FITC imaging of serial sections of the brain showed thrombi and infarct-related auto-fluorescence in the same vascular bed/territory. The Cy5.5 signals and FITC auto-fluorescent areas corresponded to the thrombi and infarcts on histology, respectively.

**Both Thrombi and Infarcts Are Heterogeneously Distributed**

Accumulation maps of thrombus and infarct locations showed the highly heterogeneous nature of the embolic stroke model (Figure 2A and Supplemental Results). The amount of thrombus-associated fluorescent signal was highly variable, ranging from 0 to 5940 pixels (2279±1270 pixels). Fluorescent thrombi signal was observed mostly in the left MCA–ACA bifurcation area. However, scattered emboli were frequently observed not only in the bifurcation area, but also in the adjacent or remote cerebral arteries. Approximately one third of the animals had emboli visualized in the contralateral right anterior circulation territory, more often in the ACAs than in the MCAs (Supplemental Results). This contralateral distribution of embolic clot is likely caused by the closer proximity of the contralateral ACA territory through vascular anastomoses through the circle of Willis compared with the
MCA; moreover, there are some variations, such as both ACAs arising from the side of the clot injection, making the ACA territory particularly vulnerable.

Total infarct size (%-infarct-area relative to the total bihemispheric area of 6 brain template slices) was also variable, ranging from 4.7 to 60.3% (21.0±10.3%). The accumulation infarct maps showed that infarcts were mainly located in the left MCA territory, particularly on template slices 2 through 5, including the sensorimotor cortex and basal ganglia. However, scattered infarcts were frequently observed not only in the left MCA territory, but also in the adjacent or remote areas of the ipsilateral hemisphere or contralateral hemisphere.

Despite the Heterogeneity of Thrombus and Infarct Distributions, a Strong Thrombus–Infarct Correlation Is Maintained

Between the subgroups with (Figure 2B; n=31) and without (Figure 2B*; n=16) emboli visualized in the right MCA–ACA bifurcation area, infarct size and distribution showed significant differences: bigger right hemispheric infarcts (% infarct area relative to the total hemispheric area of the brain template slices 2–5: 32.5±16.2% versus 45.2±20.8%; P=0.04) in the former than in the latter. In template slices 2 to 5, right

Figure 1. Optical molecular imaging of thrombi, allowing visualization of cerebral thrombus burden. Near-infrared fluorescent (NIRF) macroscopic A microscopic imaging B confirms the specific and tight association of the C15 probe with the thrombus. In A, the control thrombus with loosely associated free Cy5.5 dye does not retain the fluorescent stain after washing, but the thrombus treated with the C15 probe does retain the fluorescent signal because of the covalent linkage of the probe to the clot as demonstrated by macroscopic imaging. In B, the same finding is demonstrated but using microscopic imaging. A representative imaging-histology colocalization study shows that NIRF imaging visualizes not only the main body of thrombus in the left middle cerebral artery (MCA)—anterior cerebral artery (ACA) bifurcation (red arrowheads in C and D) but also scattered thromboemboli bilaterally in the adjacent or remote cerebral arteries, predominantly of the anterior part of the circle of Willis, 24 hours after preformed labeled clots were injected. A magnified view (inlet) of a cut section through the site of Cy5.5 signal in the MCA—ACA bifurcation (red arrowheads in C) reveals thrombi filling the bifurcating cerebral arteries (left), which is confirmed by the corresponding fluorescence microscopy image (right). In the ex vivo fluorescent isothiocyanate (FITC) channel C, bright auto-fluorescent signal is observed corresponding to infarcted areas. Both hemispheres have infarcts even though the clot was injected only on the left (though the left infarct is larger). Also note the correspondence of auto-fluorescent infarct-related signal (green arrowheads in C) and infarcted area on H&E staining (green arrowheads in D). Yellow scale-bar=1 mm. Black scale-bar=200 μm.

