A MICRODOSE EVALUATION STUDY OF ABY-029 IN RECURRENT GLIOMA

A Phase 0 open label, single-center clinical trial of ABY-029, an Anti-EGFR Fluorescence Imaging Agent via single intravenous injection to subjects with recurrent glioma.

Principal Investigator:	**David W. Roberts, MD** Department of Surgery Section of Neurosurgery Dartmouth-Hitchcock Medical Center One Medical Center Drive Lebanon, NH 03756 603-650-8618
Co-Investigators:	**Keith Paulsen, PhD** **Nathan Simmons, MD**
Regulatory Sponsor:	**Keith Paulsen, PhD**
Funding Sponsor:	NIH/NCI Bethesda, MD 20892 (240) 276-6315
Study Product:	ABY-029 (Z03115-Cys-Mal-IRDye800CW)
Protocol Number:	D16107
eIND Number:	122681

Initial version: April 19, 2016
Revised version: May 25, 2016
Revised version: June 3, 2016
Revised version: June 3, 2016 rev
Revised version: June 30, 2016
Revised version: November 4, 2016
Revised version: November 21, 2016
Revised version: December 22, 2016
Revised Version: July 7, 2017
Revised Version: August 11, 2017
Revised Version: November 28, 2017
Revised Version: January 29, 2018
Revised Version: February 20, 2018 rev 1
Revised Version: April 24, 2018
Revised Version: October 23, 2018
Revised Version: February 14, 2019

Table of Contents

List of Abbreviations

ABY-029	Affibody
AE	Adverse Event/Adverse Experience
CFR	Code of Federal Regulations
CPHS	Committee for the Protection of Human Subjects
CRF	Case Report Form
CSOC	Clinical Study Oversight Committee
DCC	Data Coordinating Center
DCE	Dynamic contrast-enhanced
dFI	Depth-detected Fluorescence Imaging
DHHS	Department of Health and Human Services
DSMAC	Data, Safety Monitoring, and Accrual Committee
FLI	Fluorescence Imaging
GCP	Good Clinical Practice
HIPAA	Health Insurance Portability and Accountability Act
ICF	Informed Consent Form
ICH	International Conference on Harmonization
iFI	Intraoperative Fluorescence Imaging
IRB	Institutional Review Board
ISM	Independent Safety Monitor
FI	Fluorescence Imaging
GBM	Glioblastoma Multiforme
HGG	High grade glioma
MRI	Magnetic resonance imaging
NIH	National Institutes of Health
OCTOM	Office of Clinical Trials Operations and Management, NIDCR, NIH
OHRP	Office for Human Research Protections
OHSR	Office of Human Subjects Research
PI	Principal Investigator
QA	Quality Assurance
QC	Quality Control
qdFI	quantitative depth-resolved Fluorescence Imaging
qFI	quantitative Fluorescence Imaging
SAE	Serious Adverse Event/Serious Adverse Experience
SOP	Standard Operating Procedure
SNR	Signal-to-Noise

Study Summary

Title	A Phase 0 open label, single-center clinical trial of ABY-029, an Anti-EGFR Fluorescence Imaging Agent, via single intravenous injection in subjects with recurrent glioma.
Short Title	A Microdose Evaluation Study of ABY-029 in Recurrent Glioma
Protocol Number	N/A
Phase	Phase 0 microdose
Methodology	Patients will be administered a single intravenous dose of ABY-029 1-3 hours before surgery. At different times during resection, the coregistered surgical microscope (Zeiss Pentero enabled with intraoperative fluorescence imaging, iFI) will be focused on a point of interest. The surgeon will switch to iFI mode to capture images of the surgical field. Quantitative measurements of fluorophore concentration will be made at the focal point of the operating microscope with an intraoperative probe and biopsy samples will be taken at the coregistered point of interest for subsequent histological analyses and ex-vivo assessments of fluorophore concentration. In addition, an imaging array mounted on second commercial, clinical microscope, will be briefly exchanged with the primary scope to take additional images of the surgical field.
Study Duration	12-18 months
Study Center(s)	Single-center, Dartmouth-Hitchcock Medical Center
Objectives	The primary study objective is to determine if microdoses of ABY-029 (up to 6X) lead to detectable signals (defined as signal-to-noise ratio, SNR $\geq$10, with wide-field iFI) in sampled tissues with an EGFR pathology score $\geq$ 1 based on histological staining. The secondary study objective is to assess diagnostic accuracy of ABY-029 detection by iFI and intraoperative probe relative to histopathology diagnosis, and other indicators (e.g. proliferation, infiltration, etc.) as the gold standard, and to measure the molecular uptake and concentration of ABY-029 in resected specimens.
Number of Subjects	A minimum of 6 and a maximum of 12 adult patients with a diagnosis of recurrent glioma will be enrolled.
Diagnosis and Main Inclusion Criteria	Operable brain tumor in adults with pre-surgical diagnosis of recurrent glioma.

Study Product, Dose, Route, Regimen	Criteria for successful iFI are locations of all EGFR positive samples (defined as having a pathology score ≥ 1 based on histological staining) having a measurable signal (defined as SNR ≥ 10). A single microdose of ABY-029 at 30 nanomoles (237µg) will be injected into the first 3 patients. If samples from these subjects meet successful iFI criteria, 3 more patients will be enrolled at the same dose, concluding the study with a total of 6 subjects. Otherwise, the ABY-029 dose in the next 3 patients will be increased to 3X (90 nanomoles, 711 µg). If samples from these subjects meet successful iFI criteria, 3 more patients will be enrolled at the 3X dose, concluding the study with a total of 9 subjects. If iFI criteria are not met, the next 3 patients will be enrolled at 6X dose (180 nanomoles, 1.42 mg). If iFI criteria are met, 3 more patients will be enrolled the 6X dose, concluding the study with a total of 12 subjects. If iFI criteria are not met in the first 3 patients at the 6X dose, enrollment will be stopped with a total of 9 subjects. Because of the microdosing levels used, the results of the Dartmouth Toxicity Study (at 10X, 100X, 1000X doses), and the safety profile of similar compounds (e.g. ABY-025), we do not anticipate any serious adverse events (SAEs) related to ABY-029.
Duration of administration	ABY-029 will be administered by IV injection on day of surgery, approximately 1-3 hours prior to the procedure.
Reference methods	Post-operative histopathology will serve as the gold standard.
Statistical Considerations	Repeated measures analyses of variance will be performed to account for multiple samples from each patient. Student's t-test will be used to evaluate ABY-029 fluorescence signal detection in tumor relative to normal brain with iFI and intraoperative probe. Empirical ROC curves and logistic regression analysis for clustered binary data will be used to evaluate the accuracy of ABY-029-specific fluorescence in discriminating between EGFR+ glioma tumor, EGFR- glioma tissue, and normal tissue when detected by iFI and/or intraoperative probe.

1 Introduction

This document is a clinical research protocol and describes a human research study. This study will be conducted according to US and international standards of Good Clinical Practice (FDA Title 21 part 312 and International Conference on Harmonization guidelines), applicable government regulations and Institutional research policies and procedures. All personnel involved in the conduct of this study have completed human subject protection training.

1.1 Background Information

The focus of this exploratory investigational new drug (eIND) development is to realize a fluorescent reporter to guide neurosurgical glioma resection using a synthetic peptide that binds to the epidermal growth factor receptor (EGFR) and provides an optical fluorescent signal of the concentration. The combined peptide and fluorophore will be called ABY-029, and is the focus of this first-in-human trial in neurosurgery. The initial focus of this Phase 0 clinical study is evaluation of drug uptake and concomitant imaging signal.

This research is funded by NIH under grant R01 CA167413. Following completion of the manufacturing and nonclinical plans, we will submit an exploratory IND (eIND) to FDA. Depending on the findings from clinical studies conducted under eIND, we anticipate the submission of a traditional IND.

Several groups have pioneered the use of fluorescence imaging in experimental surgery [1] and a platform of targeted compounds for surgical applications [2-5] in which the surgeon's white light visual field is augmented with fluorescent-guided information. Perhaps the largest clinical experience with fluorescence-guided resection has been gained in neurosurgery with aminolevulinic acid (ALA) induced protoporphyrin IX (PpIX). The approach has been evaluated in a prospective randomized Phase III trial [6] with sufficiently positive outcomes to alter standard-of-care in Germany for surgical resection of high grade glioma [7] and related studies have been completed and are ongoing at Dartmouth under FDA IND #77,751 for oral administration of ALA [8-12].

Clinical motivation exists for pursuing new brain tumor fluorescent probes beyond PpIX for guiding surgical resection. First, ALA-induced PpIX fluorescence is enhanced by non-tumor-specific molecular processes related to increased metabolism and blood-brain-barrier breakdown. Second, while these processes provide reliable enhancement in high-grade glioma tumors, enhancement at the margin and in low-grade glioma is relatively poor. Third, the anti-EGFR ABY-029 molecule we are developing has potential to guide a range of oncologic surgeries, given the large number of tumor types overexpressing EGFR. Most importantly, a wealth of information exists on EGFR fluorophore targeting [13-25] [26-28] which suggests our approach is likely to be successful and could benefit patients with malignant glioma and other cancers whose prognosis is improved by complete surgical resection.

