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**Title:** Towards in-vivo label-free detection of brain tumor margins with epi-illumination tomographic quantitative phase imaging

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Towards in-vivo label-free detection of brain tumor margins with epi-illumination tomographic quantitative phase imaging – Suplementary material (methods, tables and figures)

**Supplemental Methods**

**Detailed qOBM system description**

The free-space qOBM system (Fig. 1) uses a conventional inverted brightfield microscope geometry with epi-illumination. Samples are illuminated sequentially with four LED light sources (Luxeon sink-PAD II) which are coupled into multimode fibers (Thorlabs FP1000ERT, 1 mm diameter, NA 0.5) using an aspheric condenser lens (Thorlabs ACL2520U-A, NA 0.6). These fibers are housed in a custom-made 3D-printed objective-adapter that holds them at 45-degree incident angle and off-axis source-detector distance of 7mm. The four sources effectively form two orthogonal shear angles that provide uniform quantitative contrast in all directions when processed with qOBM. The LEDs output light at 720 nm. In this work we use a 60x (NA 0.7) microscope objective with a FoV of 240µm x 240 µm and resolution of 0.6 µm, and a 20x (0.45 NA) objectives with a FoV of 720 µm x 720 µm and resolution of 1 µm. Light is detected with a sCMOS camera (sCMOS pco.edge 42L T) at 20 Hz, for a net acquisition rate for qOBM images of 5Hz (four acquisitions, one from each LED). Illumination and camera triggering are coordinated with custom software and a low-cost USB triggering unit. Image processing is done in real-time with a typical computer. Together, an existing brightfield microscope can be modified to deliver 3D quantitative phase imaging with qOBM for <$2,000. The same overall configuration is used for the fiber-based qOBM system, except the objective is a 0.5 NA GRIN objective (GRIN Tech NEM-100-06-08-520-DS) and it is attached to an imaging fiber bundle (Fujikora FIGH 30-850N). On the proximal end, the fiber bundle is SMA connected and here we use a compact CMOS camera (Thorlabs, DCC3240M) for detection.

**Quantitative image reconstruction with qOBM**

Differential phase contrast (DPC) with OBM is achieved by subtracting two images with diametrically opposed illumination (here denoted as right and left), and normalized by their sum,

\[ I_{DPC} = \frac{I_R - I_L}{I_R + I_L} \]  

(1)
To quantify phase (φ) with qOBM, a second DPC image is taken with orthogonal illumination, and then we apply a Tikhonov regularized deconvolution, given by,

$$\phi = \mathcal{F}^{-1} \left\{ \frac{\sum k \hat{I}_{DPC} \cdot \hat{C}_{DPC}}{\sum |C_{DPC}|^2 + \alpha} \right\}. \quad (2)$$

Here \( \mathcal{F}^{-1} \{ \cdot \} \) is the inverse Fourier transform, \( k = 2 \) for the two orthogonal DPC images, \( \hat{I}_{DPC}^k \) is the Fourier transform of the \( k^{th} \) DPC image, \( \alpha \) is the regularization parameter, and \( C_{DPC}^* \) is the complex conjugate DPC transfer function given by [1],

$$C_{DPC} = \frac{-i \cdot \int [S(u)-S(u')]P(u^*+q)P^*(u)d^2u}{\int [S(u)]P(u)P^*(u)d^2u}. \quad (3)$$

Here, \( u \) and \( q \) are 2-dimensional spatial frequency coordinates, and \( u' \) represents the same coordinates as \( u \) but inverted in the shear direction. Variable \( P \) represents the pupil function of the system, and \( S \) is the effective light source angular distribution at the focal plane, which is estimated using Monte Carlo photon transport program (performed with MCXLAB in MATLAB) [2]. Optical properties for tissues were obtained from established values in the literature [3]. The transfer function was kept constant for each system. More details regarding image quantification with qOBM can be found in Ref [1,4].

9L gliosarcoma rat tumor model protocol

The 9L gliosarcoma rat tumor model has been the most widely used rat brain tumor model and has provided important information relating to brain tumor biology and therapy [5]. This tumor model presents characteristics that recapitulate human high-grade gliomas, including high proliferative capability, high vascularization, and infiltration at the margin [6]. Thus, this 9L tumor model provides a well-controlled and understood model to assess the sensitivity of qOBM to differentiate between the normal and cancerous tissue, as well as infiltrating tumor.