Figure 2. Accumulation lesion maps to show not only the heterogeneity of cerebral thromboembolism and infarction but also how well correlated thrombus and infarcts remain throughout this heterogeneity. Accumulation maps of total thrombi and infarcts (A, n=47), pseudocolor overlaid to indicate percentage of animals with lesions overlapping a specific pixel, show that thromboemboli and infarcts are located mainly but not exclusively in the left MCA or ACA territory, as expected. Between the subgroups with (B, n=31) and without (B*, n=16) emboli visualized in the right MCA–ACA bifurcation area (contralateral to the thrombus injection), infarct size and distribution show significant differences as pointed out in the third template slice (arrows and arrowheads): bigger right hemispheric infarcts (21.5±21.6% and 37.1±24.0%) than in the latter (8.2±17.2% and 51.1±21.1%; P=0.03 and P=0.05, respectively). C The group with the left-to-right embolization has lower thromboemboli burden in the left hemisphere (1204±623 pixels) than the group without (11915±1261 pixels, P=0.04), indicating that large left thrombi caused large left infarcts (B*), whereas bilaterally distributed thrombus gave bilateral infarcts with the main left infarct generally being smaller B. In the accumulation maps, blank regions mean ‘areas without lesion’ and blue (that corresponds to 0% at the lowest part of the pseudocolor-bar) regions mean “areas with lesion, but without overlap.”
hemispheric infarcts were nonsignificantly bigger in the animals with the left-to-right embolization than those without (15.3±10.3%; versus 9.1±15.5%; P=0.17).

The group with the left-to-right embolization had lower thromboemboli burden in the left hemisphere than did the group without († versus ‡ in Figure 2C; P=0.04). Considering that the same volume of thrombus was injected into each animal, left-to-right migration of some thrombus fragments likely resulted in increase of the right hemispheric infarct size; this corresponds to a reduction of thrombus burden in the left MCA–ACA bifurcation, resulting in a decrease of the left hemispheric infarct size. The other subgroup analyses (Figure 3A–D; groups with/without thrombi in the left proximal MCA or ACA) also demonstrated that thrombus imaging is a useful tool for characterizing the heterogeneous nature of embolic cerebral infarction in mice.

**Laser-Doppler Arterial Flow Measurement Has Modest Predictive Power for Infarct Size and Correlates With Clot Imaging Findings**

CBF variably decreased immediately after the placement of a thrombus in the left MCA–ACA bifurcation area (36.1±16.3%; range, 10.1–72.0%), followed by gradual increase over 5 minutes reaching a plateau to 43.9±17.3% (Figure 4A). Between the rCBF at 5 minutes and the left hemispheric infarct size at 24 hours (41.2±20.2%; range, 10.5–84.3%; template slices 2–5), there was an inverse linear correlation (P=0.02; r=−0.34; Figure 4B). When dichotomization was performed based on the median value (42%) of the rCBF, the infarct size was nonsignificantly bigger in the low rCBF group than in the high rCBF group (46.8±17.5% versus 36.2±22.1%; P=0.08). In the template slice-3 (approximately 1 mm posterior to the bregma), the low-rCBF group had significantly bigger infarcts (54.9%) than did the high rCBF group (39.1%; P=0.02).

**Infarct Size Could Be Better Predicted by Incorporating Both Thrombus Burden and rCBF Than by Considering Either One Alone**

Total thrombus burden (including both hemispheres) at 24 hours showed a linear correlation with the final bihemispheric infarct size (Supplemental Figure S2; P=0.016; r=0.35). In addition, thrombus burden in the left hemisphere showed a linear correlation with the final left hemispheric infarct size (Figure 4C; P<0.001; r=0.50). When dichotomization was performed based on the median value of the left hemispheric thrombus burden (1865 pixels), infarct size was significantly bigger in the big thrombi group than in the small thrombi group (50.6±18.7% versus 31.3±17.0%; P=0.001). Here, we focused on the left hemispheric thrombus burden because the main infarcts are located in the targeted left MCA territory, and LDF does not cover the right MCA territory.

The main left infarct size was best predicted by thrombus imaging combined with rCBF measurement (Figure 4D): the big thrombi–low rCBF group had the biggest infarct volume (56.9±10.4%, followed by the big thrombi–high rCBF group (45.9±23.5%), the small thrombi–low rCBF group (35.7±17.3%), and the small thrombi–high rCBF group (27.3±16.4%; Kruskal-Wallis test, P=0.002). The big thrombi–low rCBF group only had relatively small variability in terms of the infarct size (Figure 4D*). Representative cases (Figure 5A) and accumulation lesion maps (Figure 5B) corroborate the quantitative data described above.