1.2 Investigational Agent

The production of the investigational drug to be used in the clinical study, ABY-029, is being coordinated by Dartmouth with project partners Affibody AB, LI-COR Biosciences, and the University of Alabama at Birmingham (UAB) Vector Production Facility, with Bachem AG (Switzerland) as the manufacturing subcontractor.

The Swedish biotechnology company Affibody AB has developed a group of molecules which are small, single domain, non-immunoglobulin proteins based on a 58 amino acid, three-alpha-helical scaffold [29, 30]. The target binding site is composed of 13 surface-exposed amino acids on helix 1 and helix 2. By randomizing these 13 amino acids, the company has created large libraries of Affibody® molecules from which ones that bind to a given target protein are selected (examples include EGFR, HER2 or PDGFRß [31-33]). Their small size and stability, high affinity and target recognition specificity, and rapid biodistribution kinetics and clearance make them ideal for in vivo targeting. Affibody molecules can be produced either by recombinant techniques in bacteria or by conventional peptide synthesis and can be modified with functional groups for fluorescent or radioactive labels. Site specific modification of Affibody molecules is achieved by introducing a single cysteine which can be used for modification of maleimide-activated functional groups leading to defined and homogenous products [34].

LI-COR is a privately held company that is committed to the development of in vivo fluorescence imaging during surgery and has been systematically developing near-infrared (NIR) fluorescent molecules with the goal of creating in vivo reporters for targeted contrast. IRDye 800CW is a NIR dye that can be functionalized with either an NHS or maleimide reactive group, allowing it to be attached to a number of biomolecules.[35] Recently, cetuximab conjugated to IRDye 800CW was used at the University of Alabama in a dose escalation study in 12 patients with squamous cell carcinoma in the head and neck. After passing through FDA safety and pharmacokinetic studies, the investigators showed positive correlation between fluorescence markers and EGFR levels, and demonstrated that fluorescently labelled molecules can be safely administered to humans to identify tumor cells with sub-millimeter resolution [36].

The ABY-029 conjugate is produced by first incubating the Z03115-Cys peptide with Dithiothreitol (DTT), to break up Affibody dimers that naturally form, and then mixing the peptide with IRDye 800 CW maleimide dye, at a ratio of one dye molecule per peptide molecule.

Z03115-Cys is a synthetically generated 59 amino acid peptide produced by Bachem (Switzerland). The peptide has a molecular formula of $C_{299}H_{468}N_{78}0_{92}S_3$ and is composed of (H-Ala-Glu-Ala-Lys-Tyr-Ala-Lys-Glu-Met-Trp-Lle-Ala-Trp-Glu-Glu-Lle-Arg-Asn-Leu-Pro-Asn-Leu-Asn-Gly-Trp-Gln-Met-Thr-Ala-Phe-Lle-Ala-Lys-Leu-Leu-Asp-Asp-Pro-Ser-Gln-Ser-Ser-Glu-Leu-Leu-Ser-Glu-Ala-Lys-Lys-Leu-Asn-Asp-Ser-Gln-Ala-Pro-Lys-Cys-OH).

The dye is coupled to the peptide molecule via maleimide chemistry at a single Cys residue at the C-terminus of the peptide, as illustrated schematically below. The conjugation reaction is followed by purification from free dye.

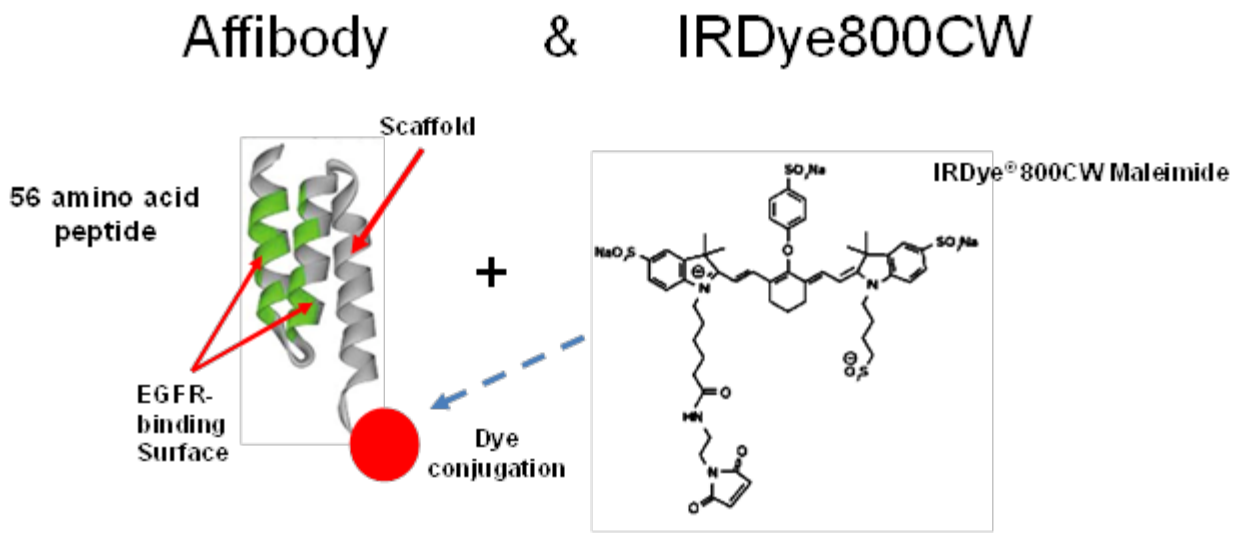


Figure 1 - Schematic view of ABY-029 (Z03115-Cys-Mal-IRDye800CW)

The solid phase peptide synthesis production of Z03115-Cys has been subcontracted to Bachem AG (Bubendorf, Switzerland). Bachem has automated systems for generation of pharmaceutical grade product, and a long history of production for human use. The maleimide linkage conjugation of Z03115-Cys with IRDye800CW has been subcontracted to the University of Alabama at Birmingham Vector Production Facility, and will be conducted in their cGMP laboratories.

Physical Form:	Clear, green-blue liquid.
Molecular Formula:	Cys-((RS)-1-[2-maleimido-ethyl]-IRDye 800CW-OH)
Molecular Weight:	7914.95 grams/mole
Solubility:	Soluble in Phosphate Buffered Saline (PBS)
pKa Value:	6.84E+05
pH Value:	7.4

Kd Value: 1 nanomolar (nM)

The drug will be supplied in vials containing 5 mL of liquid Phosphate Buffered Saline (PBS).

1.3 Preclinical Data

1.3.1 Pharmacology studies (non-GLP)

The following studies (performed with a non-GMP batch of ABY-029) confirm that fluorescently labeled anti-EGFR Affibody molecules delineate tumor margins better than a fluorescently labeled anti-EGFR antibody. [37,38]

- **Target binding assay (Biacore)**

Using 3 human tumor models with varying EGFR positive expression, and 1 EGFR negative tumor line for rats, Dartmouth determined the EGFR concentration in each tumor model using ABY-029, along with a simultaneously delivered reference agent (control affibody-IRDye680RD conjugate). We found that in vivo receptor concentration, calculated through receptor concentration imaging (RCI), was strongly correlated with ex vivo pathologist-scored immunohistochemistry and computer-quantified ex vivo immuno-fluorescence. Weak correlation was observed with ex vivo Western blot or in vitro flow cytometry assays.

- **Cell binding assays**
  - **Isolated receptor binding assay**
    The dissociation constant (K_D) and receptor-ligand binding on and off rates (K_{on} and K_{off}) were determined using surface plasmon resonance (Biacore, GE Life Sciences). Sensograms were acquired and evaluated using the Biacore evaluation software to determine K_{on} and K_{off} using a 1:1 binding ratio kinetic fitting scheme. The K_D value was subsequently calculated using the experimentally determined rate constants. We demonstrated that the binding affinity of anti-EGFR Affibody with IRDye 800 CW with human and rat EGFR was maintained as compared with anti-EGFR Affibody alone.

- **EGFR phosphorylation assay**

LI-COR Biosciences conducted a study in 2010 in which they labelled an EGFR-specific Affibody molecule (Eaff) with IRDye800CW maleimide and tested the molecular binding in cell culture and xenograft mouse tumor models. This study showed the dye labelled Affibody was bound and taken up specifically by EGFR-overexpressing tumor cells. When the fluorescent molecule was intravenously injected into nude mice with xenograft tumors, the tumor could be identified 1 hour after injection and was prominent after 1 day. Following dissection of tissue sections, they saw the labelled molecule accumulated highest in the liver, followed by the tumor and kidney. This study demonstrated that an Affibody labelled with a NIR fluorophore holds promise as a molecular imaging agent for EGFR-overexpressing tumors.

- **In vitro cytotoxicity study**

Phototoxic potential of the infrared light source when cells are exposed to ABY-029 was evaluated, compared to a known FDA approved phototoxic agent benzoporphyrin derivative monoacid A (BPD-MA) and found to be negligible.