All animal experimental protocols were approved by Institutional Animal Care and Use Committee (IACUC) of the Georgia Institute of Technology and Emory University. Intracranial tumor implantation of 9L gliosarcoma cells in Fischer rats were carried out in the following manner: **Sterilization:** Prior to surgery the instruments and supplies were sterilized. Materials such as gauze pads and instruments
were sterilized in an autoclave. **Anesthesia**: The animals were anesthetized by administering an intraperitoneal injection of a mix of Ketamine and Xylazine. Animals were anesthetized for 30-60 minutes. **Positioning of Animals in the Stereotactic Frame**: After carefully shaving the scalp with an electric razor, anesthetized animals were secured in the stereotactic frame following standard procedure. **Cleaning the Incision Area**: Scalps were swabbed with a pad moistened in 70% alcohol to remove the hair clippings and to clean the incision areas. **Incision**: A sagittal midline incision was made from 2 mm anterior to the bregma to the occiput. The scalp was bluntly elevated in the subgaleal plane on the right side of the midline. A 1 mm drill was used to make a burr hole in the skull 3 mm to the right and 1 mm anterior to the bregma. **Tumor Cell Implantation**: The dura was pierced with a 23 Hamilton syringe containing 5 µl of tumor cell suspension (containing c.a. 50,000 cells). The needle was advanced to a depth of ~4.5 mm over a period of one minute, and then retracted 0.5 mm in order to form a pocket for injection. Suspension was injected over a period of two minutes. After injection, the needle was retracted over a period of one minute, and the burr hole was filled with sterile bone wax. The surface of the skull was then washed with sterile water. **Suturing**: The scalp was closed with 3-0 running absorbable suture. The animals were removed from the head holder and allowed to recover in a cage warmed by a heating pad. Animals were returned to their regular cages once they are able to right themselves. **Housing**: All caging and housing for the animals were sterile. Routine daily animal care (food, water, and cage changes) were provided. **Euthanasia details**: CO2 was delivered via a compressed gas source and the euthanasia chamber was used, allowing for the visualization of animals during the procedure. **Other**: Procedures following euthanasia are described in the main text.

**Feature extraction**

All image processing and machine learning algorithms were done on MATLAB (2019b). A total of 482 features were extracted from each image. We considered a broad number of statistical and textural feature extraction algorithms as described below.

**First-order histogram-based features:**
The histogram shows the pixel value distribution of the image. Histogram based features can give us information related to the refractive index distribution which is proportional to dry mass [7]. Even though first-order histogram features do not take the relationship between pixels into account, they hold valuable information about the physical contents of the image. The histogram-based features calculated here were the mean, standard deviation, skewness, kurtosis, and entropy.
Second-order histogram-based features:

The Gray Level Co-occurrence Matrix (GLCM) shows the different combinations of the gray levels found in an image. The matrix can be denoted as $G(i, j, \theta, d)$, where $\theta$ is the direction, and $d$ is the distance between the pixel of interest and its neighbor. The GLCM measures how often a pixel of value $i$ appears at $d$ distance in the $\theta$ direction of a pixel with the value $j$. One image can have many GLCMs for different offsets and angles. In this study, we calculated GLCMs at 4 angles (0, 45, 90 and 135 degrees) and 10 offsets (1, 3, 5, 7, 9, 11, 13, 15, 17, 19 pixels). For each GLCM, the correlation, contrast, energy, and homogeneity were extracted using the MATLAB Image Processing Toolbox, and the angular second moment, sum of squares, inverse difference moment, sum average, sum variance, sum entropy, entropy, contrast, difference variance, difference entropy, first and second information measures of correlation, and maximal correlation coefficient were extracted following the Haralick et al. definitions [8]. The mean and range of these descriptors across the 4 different GLCM angles were calculated as the texture features for each offset.

Fractal features:

The texture of an image can be understood as a combination of granularity and patterns. The fractal dimension is a commonly used parameter to measure the roughness of an image. We computed the multi-level fractal dimension of each image following the Segmentation-based Fractal Texture Analysis (SFTA) algorithm [9]. This algorithm computes two sets of binary maps of each input image by applying a Two-Threshold Binary Decomposition (TTBD). The TTBD computes binary maps using each individual threshold as well as all pairs of continuous thresholds; thus, the number of binary maps computed is twice the number of thresholds. We set the number of thresholds to 8. Then, the Hausdorff’s fractal dimension of each binary image is computed, as well as the mean grayscale value of input image masked by the binary map, and area of the binary image edge map. The area of the binary map edges is an indicator of the fineness of the structures at that phase level. For each input image, a total of 48 features are computed by the SFTA algorithm.