A regression analysis to include both parameters revealed that the thrombus burden at 24 hours was statistically significant in predicting final infarct size at 24 hours (P=0.002), whereas rCBF at 5 minutes lost significance (P=0.34); this suggests that the residual thrombus burden appeared to be more influential than the initial rCBF in determining the final infarct size. According to the multivariate model, after adjusting for the rCBF at 5 minutes, the final infarct size was
estimated to increase by approximately 8% as the residual thrombus burden increased by 1000 pixels (Supplemental Table S1 and Supplemental Figure S1).

**Computer Simulations Show That False Negative Results in Neuroprotection Research Could Be Reduced by Selecting More Homogenous Groups Based on the Values of Both rCBF and Thrombus Burden**

When the animals with low rCBF and big thrombi \((n=12)\) were included, false-negative results became 0% after applying 35% or higher neuroprotection rate. When neither of these variables were considered, a 4-fold increase of sample size \((n=46)\) or either low rCBF \((n=12)\) or big thrombi \((n=12)\) alone was considered, false-negative results became 0% after applying approximately a 60% or higher neuroprotection rate. Notably, even if LDF showed low rCBF, with a low thrombus burden on thrombus imaging, it still had false-negative results becoming relatively frequent (Figure 6).

**Discussion**

In the present study, we show that a widely used embolic mouse model of stroke gives highly variable results. Using a sensitive NIF thrombus imaging technique, we observed bilateral thromboemboli after a unilateral injection in one third of cases. The exact distribution of emboli in an individual animal would be a function of: the variable anatomy of the circle of Willis in that animal, and the degree to which the clot fragmented in that animal. We also found significant variability in the volume of the target left hemisphere infarct, ranging from 10.5–84.3%. This represents a significant problem for stroke researchers, as final infarct volume is frequently used as a measure of outcome, and this variable needs to be as predictable as possible, or else subtle therapeutic effects might not be detected.

The solution to the problem of infarct variability is to have feedback mechanisms in place that allow one to know what sort of infarct one has achieved. We explored an optical molecular imaging approach to manage the variability in mouse embolic models of stroke, specifically by using direct thrombus imaging that attempts to image the root cause of embolic infarct: the thrombus itself.\(^1\) We compared direct thrombus imaging to laser-Doppler flowmeter measurement in the cerebral vasculature, a novel imaging parameter contrasted to a well-known pathophysiological flow measure-
Figure 5. Representative cases A and accumulation lesion maps B to corroborate the quantitative data in Figure 4. The first animal has a large thrombus, and a persistently low CBF, and has a large infarct, as expected. The second animal also has a big thrombus, but CBF did not stay down, giving rise to an infarct, but smaller than in the first case. The third animal has a low and fragmented thrombus burden (arrowhead), in the face of a CBF that stays low, and a small infarct results. The fourth animal has a fragmented and low thrombus burden and a CBF that quickly recovers, and has a small infarct. Lesion maps show this cumulatively for groups of animals. In the first group, with large thrombus burden and persistently reduced CBF, large infarcts are seen in nearly all animals (note the red areas on lesion maps indicating all animals having lesions at that location). In the second group with large thrombus but a CBF that recovers, we again see ipsilateral infarct in all animals, but less extensive than in the first group. The third and fourth groups indicate animals with low thrombus burden and persistently low and recovered CBF values respectively. In both these groups, the penetrance of infarct is much less and quite variable. The pseudocolor bar indicates percentage of animals with lesions overlapping a specific pixel. In the accumulation maps, blank regions mean “areas without lesion” and blue (that corresponds to 0% at the lowest part of the pseudocolor-bar) regions mean “areas with lesion, but without overlap.”
ment. LDF has been widely used as a way to assess the effectiveness of vessel occlusion, and in the setting of suture MCA occlusion models, correlates highly with final infarct volume. However, this method of monitoring was reported to be deficient in predicting the outcome of embolic stroke models, which is unfortunate, as embolic models are those that most closely mimic the process of infarction commonly seen in humans.