- **Imaging study for localization in U251 orthotopic xenograft glioma in mice and rats**

Rats or mice were inoculated with orthotopic implantations of a human glioma cell line (U251, F98 or F98-EGFR) or with PBS (control) in the brain. Tumors were allowed to grow for 2 – 3 weeks before carrying out fluorescent tracer experiments.

We demonstrated that ABY-029 penetrated further into the margin of a U251 orthotopic xenograft mouse model than did a fluorescently labelled antibody (IRDye 680RD-Erbitux) (Sexton et al, PLoS One 2013). Longitudinal imaging of mice bearing U251 xenografts grown orthotopically in the brain was performed by tracking the uptake and retention over 0-3 hours in order to mimic in vivo imaging in humans. A reference tracer composed of a non-specific Affibody molecule bound to IRDye 680RD (LI-COR Biosciences, Inc) was used to quantify blood vessel leakage versus ABY-029 binding to the tumor. We also quantified the

tumor to normal brain uptake as a function of time, and compared these results to direct fiber optic probe measurements in situ and ex vivo. We validated the concept that sufficiently high signal to noise is achievable for ABY-029 uptake in the xenograft tumor model for microdose injection levels. This study suggests that microdoses of the compound will be detectable via imaging in human studies.

We used the U251 orthotopic xenograft rat model to demonstrate that distribution and uptake of microdose levels of ABY-029 is heterogeneous within a tumor, and that high fluorescing regions closely match with highly overexpressing regions of EGFR, as determined by immunohistochemistry (D'Souza et al, submitted 2016). We evaluated the uptake of the preGMP batch of ABY-029 in glioma after intravenous injection in normal rats. Fluorescent imaging of ex vivo brain slices from rats was conducted at different time points after ABY-029 administration (1 h – 48 h) to verify which time points provided maximal tumor to normal contrast. Although U251 tumor was most clearly visualized 1 hour after ABY-029 injection, tumors could be differentiated from the background after 48 hours. These results suggest that ABY-029 shows excellent potential to increase surgical visualization of confirmed EGFR positive tumors, with tumor to normal tissue contrast levels between 10 and 15.

We compared uptake of pre-GMP ABY-029 and in orthotopic rat model using both an EGFR negative tumor, F98, and EGFR positive tumor line, F98-EGFR, which was transfected with EGFR. In addition, we compare the uptake of ABY-029 with protoporphyrin IX (PpIX, administered as 5-aminolevulinic acid, ALA), a fluorophore currently under investigation for surgical guidance during glioma resection at Dartmouth College, and the standard of care for glioma patients in Germany. It was shown that in the EGFR positive tumor (F98-EGFR) ABY-029 outperforms PpIX in both the bulk and the margins of the tumors, while they performed the same in the bulk EGFR negative tumor (F98) and PpIX showed a slight advantage in the margin of the EGFR tumor (Elliott et al, 2016 submitted). These results show a clear indication that ABY-029 provides a surgical advantage in EGFR positive tumors.

1.3.2 Toxicology study (GLP)

The preclinical toxicology assessment is based on a single-dose toxicity study in one species conducted at Dartmouth.

- **Extended single-dose Safety Study in rats**

We conducted a definitive, extended single-dose toxicity study of ABY-029 in Sprague-Dawley rats, consistent with the principles outlined in "Guidance for Industry, Investigators, and Reviewers: Exploratory IND Studies" (2006). This study used the non-GMP-manufactured ABY-029 batch created at UAB, which was also used to develop the GMP process.

A maximum microdose is 30 nmol per 60 kg human subject (or ~237 µg for a protein drug, or 3.95 µg/kg in a human), and this study used the equivalent rat dose of 4.898 µg for a 0.20 kg rat (allowing for a 6.2X rat-to-human correction for skin surface area). Following review of FDA guidelines and pre-IND meetings with FDA, doses of 10X, 100X and 1000X were studied to evaluate whether pharmacologically relevant effects exist. The dose levels tested were 0.049 mg, 0.49 mg and 4.9 mg in the rat toxicity study, along with a control group.

Total Study Duration	1 day and 14 day post-injections
Recovery	Control and high dose group, sacrifice and analysis on day 15
Route	IV (bolus)
Frequency of dose	single
No of doses	1
Groups	Control and 3 dose groups
No of animals	6 male + 6 female per group
Dose levels (preliminary)	0 µg/kg 244.9 µg/kg 2449 µg/kg 24490 µg/kg

Main study	Day 0 - ABY-029 administration
Recovery period	1 or 14 days
Analysis	All standard parameters: Clinical signs, post-dosing observations, standard ophthalmology, body weight, haematology, bone marrow smears, clinical chemistry, macroscopic examination and organ weights, histopathology

A comprehensive set of protocol tissues was collected during necropsy and sent to a veterinary pathologist for microscopic examination. No histopathologic evidence of systemic toxicity was associated with the single-dose (10X, 100X, 1000X) intravenous administration of ABY-029 after 24 hours or 14 days in the study rats. Further, no toxic effects were observed among any of the animals during the routine daily checks.

- **Biodistribution Study in normal rats**

Biodistribution of the 100X microdose of ABY-029 was assessed in normal rats and analysis was performed at 1 hour after injection. One hour post-intravenous administration of ABY-029 the rats were sacrificed by cervical dislocation, and the organs of interest were removed and then imaged on the Pearl Imaging System (LI-COR Biosciences). We found a high accumulation of ABY-029 in the liver, skin, lungs, and kidney, which are all organs with high endogenous EGFR expression and/or that are involved in the route of excretion. No significant difference occurred between ABY-029 accumulation in male and female non-sex organs, although males had slightly higher values, possibly due to size of organ versus body weight, as injected dose was normalized to body weight. Rats without brain tumors had comparably very low levels of ABY-029 accumulation in normal brain tissue, due to the blood brain barrier.

- **Pharmacokinetic Study in normal rats**

Plasma pharmacokinetics of the dye was assessed as function of time by sampling blood from a jugular vein catheter at various time points after administration. Pharmacokinetic data was collected from normal Sprague Dawley rats at time points from 1 minute to 48 hour) after intravenous injection of 100X microdose ABY-029 (244.9 μg/kg). Blood plasma was read on a Fluoromax-3 fluorimeter (Horiba Jobin Yvon), a standard curve was created, and all fluorescent data was converted to concentration using the standard curve and the bi-exponential plasma curve. The plasma curve created in this study matched closely with the previously determined pre-clinical plasma curves, indicating that the physical and biological characteristics of ABY-029 were closely retained. The plasma half-life of ABY-029 in normal rats is approximately 15-20 minutes and less than 10% remains in the plasma at 24 hours and is undetectable at 48 hours. In pre-clinical rat and mouse studies, ABY-029 has been detectable in tissues (tumor, liver, kidney) for up to 72 hours.

1.4 Clinical Data to Date

While no clinical data is available to date on the experimental investigational product, the component molecules Z03115-Cys and IRDye800CW have been used in clinical trials, and products similar to ABY-029 have gone to clinical trial as well.

1.4.1 Related Affibody product ABY-025

ABY-025 ($Z_{HER2:2891}$-Cys-MMA-DOTA), is a HER2-specific Affibody® molecule that targets a radionuclide to HER2-receptor expressing tumors, for in vivo diagnosis of HER2-expressing cancer. ABY-025 is a 61 amino acid peptide that is modified site-specifically with one DOTA molecule at the side chain of the C-terminal cysteine. DOTA acts as chelator for the radionuclide Indium-111 (^{111}In) or Gallium-68 (^{68}Ga). An Investigational Medicinal Product Dossier (IMPD) for ABY-025 labeling with ^{68}Ga or ^{111}In has been filed for clinical studies in Sweden and Denmark, as outlined below.

1) ABY-025-MI103 (EudraCT 2010-021078-12, NCT01216033)

An exploratory study to evaluate the distribution of [^{111}In]ABY-025 uptake for SPECT imaging in subjects with metastatic breast cancer. This study included 7 patients and was completed in Sweden in 2011, with results published [39]

2) ABY-025-MI105 (EudraCT 2012-005228-14, NCT01858116).
An exploratory study to evaluate [^{68}Ga]ABY-025 for PET imaging of HER2 expression in subjects with metastatic breast cancer was due to have been completed in December 2014. This study was initiated 2013 in Swede68n and 16 patients were enrolled to receive at least 2 doses of ABY-025. No adverse events with possible or probable relationships to the study drug were reported, and seven non-serious adverse events with "unlikely" relationships to the study drug were noted [40].

3) MA 1021 (EudraCT 2010-024353-36, NCT02095210)
An exploratory study of HER2 PET imaging in breast cancer patients using [^{68}Ga]ABY-025 was completed. This study was conducted in Denmark from October 2010 to April 2013 with 8 patients enrolled. Results have been published [41]

1.4.2 IRDye 800CW in Clinical Trials

As of March 31, 2016, seven trials are listed on http://clinicaltrials.gov/ involving the use of IRDye 800CW, two that were completed and five are ongoing with patients being recruited.

