SUPPORTING INFORMATION:

Reducing Frost during Cryoimaging Using a Hygroscopic Ice Frame

Adam W. Lowery\textsuperscript{1,2}, Ashwin Ambi\textsuperscript{2,3}, Lisa M. Miller\textsuperscript{2,3}, Jonathan B. Boreyko\textsuperscript{1*}

\textsuperscript{1}Department of Mechanical Engineering, Virginia Tech, Blacksburg, VA, 24060, USA
\textsuperscript{2}National Synchrotron Light Source II, Brookhaven National Laboratory, Upton, NY, 11973, USA
\textsuperscript{3}Department of Chemistry, Stony Brook University, Stony Brook, NY, 11794, USA

*Corresponding author.
Email: boreyko@vt.edu (J.B. Boreyko)

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- Supplementary Text
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Other Supporting Materials for this manuscript include the following:

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Supplementary Text

Illuminated Dot Feature Array: Greyscale Image Processing

All photos taken during testing of the dot array were first white balanced to remove the yellow hue created from the transmitted light through the silicon nitride membrane. A computer program was written so that the user could define the regions of interest (ROI) for each dot feature and the background film paper. Because the ROI for the background encapsulates the dots, the intensity of the translucent paper background was found by segmenting the dot features out of the photos and averaging the remaining pixel values in the selected region of interest. The value for each of the pixels that comprise the individual dots in the photos was interpolated into greyscale intensity, which ranges anywhere from 0-255. The pixel intensities within the ROIs were averaged (Figure S2). The code was used to evaluate the dot feature intensities over the 3 h test period, in 30 min intervals (Figure 4A).

Microvessel and Amyloid Deposits: Luminance Measurements

A code prompted the user to first crop the photo to reduce background artifacts on the image and establish the remaining selected area surrounding the microvessel as the ROI. After an appropriate ROI was chosen, the image color space was converted from RGB to CIELAB. The CIELAB color space pixels in the resultant image were segmented into multiple clusters through use of k-means. K-means is a machine learning method that partitions images into different clusters based on observations from the computer. Two clusters were specified: one for the feature (blood vessel or amyloid deposits) and the other for the remaining background (Figure S9). The luminance metric from pixels in the feature cluster for each of the individual vessels were then averaged over the 3 h test period, in 30 min intervals.
Figure S1. Microscopy of silicon nitride window during frost growth at cryogenic temperatures. Focusing on the silicon nitride window (not the underlying dot array), transmission bright-field microscopy was used to capture a high-resolution image every 15 min over the course of 3 h. The window was held at cryogenic temperatures and frost slowly propagated on its substrate surface, obscuring its transparency. Four different experimental conditions were tested: (1) no nitrogen purge and no ice frame, (2) nitrogen purge and no ice frame, (3) nitrogen purge with an ice frame, and (4) no nitrogen purge with an ice frame. The corresponding images where the microscope was focused on the dot array (not the silicon nitride window) comprise Figure 2 of the main manuscript.
**Figure S2.** Grayscale intensity measurements of the dot feature array. A Nikon Eclipse LVDIA-N microscope was used to observe the visibility of a printed dot feature array through a silicon nitride membrane with a 5x objective. Using the microscope’s camera and transmission brightfield illumination, high resolution images focused on the array and membrane surface in 15 min intervals over the course of 3 h. The dots and background were segmented by a computer program to obtain the grayscale intensity of each of the features. The above photograph is a representative example of measuring the grayscale intensities, taken at room temperature prior to frost growth.
Figure S3. Epi-bright-field microscopy of isolated rat brain vessels with amyloid deposits. (A) Mosaic of various rat brain vessels stitched together from 16 separate images. The three vessels used to evaluate the impact of frost growth on image clarity are highlighted with rectangles. (B-D) 50x magnified view of the three microvessels selected for characterization. Each vessel additionally contains amyloid deposits.
Figure S4. Transmission bright-field microscopy of isolated rat brain vessels with amyloid deposits. (A) The same mosaic shown in Figure S4, but now using transmission microscopy. (B-D) 50x magnified view of the three microvessels selected for characterization.
**Figure S5.** Dark-field fluorescent images (DsRed) of rat brain vessel 3 (cf. Figure S3D) during 3 h of cryomicroscopy. The clarity of the fluorescently stained blood vessel, and its attached amyloids, is shown in 1 h intervals for all four types of experimental conditions.
**Figure S6.** Dark-field fluorescent images (GFP) of rat brain vessel 3 (cf. Figure S3D) during 3 h of cryomicroscopy. The clarity of the fluorescently stained blood vessel, and its attached amyloids, is shown in 1 h intervals for all four types of experimental conditions.
Figure S7. Schematic of the experimental setup incorporating the dot array. The silicon nitride window was adhered to one face of the sample holder. The dot array, printed on translucent film paper, was adhered to the opposite face. A transmission light source from the microscope illuminates the array to be observable through the transparent substrate surface of the window over the sample holder’s aperture. The opposite end of the sample holder is in contact with the cryostage’s cooling block to establish cryogenic temperatures on the window during testing.
Figure S8. Schematic of the experimental setup. The cryostage houses the sample setup and facilitates cryogenic temperatures for testing through thermal conduction with the sample support. The ice frame is developed on top of the silicon nitride window. The dot array is illuminated from the microscope light source and observed through the silicon nitride window (cf. Figure S7).
Figure S9. Image segmentation through k-means clustering. A darkfield fluorescent image of the biological sample (i.e., rat brain vessel 1) was cropped to shrink the background and minimize artifacts. The cropped image is then converted to the CIELAB color space and segmented, via K-means, into two specified clusters (immunostained vessel and background). The image cluster that contains the vessel has the luminance value of its total pixels averaged and tabulated.
Table S1. Dot array test intensity measurements
This table shows the measured intensity for each dot and the background over the course of the 3 hr examination. Each sheet corresponds to an experimental condition used to evaluate the performance of the ice frame.

Table S2. Average luminance of the rat brain vessels
This table list the calculated average luminance of each of the three microvessels for a given experimental condition. Each sheet corresponds to a particular darkfield imaging modality. The tables contain the luminance measurements for both the vessels and the resultant background (Figure S9).

Movie S1. Example of dot array clarity change over a 3 h period.
A montage showing a 3 h evolution of frost on the silicon nitride membrane in a 2x2 tile grid. Each tile is a photo of the dot array for a given experimental condition at specified timestamps. As time increases, the conditions without a hygroscopic ice frame show a clearly observable reduction in visibility of the dots placed behind the silicon nitride membrane.

Movie S2. Luminance observation of rat brain vessel over a 3 h period
A montage showing the 3 h evolution of the luminance for a sample rat brain vessel in a 2x2 tile grid. Each tile is focused on the same vessel, but for a specific experimental condition starting at room temperature before proceeding to 1 h intervals at cryogenic temperatures. The clip shows the vessel under the four different forms of visible light (epi-illumination and transmission brightfield, and darkfield (GFP and DsRed filters)) it was observed under for optical microscopy.