In our work, we have showed that thrombus imaging allowed ex vivo visualization of cerebral thrombus burden and distribution, which was closely correlated with final infarct volume and distribution. LDF enabled useful and immediate feedback, but its predictive capability for final infarct volume was more modest than was imaging thrombus burden, as was shown by the multivariate analysis.

The combination of thrombus imaging and rCBF allowed the best predictive results, and allowed the identification of large-volume, low-variability infarcts in approximately a quarter of animals: the subgroup with low rCBF at 5 minutes and big thrombi at 24 hours. By contrast, animals with low rCBF on LDF, when combined with small thrombus burden on thrombus imaging, had highly variable infarct sizes. In these animals, spontaneous thrombolysis with or without distal embolization likely occurred. We observed that rCBF immediately following the placement of a thrombus in the MCA was variable, but generally we saw a sharp decrease of flow followed by a slow increase of flow, probably caused by spontaneous thrombolysis.

Unmanaged infarct variability can cause great hardship to the stroke researcher and raise noise levels in mouse embolic infarct experiments to such high levels that therapeutic effects might be drowned out. We performed a simulation experiment for various cases where infarct variability was and was not controlled for, and found that therapeutic effects could be detected with far greater reliability if thrombus imaging and rCBF measurement were both used to select the best infarct subgroup. The implications in real-life research are obvious: by excluding highly variable animals, the discriminating power of experiments will increase, and false-negative results can be avoided. In addition, researchers could use thrombus imaging data for predefined posthoc adjustment or stratification of original outcome data.

This study, the first report on performing NIRF thrombus imaging and mapping in mouse embolic stroke, should be considered in the light of the following limitations. First, although thrombus imaging was found to be useful in our work, it still is an ex vivo, postmortem, imaging technique, not allowing more than a snapshot of time. Using transcranial window or NIRF tomography might allow in vivo serial monitoring of clot evolution in the future. Future advances in imaging may yet make noninvasive in vivo imaging of thrombus burden possible in the intact animal. Second, it is uncertain how much the labeling procedure alters natural clot biology. Last, late behavioral studies were not performed. Improved in vivo imaging studies might allow better behavioral outcome research to be conducted.

In summary, we have shown that embolic models of infarct in mice, although faithful mimics of infarcts in human patients, are beset by the same heterogeneity of tissue outcome that we also observe in humans, making research using these models difficult. We offer a helpful research tool to researchers hoping to control for infarct variability: direct thrombus imaging and laser-Doppler flowmeter measurements. These 2 tools in conjunction allowed for stratification of animals and selection of a subset of homogenous infarcts that were suitable for the detection of subtle therapeutic effects.

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Disclosures
None.

References
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Supplemental Methods

Synthesis of the C15 Near-Infrared Fluorescent Thrombus Marker

Factor XIIIa substrate peptide (GNQEQVSPLTLKWC, 1mg) was synthesized using standard solid-phase Fmoc peptide chemistry (Peptron, Daejeon, Korea). To obtain Cy5.5-maleimide, Cy5.5 N-hydroxysuccinimide (Thermo Fisher Scientific, Rockford, IL; 1mg, excitation / emission = 675nm / 695nm) dissolved in phosphate buffered saline (PBS, 400 μl, pH 7.0; Sigma-Aldrich, St. Louis, MO) was mixed with N-[β-maleimidopropionic acid] hydrazide, trifluoroacetic acid salt (Thermo Fisher Scientific, 3mg) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (Sigma-Aldrich, 2mg) dissolved in dimethyl sulfoxide (DMSO, 100 μl; Sigma-Aldrich) for 4h. The product was purified by C18 semi-preparative reversed-phase HPLC: 40 % to 70 % acetonitrile vs. 0.1 M triethylammonium acetate over 20min at a flow rate of 4.0 ml/min. Purity (> 95 %) was confirmed by HPLC. Cy5.5-maleimide fractions were collected and lyophilized. For site-specific conjugation of Cy5.5 to the thiol of the cysteine adjacent to the recognition sequence of FXIIIa substrate peptide, the peptide (2.5 μmol) was reacted with Cy5.5-maleimide (2.5 μmol) in PBS (100 μl, pH 7.0) for 4h. The product
was further purified by C18 semi-preparative reversed-phase HPLC: 40 % to 70 %
acetonitrile vs. 0.1 M triethylammonium acetate over 20min at a flow rate of 4.0 ml/min.
Purity (> 95 %) was confirmed by HPLC and MALDI-TOF mass spectrometry, and C15
NIRF imaging probe fractions were collected and lyophilized.