1) A Phase 1 study was completed (NCT01508572) (**VEGF-targeted Fluorescent Tracer Imaging in Breast Cancer**) (Oct 2011 – Sept 2012), using a VEGF targeted fluorescent tracer to breast cancer, imaging with diffuse tomography, opto-acoustic tomography and fluorescence reflectance imaging, with administration of 4.5 mg of bevacizumab-IRDye800 at 1-3 days prior to imaging. The trial enrolled 20 subjects total beginning in October 2011 and was completed in January 2015, but no results have been published.

2) A Phase 1 study was completed (NCT 02113202) (**Molecular Fluorescence Endoscopy in Patients with Familial Adenomatous Polyposis, Using Bevacizumab-IRDye800CW**) between March 2014 and January 2016. Seventeen patients with Familial Adenomatous Polyposis were administered Bevacizumab-IRDye800 at 4.5mg, 10mg or 25mg at 1-3 days prior to endoscopy. The study's primary objective was to determine the sensitivity of the fluorescent tracer bevacizumab-IRDye800CW, a NIR fluorescence endoscopy platform, in identifying adenomatous polyps. Results have not been published.

3) A Phase 1 study is recruiting patients (NCT 01972373) (**Visualization of Rectal Cancer During Endoscopy, Using a Fluorescent Tracer**), as of 2014, imaging rectal cancer with endoscopy and optical tomography methods, using two imaging sessions, each with a dose of 4.5mg of Bevacizumab-IRDye800, imaged before and then again 3 weeks after the initiation of chemotherapy. In these cases fluorescence imaging is performed 2 days after administration of the conjugate. 30 patients were estimated for enrolment.

4) Another Groninigen study is a Phase 1 trial for esophageal imaging (NCT 02129933) (**VEGF-targeted Fluorescence Near-Infrared (NIR) Endoscopy in (Pre)Malignant Esophageal Lesions**), approved for April 2014 – June 2016. In this study, 4.5 mg of Bevacizumab-IRDye800 is given by IV at 2 days prior to fluorescence endoscopy imaging in 10 patients.

5) An ongoing Phase 1 trial in the United States with IRDye800CW targeted to the EGF receptor, (NCT 01987375) (**Cetuximab IRDye800 Study as an Optical Imaging Agent to Detect Cancer during Surgical Procedures**) is a dose escalation trial at the University of Alabama Birmingham. Head and Neck cancer is imaged during surgery, with Cetuximab-IRDye800CW conjugate as the administered agent. This study is directed by Dr Eben Rosenthal, planned for November 2015 – December 2016. Fifteen patients must be shown to tolerate a test dose of unlabelled Cetuximab of 100 mg, in the week prior to entering this study. The second phase of this study will reduce the test dose to 10 mg.

6) Radboud University in the Netherlands is conducting a Phase 1 trail (NCT 02497599) (**Intraoperative Dual-modality Imaging in Renal Cell Carcinoma**) with Indium-111-DOTA-girentuximab-IRDye800CW in renal cell carcinoma patients. The study will assess the feasibility and safety of intraoperative dual-modality imaging with Indium-111-DOTA-girentuximab-IRDye800CW in 22 patients between June 2015 and December 2016. Patients will receive a single IV dose of the drug, followed by a whole body planar scan and SPECT/CT scan and a partial nephrectomy. The study will be extended by use of dual-modality imaging.

7) A Phase 1 and 2 study at the University Medical Center Groningen (NCT 02583568) (**Fluorescence Guided Surgery in Breast Cancer**) is recruiting patients. This study will enrol 26 patients from October 2015 to December 2016 to define the optimal dose of the fluorescent tracer Bevacizumab-IRDye800CW for intraoperative delineation of breast cancer tissue using an improved and optimized fluorescent tracer and camera system.

1.5 Dose Rationale

We plan a sample size of 6-12 patients in this open label, single center, clinical trial of ABY-029. Administration will occur as a single intravenous injection to subjects with recurrent glioma, approximately 1-3 hours prior to surgery. The protocol is not a safety study since no physiological effects are expected at microdose levels of ABY-029. Rather, doses have been selected to determine if a fluorescence signal can be detected by wide-field imaging technology with a signal-to-noise ratio of 10, which is considered necessary for subsequent assessment of diagnostic performance of ABY-029 as a tumor biomarker sufficient to guide surgical resection in the future. No diagnostic or therapeutic intent is proposed, and administration of the study drug is not intended to alter the extent of planned brain tumor resection during the surgical procedure.

Dosing Scheme: A single microdose of ABY-029 at 30 nanomoles (237µg) will be injected into the first 3 patients. If samples from these subjects meet successful iFI criteria (defined as SNR ≥10 at locations of samples with EGFR pathology score ≥ 1 based on histological staining), 3 more patients will be enrolled at the same dose, concluding the study with a total of 6 subjects. Otherwise, the ABY-029 dose in the next 3 patients will be increased to 3X (90 nanomoles, 711 µg). If samples from these subjects meet successful iFI criteria, 3 more patients will be enrolled at the 3X dose, concluding the study with a total of 9 subjects. If iFI criteria are not met, the next 3 patients will be enrolled at 6X dose (180 nanomoles, 1.42 mg). If iFI criteria are met, 3 more patients will be enrolled the 6X dose, concluding the study with a total of 12 subjects. If iFI criteria are not met in the first 3 patients at the 6X dose, enrollment will be stopped with a total of 9 subjects.

1.6 Potential Risks and Benefits

The risks to subjects are reasonable in relation to anticipated benefits and/or knowledge that might reasonably be expected from the results of this clinical trial.

1.6.1 Potential Risks

Because of the microdose levels to be used, the results of the Dartmouth Toxicity Study, and the safety profile of similar compounds (e.g. ABY-025), serious adverse events (SAEs) related to ABY-029 are not expected to occur.

1.6.2 Potential Benefits

Patients enrolling in this study will not benefit directly because no diagnostic or therapeutic decisions will occur based on the study drug, and thus, administration of the study drug is not intended to alter the extent of planned brain tumor resection during the surgical procedure. However, future patients may benefit from the knowledge gained from the study. Current literature shows that maximum cytoreductive surgery is of significant benefit to patients with malignant gliomas. Patients with complete resections also experience survival benefits from post-operative radiation and/or chemotherapy. Unfortunately, achieving a high degree of tumor removal is often limited by the surgeon's ability to distinguish residual disease from intervening parenchyma under conventional white light illumination, and rates of complete resection have

been reported to be as low as 20% in high grade glioma. iFI augments the surgeon's white light visual field with fluorescent information, and in the case of ALA-induced PpIX, improved patient outcomes in a prospective randomized Phase III trial sufficiently to alter standard-of-care in Germany for surgical resection of high grade glioma. While ALA appears to be effective, motivation exists for pursuing new fluorescent probes beyond PpIX for guiding surgical resection. First, ALA-induced PpIX fluorescence is not tumor-specific. Second, fluorescent enhancement at the margin and in low-grade glioma is relatively poor. Third, ABY-029 has potential to guide a range of oncologic surgeries, given the large number of tumor types that overexpress EGFR. Fourth, a wealth of information exists on EGFR fluorophore targeting which suggests our approach is likely to be successful and could benefit patients with malignant glioma and other cancers whose prognosis is improved by complete surgical resection.

2 Study Objectives

The primary study objective is to determine if microdoses of ABY-029 (up to 6X) lead to detectable signals (defined as signal-to-noise ratio, SNR ≥10, with wide-field iFI) in sampled tissues with an EGFR pathology score ≥ 1 based on histological staining.

The secondary study objectives are to assess the diagnostic accuracy of ABY-029 detection by iFI and an intraoperative probe relative to histopathology as the gold standard, and to measure the molecular uptake and concentration of ABY-029 in resected specimens.

3 Study Design

3.1 General Design

This study is a Phase 0 open label, single-center clinical trial of ABY-029, an Anti-EGFR Fluorescence Imaging Agent, via single intravenous injection to subjects with recurrent glioma. The expected duration for enrolling study participants is 12 to 18 months, and up to 12 subjects may be enrolled. Doses of 1X (30 nanomoles or 237µg), 3X, and 6X may be considered depending on strength of signal detection with iFI in tissues within the surgical field, subsequently sampled and determined post-operatively to have positive EGFR scores based on histological staining.

3.2 Primary Study Endpoints

The primary study endpoint is signal detection (defined as SNR with wide-field iFI) in vivo in brain tissues within the surgical field intended for resection that are subsequently sampled during surgery and assigned an EGFR pathology score based on histological staining.

3.3 Secondary Study Endpoints

Secondary endpoints of the study are diagnostic accuracy of ABY-029 detection by iFI and intraoperative probe relative to histopathology tissue diagnosis, and other indicators (e.g. proliferation, infiltration, etc.) as the gold standard, and measurement of molecular uptake and concentration of ABY-029 in resected specimens.

4 Subject Selection and Withdrawal

4.1 Patient Selection

Patients with recurrence of presumed high grade glioma having EGFR positive tissue (score ≥ 1 based on histological staining or molecular pathology analysis) obtained from prior surgery will be eligible to participate.