Phase density features:

The fractal SFTA algorithm applies a set of multilevel thresholds to the images, these thresholds are based on the histogram of each individual image, meaning that the phase values of each level varies from image to image. While this algorithm is effective when extracting textural information from the
images, it does not access information about the quantitative phase values. Thus, an additional multilevel thresholding algorithm was applied based on a set of global phase thresholds to extract features related to the concentration of different phase values. By setting 8 linear global phase thresholds, we are again able to compute two sets of binary maps from each image, in a similar way to TTBD. These binary images are then used to calculate the masked mean phase value as well as the binary area, which represents the density of phase values at different levels. A total of 32 phase density features are computed.

**Autocorrelation features:**
The autocorrelation matrix (ACM) yields second-order geometrical information (i.e., morphological covariance) and can produce metrics that are invariant to translation, scale, and rotation [10–12]. Examples of the ACM of healthy and tumor images can be found in Fig. S2. The ACMs extracted from the input phase images appear to have clear, repeated patterns of different sizes and granularities depending on the texture of the input image. The roughness of these patterns are indicative of the overall texture of the tissue structure; therefore, we extracted the same fractal features directly from the ACM. The variance and entropy of the ACM were also considered as individual features. Two more features were extracted from the ACM: the coarseness [13], and the radial density of the Power Spectral Density (Fourier transform of ACM).

**Edge detection feature:**
The Laplacian of the input image is given by

$$L(x, y) = \frac{\partial^2 I(x, y)}{\partial x^2} + \frac{\partial^2 I(x, y)}{\partial y^2}$$

The Otsu threshold of the Laplacian was found. The output of the thresholded Laplacian image gives the edges of the input image. The entropy of these edges was computed as a measure of image sharpness and use as a feature.

**Spectral features:**
As previously discussed, image texture can be understood as a combination of granularity and patterns. These patterns have some degree of regularity, meaning that they can be described in terms of the
Fourier space. Five features were extracted from the Fourier transform of the image. The Fourier transform of the input image $\mathcal{F}\left(I(i,j)\right)$ can be expressed in polar coordinates, $S(\theta, r)$. We can obtain unidimensional radial and angular functions, $S(r)$ and $S(\theta)$, by summing across all angles and radii. The mean and standard deviation of each of $S(r)$ and $S(\theta)$ we used as descriptors of texture. The slope of the line described by the radial power spectrum, $|S(r)|^2$ vs. $r$, was used as a descriptor of image smoothness.

**Feature selection and image classification and segmentation**

Neighborhood Component Feature Selection (NCFS) is a nearest neighbor-based feature weighting algorithm that focuses on maximizing the leave-one-out nearest neighbor classification accuracy of the training data. The algorithm assigns weights for each feature based on classification accuracy, then the features within 2% of the highest weight are selected as the most relevant features.

Feature selection was performed for each objective/magnification and ROI size, individually. The number of selected features per ROI, as well as the representation of the most significant features, can be found in Fig. S3 and Table S2.

A gaussian SVM was trained as a binary classifier to identify healthy and cancerous tissue. The kernel function used for the SVM is of the form, $G(x_j, x_k) = e^{-||x_j - x_k||^2}$, and its scale was adjusted by applying a Bayesian Optimizer to the loss function of a 30 fold cross-validated SVM within the training data set. The classifier was then trained with the optimized kernel scale.

The SVM finds the hyperplane in a kernel space that best separates the data (in this case, the features extracted from the data) into the two classes: tumor and healthy. When classifying an image, the SVM finds the score of that data point, i.e., the distance between the data point and the hyperplane in the kernel space, with a positive or negative side indicating which side of the hyperplane the data point lands. It can be understood that a data point far away from the hyperplane is more confidently classifiable than one laying closer to the plane. Therefore, the SVM score can be used to not only make a binary decision, but to measure its own level of confidence. These scores can then also be used to colorize an image based on its contents as a form of segmentation. Moreover, it may be used to classify tumor border regions which are expected to fall near zero, and in between the scores of the two
discrete classes (healthy and tumor). To generate the segmented images shown in Fig. 4, we applied a sliding window of 240µm to the input images with a sliding resolution of 50 pixels (~20µm). Each window was classified and averaged into the final image.