**Mouse Embolic Stroke Model**

Mice were anesthetized with 2% isoflurane using an inhalation mask, and cerebral
blood flow was monitored using a laser-Doppler flowmeter (Omegawave, Tokyo,
Japan). A homeothermic blanket (Panlab, Barcelona, Spain) was used to keep body
temperature at 36.5°C. The left common carotid artery (CCA), external carotid artery
(ECA) and internal carotid artery (ICA) were exposed through the midline neck incision
and dissection of the peri-vascular tissue. The ECA was ligated with coagulation of the
origin of superficial temporary artery. The CCA was clamped using a microvascular clip
(S&T AG, Neuhausen, Switzerland), and then the ICA was clamped after separating it
from the adjacent vagus nerve. After making a puncture in the ECA proximal to which it
was tied, a tapered PE-45 catheter containing the clot was inserted via the small hole,
then turned toward the ICA. Immediately after the microvascular clip clamping the ICA
was removed, the catheter was advanced about 9mm toward the MCA—ACA
bifurcation area. After the injection of the thrombus, the catheter was carefully removed, followed by the ligation of the ECA and declamping of the CCA.

**NIRF Macroscopic / Microscopic Imaging**

NIRF imaging was performed as published previously.\textsuperscript{1-3} Briefly, the excised brains or brain sections were imaged by using a NIRF imager (KOS. Inc., Seoul, Korea) with a charge-coupled device camera (CoolSnap-EZ, Roper-Scientific, Tucson, AZ). White light, FITC-channel (excitation/emission, 492nm/520nm; 1 second acquisition), and Cy5.5 NIRF-channel (excitation/emission, 675nm/690nm; 1 second acquisition) images were acquired. Fluorescence microscopy (Olympus-BX61, Tokyo, Japan) was used to visualize the distribution of Cy5.5 fluorescence (2 second acquisition) in the brain cryosections. After the microscopic imaging, the sections were H/E stained to confirm that the NIRF signals co-localized with intra-arterial thromboemboli.

**Quantitative Lesional Topography Analyses**

Cy5.5 thrombus signals on the NIRF images were mapped on a template (Supplemental Figure 1) that was prepared by using a published figure of brain vasculature, including proximal / distal MCA (M1/M2) and ACA (A1/A2), at the circle of Willis in a C57/BL6
A custom-built software package was used for the mapping; the software allows semi-automatic segmentation and transfer of lesions from a subject’s preclinical or clinical brain images onto a standard brain template set. After the storage of the final registration results, the left or right hemispheric thrombus burden (pixel numbers) is automatically calculated and provided on queries. In addition, color-coded maps can be produced by summation of multiple maps with color coding to indicate the amount of lesion overlap.

To analyze the distribution of ischemic brain injury, whitish infarct areas on the TTC stained sections were mapped on a template set that consists of six brain sections (slice numbers 1 ~ 6 at 2.96, 0.98, -1.06, -3.08, -4.60, -6.36mm distant from the bregma, respectively). The mean TTC images were acquired using the software package described above, and the left or right hemispheric infarct area (pixel numbers) could be obtained. The infarct size was calculated as a percentage of the left-sided or bilateral hemispheric area of selected template slices. The data of multiple template slices were averaged for the correlation studies or multivariate studies between the final total (slice numbers 1—6) or left hemispheric (2—5) infarct size vs. rCBF (% cerebral blood flow relative to the baseline value) and / or thrombus burden in the bilateral or left-sided circle of Willis and nearby arteries of the basal brain.
Computer-simulated Virtual Neuroprotection Research