4.2 Inclusion Criteria

The following criteria must be met by study subjects to be eligible for study enrollment:

1) Preoperative diagnosis of recurrent high-grade glioma having EGFR positive tissue from prior surgery.

2) Tumor judged to be suitable for open cranial resection based on preoperative imaging studies.
3) Valid informed consent by subject.
4) Age ≥ 18 years old.

4.3 Exclusion Criteria

The following criteria would exclude a subject from study enrollment:

1) Pregnant women or women who are breast feeding.
2) Patients on any experimental anti-EGFR targeted therapies

4.4 Subject Recruitment and Retention

Participation in this research requires informed consent according to Institutional Review Board (IRB) guidelines and a signed IRB-approved Consent Form as the means of documenting this understanding. Potential recruits are instructed that their participation is completely voluntary and that their medical care will not be altered in any way should they elect not to participate at any time prior to surgery. Subjects are recruited from patients presenting or referred to the Section of Neurosurgery at Dartmouth-Hitchcock Medical Center for surgical resection of intracranial tumor of the types meeting protocol inclusion criteria. Potential subjects may learn about the study through its posting on clinicaltrials.gov and contact Dr. Roberts or another surgeon in the Neurosurgery Section about participation. Subjects will be invited to participate in this study by a member of the Neurosurgery Section which will occur either at the time of consultation with the surgeon about the candidate's standard-of-care procedures or at another time agreed to by the potential participant, the candidate's surgeon and/or Dr. Roberts or Dr. Roberts's designee. No advertisements or other promotional material will be used. No finder fees or recruitment incentives will be offered. Women of child bearing potential are eligible for enrollment into this study because ABY-029 administration is not considered to present any additional risk for these women. The study will exclude women who are pregnant or breast-feeding as indicated in the exclusion criteria. Women of child-bearing potential, if asked to participate, will be given a pregnancy test at no cost to confirm pregnancy status before administration of ABY-029.

4.5 Early Withdrawal of Subjects

4.5.1 When and How to Withdraw Subjects

Subjects participate in a "one-time" event (i.e., surgery) as part of this study and are not expected to be withdrawn because of subsequent post-surgical conditions and/or scenarios (e.g. the need for alternative and/or additional treatment). Subjects may withdraw their consent at any time prior to surgery. Subject data collected per protocol may subsequently not be included in the final analysis, if IV administration of ABY-029 is found not to have occurred at the proper dose (237µg, 711µg or 1.42mg, depending on the dose group) approximately 1-3 hours prior to surgery or the definitive post-operative diagnosis does not meet the presumed entry criteria.

4.5.2 Data Collection and Follow-up for Withdrawn Subjects

No post-operative end-points or follow-up information is needed to meet study objectives; hence, consenting subjects who complete surgery will not be lost to follow-up.

5 Study Drug

5.1 Description

Patients will be administered ABY-029 by intravenous injection approximately 1-3 hours prior to induction of anesthesia. The range of approximately 1-3 hours is acceptable given pre-clinical data indicating that imaging signal levels appear to be stable over this time period. Time from ABY-029 administration to surgery is collected. Subjects are considered enrolled in the study once ABY-029 has been injected.

5.2 Treatment Regimen

Groups of up to 6 patients (as described in Section 1.5) will receive a single injection of ABY-029 at 1X or 3X or 6X of the microdose of 30 nanomoles (237µg). The dose will be administered by intravenous injection on the day of surgery, approximately 1-3 hours prior to the procedure.

5.3 Method for Assigning Subjects to Treatment Groups

No separate treatment groups are part of the study design.

5.4 Preparation and Administration of Study Drug

ABY-029 used in this study is stored, prepared, and dispensed by the Investigational Pharmacy at Dartmouth-Hitchcock Medical Center. (Address: Dartmouth Hitchcock Medical Center, Investigational Pharmacy, Rubin-463N3, One Medical Center Drive, Lebanon, NH 03756; email address: investigational.pharmacy@hitchcock.org; Tel: 603-650-7890).

ABY-029 Preparation and Administration Procedures:

1) ABY-029 is prepared by the Investigational Pharmacy at the appropriate dose, typically on the day of or day before surgery, depending on the scheduled surgery date and time.
2) From the vial containing ABY-029, the required dose is withdrawn into a syringe, labeled, and placed into a light-impermeable bag.
3) Once prepared at the requested dose, the drug in the light-impermeable bag is either held by the Investigational Pharmacy until picked up by a member of the study team, or stored in a refrigerated and locked box in the Same Day Surgery patient holding area. The key to the lock-box is placed in an associated Acudose Unit.
4) The dose of ABY-029 is administered intravenously through an IV port followed by a saline flush.

Most study subjects will check in to the Same Day Surgery Program on the day of their surgery, though some may be admitted inpatients. Study subjects generally have a pre-surgical MRI. ABY-029 will be administered to patients approximately 1-3 hours prior to surgery, in the Same Day Surgery Program area, the MRI area, or in an inpatient unit. Patients will be under the care and observation of a research nurse or Same Day Surgery nurse, or by an inpatient nurse if ABY-029 is administered in the inpatient area, during the time between ABY-029 administration and the start of surgery, and will be monitored accordingly and appropriately. If ABY-029 is administered prior to the pre-surgical MRI, a research nurse will accompany the patient to the MRI and monitor the patient until he or she is in the Same Day Program holding area.

5.5 Subject Compliance Monitoring

Subjects participate in a “one-time” event (i.e., surgery) as part of this study and are monitored through the in-patient surgical service during the time of their participation.

5.6 Prior and Concomitant Therapy

This study is a Phase 0 diagnostic trial of ABY-029 fluorescence as a possible intraoperative imaging biomarker during open cranial surgery for tumor resection. Prior and/or concurrent medical therapies do not alter the diagnostic intent and/or endpoints associated with the study.

5.7 Packaging

ABY-029 is packaged as a transparent, green-blue liquid in 5mL glass vials at a concentration of 0.27 mg/mL, in PBS (phosphate-buffered saline). It is stored at the Investigational Pharmacy at Dartmouth-Hitchcock Medical Center (Address: Dartmouth Hitchcock Medical Center, Investigational Pharmacy, Rubin-463N3, One Medical Center Drive, Lebanon, NH 03756). ABY-029 is shipped in clear glass vials, each with a rubber stopper in boxes containing 10 mL units.

5.8 Blinding of Study Drug

This study is a Phase 0 diagnostic trial of ABY-029 fluorescence as a potential intraoperative imaging biomarker during open cranial surgery for tumor resection and does not include comparison treatment groups. Because all subjects receive ABY-029, blinding of the surgeon to the study drug is not practical or appropriate.

5.9 Receiving, Storage, Dispensing and Return

5.9.1 Receipt of Drug Supplies

ABY-029 will be shipped to the Investigational Pharmacy at Dartmouth-Hitchcock Medical Center by UAB. Investigational pharmacy staff will count and verify that the shipment contains all the items noted in the shipment inventory, providing temperature monitoring data to UAB if necessary. Investigational pharmacy staff will document receipt into their electronic investigational drug management database that keeps a perpetual inventory. Any damaged or unusable study drug in a given shipment will be documented in the study files, and destroyed on site following standard procedures.

5.9.2 Storage

ABY-029 will be stored as a transparent, green-blue liquid in its shipment vials and outer carton in a freezer at temperatures between -15^{o}C and -25^{o}C. Vials will not be removed from the outer carton until drug is prepared as ABY-029, which must be stored under dark conditions.

5.9.3 Dispensing of Study Drug

Once drug assignment is performed, a study authorized staff member (e.g., Dr. Roberts, his designee) will order ABY-029 through the institution's electronic medical record. This order will specify the dose of 1X (237 µg), 3X (711 µg) or 6X (1.42mg) for ABY-029. Since the concentration in each vial of ABY-029 is 1.35 mg or 5.7X, in order to conserve our supply of ABY-029 we will administer a 5.7X dose to the 6X dose group in this study, and will call this the "6X dose" throughout this protocol. One dose of ABY-029 will be administered to each subject. The investigational pharmacy pharmacist will process this order and a pharmacy technician will prepare the ABY-029 dose as described in Section 5.4 (above)

5.9.4 Drug Accountability

When processing an order for ABY-029, an investigational pharmacy pharmacist will make an entry into the electronic investigational drug management database to remove vial(s) used to prepare a dose from ABY-029 drug inventory. A member of the study team will document the amount of ABY-029 administered and any amount remaining in the institution's electronic medical record.

5.9.5 Return or Destruction of Study Drug

At the completion of the study, a final reconciliation of drug shipped, drug consumed, and drug remaining will occur. This reconciliation will be logged on the drug reconciliation form, signed and dated. Any discrepancies noted will be investigated, resolved, and documented prior to return or destruction of unused study drug. Site policy (i.e., Investigational Pharmacy) is to destroy any used, open drug containers on site. Drug destroyed on site will be documented in the study files.