**Supplemental tables and Figures**

| Objective (µm) | 240 | 120 | 60 | 30 | 720 | 240 | 120 | 60 | 30 |
|---------------|-----|-----|----|----|-----|-----|-----|----|----|
| # features    | 5   | 17  | 15 | 16 | 17  | 11  | 8   | 8  | 8  |
| Accuracy      | 1   | 0.994 | 0.982 | 0.963 | 1   | 0.999 | 0.993 | 0.961 | 0.918 |
| Sensitivity   | 1   | 1   | 0.978 | 0.964 | 1   | 1   | 1   | 1   | 0.955 |
| Specificity   | 1   | 0.988 | 0.985 | 0.962 | 1   | 0.991 | 0.994 | 0.983 | 0.861 |
| AUC           | 1   | 0.994 | 0.982 | 0.963 | 1   | 0.999 | 0.993 | 0.983 | 0.909 |
| Accuracy      | 1   | 0.99  | 0.973 | 0.956 | 1   | 0.998 | 0.999 | 0.983 | 0.796 |
| Sensitivity   | 1   | 0.991 | 0.966 | 0.833 | 1   | 0.998 | 0.999 | 0.983 | 0.963 |
| Specificity   | 1   | 0.999 | 0.977 | 0.776 | 1   | 0.999 | 0.997 | 0.983 | 0.733 |
| AUC           | 1   | 0.998 | 0.971 | 0.879 | 1   | 0.998 | 0.997 | 0.983 | 0.848 |

*Table S1. SVM classification results per field of view per magnification.*
Figure S1. Original (non-filtered) stitched qOBM images (21X31 images, covering ~2.3cm x 1.5cm). The whole slice took ~45 minutes to image, including stage translation, real-time phase computation and display, as well as reading and writing files onto a server. If optimized, same area could be acquired in ~10 min (1 sec per image). In order to maintain the brain fresh, it was imaged while submerged in media. Over time, some debris accumulates around the tumor. The blue line denotes a manual mask applied to the final image shown in the main text. To obtain the enhanced version of this image shown in fig. 4e, a gaussian filter (standard deviation $\sigma=0.5$ pixels) and histogram equalization were sequentially applied three times to the original stitched image.
**Figure S2.** Representation of autocorrelation matrices (in log scale) of healthy tissue and tumor tissue. FoV: 240x240µM

|       | 20x          | 60x          |
|-------|--------------|--------------|
| **Tumor** | ![Image](image1.png) | ![Image](image2.png) |
|       | ![Image](image3.png) | ![Image](image4.png) |
| **Healthy** | ![Image](image5.png) | ![Image](image6.png) |
|       | ![Image](image7.png) | ![Image](image8.png) |
Table 2. Selected features for 240 µm SVMs, in order of relevance. See Methods section in main text for detailed description of features.

| Magnification | Selected features | Description |
|---------------|------------------|-------------|
| **60x**       | Autocorrelation width | Coarseness |
|               | AC fractal dimension – 15<sup>th</sup> level | Fractal dimension of autocorrelation function (from image 15 of binary set) |
|               | Correlation mean offset 3 | Correlation from GLCM, offset = 3 |
|               | Kurtosis | Histogram kurtosis |
|               | Correlation mean offset 1 | Correlation from GLCM, offset = 1 |
| **20x**       | Binary edge area - 1<sup>st</sup> level | Area of binary edge image (from image 1 of binary set). Represents fineness of the texture |
|               | AC binary edge area – 13<sup>th</sup> level | Area of binary edge image in autocorrelation (from image 13 of binary set) |
|               | Phase Density_16 | Phase density from highest global threshold, represents high phase value concentration |
|               | Mean Phase value_31 | Mean thresholded phase value, highest global dual threshold |
|               | AC binary edge area - 1<sup>st</sup> level | Area of binary edge image in autocorrelation (from image 1 of binary set). Represents the fineness of the AC |
|               | AC fractal dimension – 1<sup>st</sup> level | Fractal dimension of autocorrelation function (from image 1 of binary set) |
|               | Thresholds 15 | Mean thresholded phase value, highest global threshold |
|               | Difference entropy mean offset 3 | Difference entropy from GLCM, offset = 3 |
|               | Inverse difference moment mean offset 1 | Inverse difference moment from GLCM, offset = 1 |
Figure S3. Scatter plots of the most relevant features from training and testing data combined.
Figure S4. SVM score box plots of each dataset (training and testing), acquired with each objective (20X and 60X) with ROI size of 240µmX 240µm. Tumor Margin data were not used in training but are included in the boxplots to facilitate comparison. All p-values are adjusted for the number of features, i.e., Bonferroni correction.
Figure S5. (a) qOBM image of clear, non-fluorescent, 10µm beads immersed in water taken with the handheld fiber based probe prototype. The field of view has a diameter of 170 µm. Quantitative phase, $\phi$, is converted to thickness, $z$, by $\phi(z) = \frac{2\pi}{\lambda} \cdot \Delta n \cdot z$, where $\lambda$ is the wavelength of light (720 nm) and $\Delta n$ is the refractive index difference between the target and medium.
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