Neuroprotection research depends critically on knowing how much infarct volume was altered by a treatment. In the absence of real data on how much infarct there is to start with (as can be obtained by performing magnetic resonance imaging (MRI) prior to treatment allocation), smaller protective effects could easily be overwhelmed by noise, and not be statistically detected. In order to quantify the potential benefits doing NIRF thrombus imaging and rCBF monitoring, we did the following computer simulation experiment: selected animals from the present study cohort were randomly assigned to either a control group or a treatment group, with this random assignment done prior to every experiment performed during the simulation. For the animals in the treatment group, infarct size was decreased from the measured values by 0–100% at 21 steps: 0, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, or 100%. This simulates the effect of neuroprotection regimens decreasing infarct size over a spectrum of effectiveness. We did these simulation runs for the following cases: no selection (n=46) or selecting a quarter of the animals—the ones with low rCBF (n=12; lowest quartile), big thrombi (n=12; highest quartile), a combined low rCBF and big thrombi (n=12; dichotomizations based on the median values), or a combined low rCBF and
small thrombi (n=11; dichotomizations based on the median values). For each group, 1,000 random divisions were made into control and treatment groups, with each assessed at 21 steps of neuroprotection. If the $t$-test for each individual experiment was $P<0.05$, then the experiment was counted as detecting neuroprotection.

**Supplemental Results**

**Quantitative Thrombus / Infarct Maps Visualize the Heterogeneous Nature of the Embolic Stroke Model**

Accumulation maps of thrombus and infarct locations showed that scattered emboli were frequently observed not only in the bifurcation area but also in the adjacent or remote cerebral arteries: left M1 (77.1%), left A1 (66.7%), left ICA (54.2%), left M2 (54.2%), left A2 (47.9%), right A2 (22.9%), right A1 (18.8%), right M2 (10.4%), right M1 (8.3%), and right ICA (6.3%). While one third of the animals had thrombi visualized in the right anterior circulation territory, small numbers of animals had fluorescent thrombus signal that was restricted to either left M1 (6.3%) or A1 (4.1%). Three animals (6.3%) had no thrombus signal at all. Scattered infarcts were frequently observed not only in the left MCA territory but also in the adjacent or remote areas of
the ipsilateral hemisphere (% of the animals with infarcts on template slices 1–6: 56.8%, 97.7%, 100%, 97.7%, 90.5%, and 41.9%) or contralateral hemisphere (31.8%, 56.8%, 57.8%, 60.5%, 69.0%, and 55.8%).
### Supplemental Table

**Supplemental Table. A Multivariable Analysis to Predict the Final Infarct Size**

\( (P=0.001, r=0.54) \)

<table>
<thead>
<tr>
<th>Variable</th>
<th>B (95% C.I.)</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>rCBF at 5min on Laser-Doppler Flowmetry</td>
<td>-0.160 (-0.4926―0.173)</td>
<td>0.338</td>
</tr>
<tr>
<td>Thrombus Burden at 24h on NIRF Imaging (per *7.706 (3.047―12.364) 1000 pixels)</td>
<td>0.002</td>
<td></td>
</tr>
</tbody>
</table>

Final infarct size: % infarct area relative to the left hemispheric area of the brain template slices 2–5.

C.I.: confidence interval.

rCBF: % cerebral blood flow relative to the baseline value before embolic stroke

*Please see the Supplemental Figure 1.
Supplemental Figures and Figure Legends

Supplemental Figure 1. A template of the circle of Willis with a thrombus of 1000 pixel size in the left middle cerebral artery (MCA) — anterior cerebral artery (ACA) bifurcation.

The vessel diameter of the template was expanded to become about 4 fold wider than the original for better visualization. ICA denotes internal carotid artery. Scale-bar=1.5mm
**Supplemental Figure 2.** A linear correlation between total thrombus burden and final bi-hemispheric (template slices 1–6) infarct size at 24h (Pearson correlation, $P=0.016$, $r=0.35$). The vertical line is at the median value (2230 pixels) of the thrombi burden.
Supplemental References