6 Study Procedures

This study is a Phase 0 clinical investigation of ABY-029 concentration measured/detected in brain tissue through its fluorescence emissions as an intraoperative biomarker of tumor during open cranial surgery for tumor resection. Subjects participate in a "one-time" event/visit (i.e., surgery) as part of this protocol. Images and data from the patient's pre- and post-operative MR scans are used for analysis in the study, but these acquisitions are part of the patient's standard-of-care, would occur independently of whether the participant is enrolled, and thus, are not considered to be research study visits. No post-surgical follow-up visit is part of the protocol data collection or analysis. Patients are monitored for possible adverse events for approximately 30 days through the routine follow-up under the care of the operating surgeon involved in

the study procedures, post-operatively, and subsequently through the medical record. Hence, this information is already being collected as part of standard-of-care and is available for adverse event surveillance.

6.1 Surgical Visit

After the study drug is administered (~1-3 hours prior to surgery), the patient will be prepared and transported to surgery as per routine at Dartmouth-Hitchcock. In the OR, patient positioning, registration of the MR image-guidance system, preparation of the surgical field, and draping will follow standard practice, as will scalp incision and craniotomy. Upon opening of the dura, the coregistered and tracked Zeiss Pentero operating microscope adapted for intraoperative fluorescence imaging (iFI) at 800 nm will be brought in to begin conventional image-guided microsurgical technique for tumor resection. At approximately 4 time points during each surgery and/or at the discretion of the surgeon, data/image and specimen acquisitions will occur. Three (3) will typically occur at first exposure of tumor, at the approximate mid-point of tumor resection (when a significant tumor tissue is in the field), and at a point nearing but prior to completion of tumor resection (when small amount of tumor tissue remains), and a fourth may occur at presumed end of resection. At each time point, iFI acquisitions will be recorded. The surgeon will then focus the coregistered microscope at discrete points in the operative field which exhibit non-, weakly, moderately, and strongly fluorescent emissions, if evident on the iFI scan. At each point, a second iFI will be acquired, optical probe measurements will be recorded and a surgical biopsy specimen will be obtained (except at presumed end of resection when no more tissue is planned for excision). Since the 2nd iFI, probe data and biopsy sample will occur each time at the focal point of surgical microscope which is coregistered, all intraoperative images, data and specimens will also be coregistered. If no signal is evident on the iFI scan, the surgeon will sample several locations in the surgical field by focusing the operating microscope on points of interest intended for resection, performing the probe measurements and biopsying the positions for later analysis. iFI and optical probe readings will also be acquired from normal brain to establish baseline/background signal levels for the case. Additionally, an imaging module mounted on a second commercial, clinical microscope, will be used to acquire images during surgery. Acquiring these images will involve moving the primary clinical scope out of the field, positioning the new scope to acquire images, and moving the primary scope back into place. This process will take approximately three minutes or less and poses no additional risk to the patient. When the case is performed in the Center for Surgical Innovation, and when indicated clinically, iMR (intraoperative MRI) may be acquired and resection may continue based on the findings. In these cases, iFI, optical probe and biopsy may occur at locations planned for resection. We have used these same coregistered image, data, and biopsy procedures in our current studies of ALA-induced PpIX fluorescence and have collected high quality information from up to 12-16 surgical specimens per case (~10 on average).

6.1.1 Specimen Preparation and Handling

Resected specimens used for standard clinical care will be analyzed according to practice. Tissues taken as part of the study will be bisected immediately in the OR into nearly equivalent pieces in terms of size and gross appearance with one part going into 10% buffered formalin and the other snap frozen in liquid nitrogen. If the specimen cannot be divided, the sample will be formalin-fixed.

6.2 Clinical Laboratory and Monitoring Data

Since the microdoses that will be administered are presumed to fall below levels needed to cause pharmacological and/or physiological responses/effects that increase subject risks, and the preclinical (rodent) results from the Dartmouth Toxicity Study showed no evidence of systemic toxicity, either histologically or in terms of blood chemistry (in the latter, some clinical chemistry values in dosed animals were statistically different from un-dosed controls at 24 hours, but all levels were well within the normal range) associated with much higher single-doses of ABY-029 (10X, 100X, 1000X), no protocol-specific clinical laboratory or monitoring data associated, for example, with renal or liver function will be collected. Data obtained in this patient group as part of standard-of-care laboratory studies and clinical monitoring procedures includes hemoglobin, hematocrit, white blood cell count, platelet count, electrolytes (sodium, potassium, chlorate, bicarbonate), glucose, BUN and creatinine prior to administration of ABY-029. Similarly, vital signs (heart rate, blood pressure, respiratory rate) are monitored pre-operatively, during

surgery and post-operatively in the Neurosurgical Special Care Unit for a minimum of 24 hours following surgery as part of standard-of-care, and these data are available for adverse event monitoring. EKGs, when collected as part of standard-of-care operating procedures on surgical patients at Dartmouth-Hitchcock, are available pre-operatively, monitored continuously during surgery and again post-operatively (typically in the evening of the day of surgery). These data will not be collected as part of this clinical protocol per se, but will be available for adverse event monitoring, as needed.

6.3 Specimen Analysis

Biopsy samples taken at the time of surgery for research purposes and processed for IHC will be scored by a pathologist according to the following criteria: 0 = no staining; 1 = low membranous staining around cells; 2 = medium membranous staining completely around cells; and 3 = high membranous staining completely around cells. Standard formalin-fixation with paraffin embedding will be used for histopathologic type and grade to confirm diagnosis of astrocytoma or glioblastoma (following WHO criteria) by the study neuropathologist. As these tumors are recurrences and patients have received radiation and chemotherapy, an assessment of reactive gliosis vs. residual/recurrent glioma will also be scored as well as tumor burden and necrosis using methods previously described. Here, histopathology will be classified as non-tumor tissue, solid tumor, infiltrating tumor, or indeterminate. Infiltrating tumor will be further graded into <33% tumor, >33% but <67% tumor, and >67% tumor). Proliferation will be assessed by quantitatively scoring KI-67 immunopositive cells. ABY-029 binding to tumor cells in the specimen will be evaluated by performing IHC with secondary antibodies directed against the anti-EGFR Affibody following the protocol. EGFR protein expression often corresponds to gene amplification, thus, we will assess gene-protein correlations on a cell-by-cell basis by performing both IHC for EGFR protein and CISH for EGFR gene amplifications using a multi-spectral analysis microscope (Nuance) to distinguish in individual tumor cells. Frozen tissue will be solubilized and evaluated for quantitative fluorescence using fluorimetry. Western and/or ELISA will be used if the signal is not sufficiently strong.

7 Statistical Plan

7.1 Sample Size Determination

Sample size determination is based on the primary objective of the study as a pilot assessment of fluorescence detection of ABY-029 localization in EGFR+ tumors with iFI. We estimate a minimum of 6, and more typically 12-16 specimens per case will be collected, and assume an average of 10 is available consistent with numbers in similar studies of ALA-induced PpIX fluorescence that have enrolled over 100 subjects. To assess the adequacy of this sample size, if only 3 patients are enrolled at a specific dose, the study will have 80% power to detect a difference in fluorescence signal mean in samples relative to normal brain as small as 3.3 standard deviations with a two-sided significance level of 0.05, and a difference in fluorescence signal mean in samples between to two EGFR pathology scoring levels of 2.7 standard deviation with the same power and significance level (assuming samples are equally distributed between EGFR pathology scoring levels). If 6 patients are enrolled a one dose, the detectable difference in fluorescence signal mean in samples relative to normal brain drops to 1.4 standard deviations (at the same power and significance level) and to 1.8 standard deviations for differences in signal mean between two EGFR pathology scoring levels.

7.2 Data and Statistical Analysis

The primary study objective is to relate ABY-029 fluorescence (resulting from a specific pre-surgical dose) measured by wide-field iFI to EGFR pathology score (based on IHC staining) of coregistered tissue specimens sampled at the same time points during surgery. Similar but more sensitive fluorescence data will be available from the intraoperative optical probe. A minimum of 3 patients will be enrolled at a given dose, and up to 6 patients may be enrolled at one dose, if iFI SNR requirements are met. Assuming 10 specimens are available for analysis on average from the surgical cases at a given dose (this value is consistent with average specimen counts in related ALA-induced PpIX studies which have enrolled more than 100 subjects), 30 specimens with corresponding probe signals and IHC pathological scoring will be available for analysis, and as many as 60 specimens may exist, if iFI SNR goals are achieved. Clustered

data analyses of variance will be performed to account for multiple samples from each patient. Student's t-test evaluation will be used to determine signal detectability relative to measurements in normal brain, and relative to the noise floor of the iFI system determined from laboratory measurements in phantoms. ANOVA will also be used assess differences in signal means when stratified by EGFR pathology score (0 = no expression, 1 = low expression, 2 = medium expression, 3 = high expression). Pearson's and Spearman's-rank correlations between iFI signal strength and EGFR pathology score will be computed. These analyses will be repeated for the optical probe data as well.

Secondary study objectives will be assessed by performing analyses on the iFI and probe data as a diagnostic test with the corresponding specimen histopathological diagnoses as the gold standard. Predictive models (for each pathology) of the odds of tumor positivity for biopsies given concentration of ABY-029 will be constructed. Histologically confirmed tumor status from biopsies will be treated as a binary outcome with a logit link function in a generalized linear mixed model (SAS GLIMMIX) with a subject-specific random effect to account for intra-individual correlations among biopsies. The iFI and/or probe fluorescence measures will be introduced first as linear terms. Interactions and polynomial fits will also be assessed via goodness of fit statistics. The predictive accuracy of the model fit will be summarized through a concordance index, i.e., area under the ROC curve formed with the fitted probabilities of tumor. Analyses will be repeated for other histopathological indicators (beyond tumor/non-tumor) such as degree of proliferation, infiltration, ABY-029 binding, among others.

7.3 Subject Population(s) for Analysis

This study will involve patients with recurrent intra-cranial malignant glioma judged to be operable. Subjects eligible for enrollment in the trial will be patients with a previously resected high grade glioma with tissue positive for EGFR expression (pathology score ≥ 1 based on histological staining).

8 Safety and Adverse Events

The study will be monitored by the Data, Safety Monitoring, and Accrual Committee (DSMAC) of the Norris Cotton Cancer Center at Dartmouth-Hitchcock Medical Center. All unexpected and/or serious adverse events will be reported to committee by the protocol PI or the designee, along with a determination of their relationship to study procedures. Given that brain tumor patients undergoing open surgical resection procedures present with post-operative complications and medical events that are expected, we will log and review all adverse events, but evaluate and report on only those with outcomes that would not routinely be expected in this surgical patient population to the NCCC DSMAC, along with a determination of their probable relationship to study procedures. Summaries of study enrollments, clinical status, and data will be submitted for annual review (or more frequently as required depending on enrollment patterns). In the case of any unexpected adverse events, the patient's medical record will be reviewed by a study physician, and if necessary, the patient will be examined by a study physician and appropriate medical action taken. The event will be recorded in the patient's study file with the date of occurrence, date of resolution, severity and the actions taken. Any unexpected or serious adverse events, or drug reaction beyond those expected from open-cranial brain tumor surgery that are determined likely to be related to the study procedures, will be reported to the Dartmouth IRB and the FDA as required.

9 Data Handling and Record Keeping

9.1 Confidentiality

Information about study subjects will be kept confidential and managed according to the requirements of the Health Insurance Portability and Accountability Act of 1996 (HIPAA). Risk of breach of confidentiality of the medical records and status of participants will be minimized. Databases which are used to store subject-sensitive information are password-protected and encrypted during file/data transfers from viewing terminals. Access will be limited to research team members who have undergone CPHS training at Dartmouth. Whenever possible and practical standard-of-care clinical data used in the research will be de-identified when under analysis.

9.2 Case Report Forms

Study case report forms (CRFs) will be the primary data collection instruments for the study. All data requested on CRFs will be recorded. Any missing data will be explained. If a space on the CRF is left blank because the procedure was not performed or the question was not asked, a written notation will be made. If an item is not applicable to an individual case, written notation will be made. Changes to the CRFs will be initialed and dated.

10 Clinical Site Monitoring

10.1 On-Site Monitoring

Clinical research monitoring for regulatory compliance and data integrity will be conducted according to the NCI-approved NCCC Data and Safety Monitoring Plan. Internal monitoring is conducted by appropriately trained staff of the NCCC Office of Clinical Research and Dartmouth-Hitchcock Medical Center Clinical Trials Office who are not involved in the study. This monitoring will include periodic assessment of the regulatory compliance, data quality, and study integrity. Study records will be reviewed and directly compared to source documents and the conduct of the study will be discussed with the investigator. Monitors may request access to all regulatory documents, source documents, CRFs, and other study documentation for on-site inspection. Direct access to these documents is guaranteed by the investigator, who must provide support at all times for these activities.

10.2 Auditing and Inspecting

The study investigators will permit study-related monitoring, audits, and inspections by the IRB, the sponsor, government regulatory bodies, and university compliance and quality assurance groups of all study related documents (e.g. source documents, regulatory documents, data collection instruments, study data, etc.). The study investigators will ensure the capability for inspections of applicable study-related facilities (e.g. pharmacy, diagnostic laboratory, etc.).

Participation as an investigator in this study implies acceptance of potential inspection by government regulatory authorities and applicable university compliance and quality assurance offices.

11 Ethical Considerations

This study is to be conducted according to US and international standards of Good Clinical Practice (FDA Title 21 part 312 and International Conference on Harmonization guidelines), applicable government regulations and Institutional research policies and procedures.

All subjects for this study will be provided a consent form describing this study and providing sufficient information for subjects to make an informed decision about their participation in this study. This consent form will be submitted with the protocol for review and approval by the IRB. The formal consent of a subject, using the IRB-approved consent form, will be obtained before that subject undergoes any study procedure. The consent form will be signed by the subject and the investigator-designated research professional obtaining the consent.

12 Study Finances

12.1 Funding Source

This study is financed through a grant from the US Department of Health and Human Services, National Institutes of Health, National Cancer Institute, grant # 5R01CA167413.

12.2 Conflict of Interest

All Dartmouth investigators will follow the Dartmouth conflict of interest policy, and will have disclosed potential conflicts-of-interest related to the study.

13 References

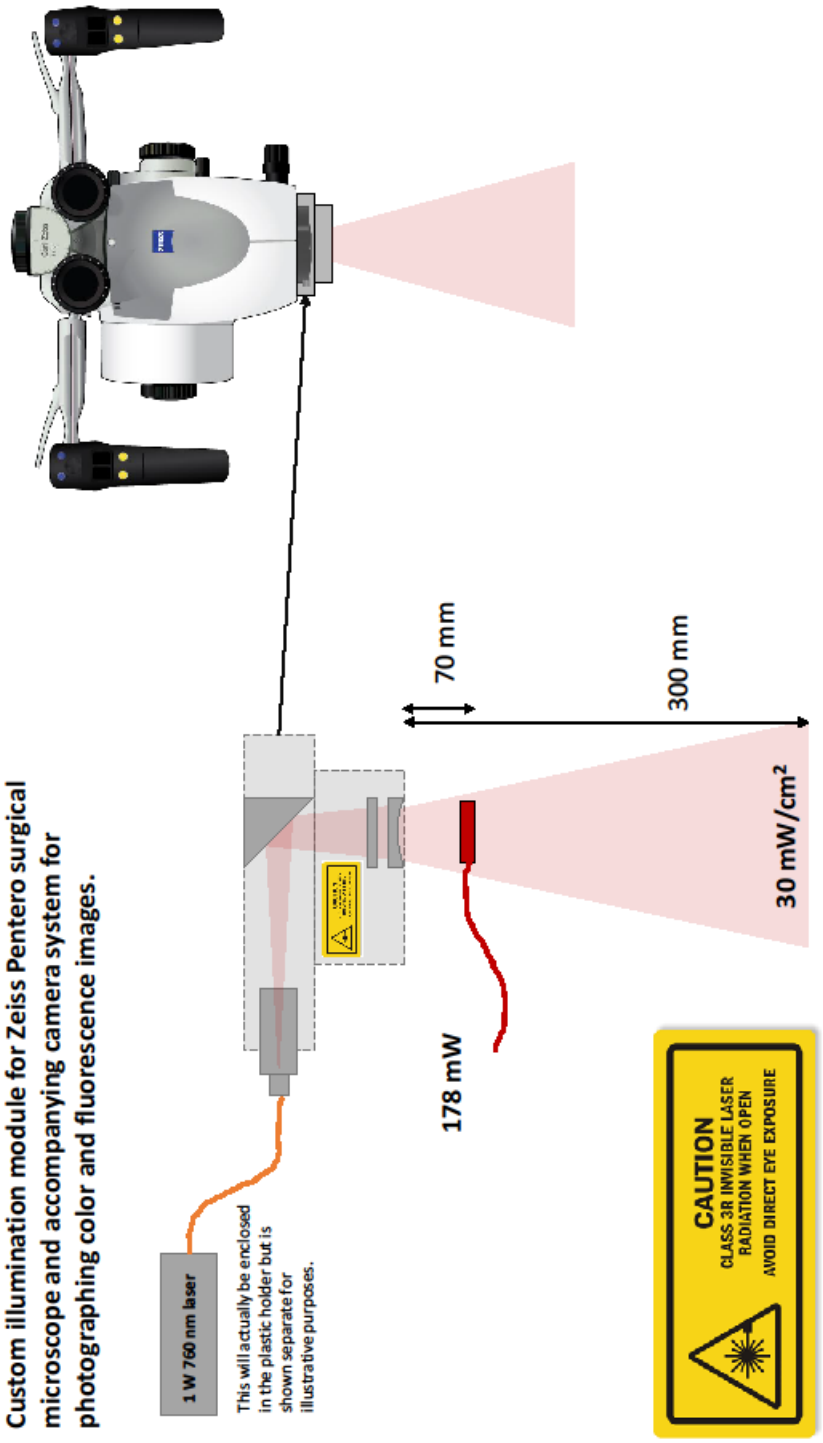
1. De Grand, A.M. and J.V. Frangioni, *An operational near-infrared fluorescence imaging system prototype for large animal surgery.* Technol Cancer Res Treat, 2003. **2**(6): p. 553-62.
2. Vahrmeijer, A.L. and J.V. Frangioni, *Seeing the invisible during surgery.* Br J Surg, 2011. 98(6): p. 749-50.
3. Orcutt, K.D., et al., *Engineering an antibody with picomolar affinity to DOTA chelates of multiple radionuclides for pretargeted radioimmunotherapy and imaging*. Nucl Med Biol, 2011. 38(2): p. 223-33.
4. Gibbs-Strauss, S.L., et al., *Nerve-highlighting fluorescent contrast agents for image-guided surgery.* Mol Imaging, 2011. 10(2): p. 91-101.
5. Orcutt, K.D., et al., A modular IgG-scFv bispecific antibody topology. Protein Eng Des Sel, 2010. 23(4): p. 221-8.
6. Stummer, W., et al., Fluorescence-guided surgery with 5-aminolevulinic acid for resection of malignant glioma: a randomised controlled multicentre phase III trial. Lancet Oncol, 2006. 7(5): p. 392-401.
7. Stummer, W., et al., Extent of resection and survival in glioblastoma multiforme: identification of and adjustment for bias. Neurosurgery, 2008. 62(3): p. 564-76; discussion 564-76.
8. Valdes, P.A., et al., 5-Aminolevulinic Acid-Induced Protoporphyrin IX Fluorescence in Meningioma: Qualitative and Quantitative Measurements In Vivo. Neurosurgery, 2014. 10 Suppl 1: p. 74-83.
9. Valdes, P.A., et al., Gadolinium- and 5-aminolevulinic acid-induced protoporphyrin IX levels in human gliomas: an ex vivo quantitative study to correlate protoporphyrin IX levels and blood-brain barrier breakdown. J Neuropathol Exp Neurol, 2012. 71(9): p. 806-13.
10. Roberts, D.W., et al., Adjuncts for maximizing resection: 5-aminolevuinic acid. Clin Neurosurg, 2012. 59: p. 75-8.
11. Valdes, P.A., et al., Quantitative fluorescence in intracranial tumor: implications for ALA-induced PpIX as an intraoperative biomarker. J Neurosurg, 2011.
12. Roberts, D.W., et al., Coregistered fluorescence-enhanced tumor resection of malignant glioma: relationships between delta-aminolevulinic acid-induced protoporphyrin IX fluorescence, magnetic resonance imaging enhancement, and neuropathological parameters. Clinical article. J Neurosurg, 2011. 114(3): p. 595-603.
13. Leung, K., IRDye 800-albumin-binding domain-fused-ZHER2:342 Affibody. 2004.
14. Chopra, A., Cy5.5 conjugated anti-epidermal growth factor receptor monoclonal antibody. 2004.
15. Leung, K., IRDye 800CW-Epidermal growth factor. 2004.
16. Helman, E.E., et al., Optical imaging predicts tumor response to anti-EGFR therapy. Cancer Biol Ther, 2010. 10(2): p. 166-71.
17. Wang, K., et al., Characterizing breast cancer xenograft epidermal growth factor receptor expression by using near-infrared optical imaging. Acta Radiol, 2009. 50(10): p. 1095-103.
18. Gleysteen, J.P., et al., Fluorescent labeled anti-EGFR antibody for identification of regional and distant metastasis in a preclinical xenograft model. Head Neck, 2008. 30(6): p. 782-9.
19. Koyama, Y., et al., In vivo molecular imaging to diagnose and subtype tumors through receptor-targeted optically labeled monoclonal antibodies. Neoplasia, 2007. 9(12): p. 1021-9.
20. Barrett, T., et al., In vivo diagnosis of epidermal growth factor receptor expression using molecular imaging with a cocktail of optically labeled monoclonal antibodies. Clin Cancer Res, 2007. 13(22 Pt 1): p. 6639-48.
21. Kulbersh, B.D., et al., Sensitivity and specificity of fluorescent immunoguided neoplasm detection in head and neck cancer xenografts. Arch Otolaryngol Head Neck Surg, 2007. 133(5): p. 511-5.
22. Rosenthal, E.L., et al., In vivo detection of head and neck cancer orthotopic xenografts by immunofluorescence. Laryngoscope, 2006. 116(9): p. 1636-41.
23. Verbeek, F.P., et al., Near-Infrared Fluorescence Imaging of Both Colorectal Cancer and Ureters Using a Low-Dose Integrin Targeted Probe. Ann Surg Oncol, 2014.
24. Ryu, J.H., et al., In vivo fluorescence imaging for cancer diagnosis using receptor-targeted epidermal growth factor-based nanoprobe. Biomaterials, 2013. 34(36): p. 9149-59.
25. Tafreshi, N.K., et al., Synthesis and characterization of a melanoma-targeted fluorescence imaging probe by conjugation of a melanocortin 1 receptor (MC1R) specific ligand. Bioconjug Chem, 2012. 23(12): p. 2451-9.

26. Huang, C.W., Z. Li, and P.S. Conti, In vivo near-infrared fluorescence imaging of integrin alpha2beta1 in prostate cancer with cell-penetrating-peptide-conjugated DGEA probe. J Nucl Med, 2011. 52(12): p. 1979-86.
27. Terwisscha van Scheltinga, A.G., et al., Intraoperative near-infrared fluorescence tumor imaging with vascular endothelial growth factor and human epidermal growth factor receptor 2 targeting antibodies. J Nucl Med, 2011. 52(11): p. 1778-85.
28. Miller, S.J., et al., In vivo fluorescence-based endoscopic detection of colon dysplasia in the mouse using a novel peptide probe. PLoS One, 2011. 6(3): p. e17384.
29. Nord, K., A combinatorial library of an alpha-helical bacterial receptor domain. Protein Eng, 1995. 8(6): p. 601-608.
30. Lofblom, J., et al., Affibody molecules: engineered proteins for therapeutic, diagnostic and biotechnological applications. FEBS Lett, 2010. 584(12): p. 2670-80.
31. Friedman, M., et al., Directed evolution to low nanomolar affinity of a tumor-targeting epidermal growth factor receptor-binding affibody molecule. J Mol Biol, 2008. 376(5): p. 1388-402.
32. Orlova, A., et al., Tumor imaging using a picomolar affinity HER2 binding affibody molecule. Cancer Res, 2006. 66(8): p. 4339-48.
33. Lindborg, M., et al., Engineered high-affinity affibody molecules targeting platelet-derived growth factor receptor beta in vivo. J Mol Biol, 2011. 407(2): p. 298-315.
34. Orlova, A., et al., Synthetic affibody molecules: a novel class of affinity ligands for molecular imaging of HER2-expressing malignant tumors. Cancer Res, 2007. 67(5): p. 2178-86.
35. Sampath, L., et al., Dual-labeled trastuzumab-based imaging agent for the detection of human epidermal growth factor receptor 2 overexpression in breast cancer. J Nucl Med, 2007. 48(9): p. 1501-10.
36. Rosenthal, E., et al., Safety and Tumor-specificity of Cetuximab-IRDye800 for Surgical Navigation in Head and Neck Cancer. Clin Cancer Res, 2015 21:3658-3666.
37. Gong, H., et al., In vivo imaging of xenograft tumors using an epidermal growth factor receptor-specific affibody molecule labeled with a near-infrared fluorophore. Neoplasia, 2010. 12(2): p. 139-49.
38. Sexton, K., et al., Fluorescent affibody peptide penetration in glioma margin is superior to full antibody. PLoS One, 2013. 8(4): p. e60390.
39. Sorensen, J, et al., First-in-Human Molecular Imaging of HER2 Expression in Breast Cancer Metastases Using the 111IN-ABY-025 Affibody Molecule. J Nucl Med. 2014 May;55(5):730-5.
40. Sorensen, J, et al., Measuring HER2-Receptor Expression In Metastatic Breast Cancer Using [68Ga]ABY-025 Affibody PET/CT, Theranostics 2016, Vol 6, issue 2, p 262-271.
41. Sandstrom, M, et al., Biodistribution and radiation dosimetry of the anti-HER2 Affibody molecule 68Ga-ABY-025 in breast cancer patients, J Nucl Med. 2016 Feb [epub ahead of print].

14 Attachments

14.1 Zeiss Pentero microscope modifications

The figure below illustrates modifications made to the Zeiss Pentero surgical microscope. The device as modified presents a non-significant risk to patients. It will not be in contact with the subject in any way, including as an implant. The device will not be used in any way to direct or manage patient care, nor to collect data, diagnose or treat the patient in any way. It is used purely as part of an observational study.



14.2 Zeiss S1 microscope modifications

The figure below illustrates modifications made to the Zeiss S1 surgical microscope. The device as modified presents a non-significant risk to patients. It will not be in contact with the subject in any way, including as an implant. The device will not be used in any way to direct or manage patient care, nor to collect data, diagnose or treat the patient in any way. It is used purely as part of an observational study.