TITLE:

Intravital Microscopy (IVM) in Human Solid Tumors

Study Number:

Initial Date:

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Confidentiality Statement

SYNOPSIS: Intravital Microscopy (IVM) in Human Solid Tumors

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Concept and Rationale: Intravital microscopy (IVM) allows real-time, direct visualization of microscopic blood vessels and calculation of blood flow. Our group has used IVM in mouse models to characterize aberrant blood vessel structure surrounding tumor implants and the associated impaired lymphocyte trafficking along these tortuous vessels.[1] These preclinical studies have been translated to human intravital microscopy (HIVM) in a pilot study that analyzed the tumor-associated microvasculature at primary melanomas.[2] The feasibility of HIVM was 90% at the primary tumor. As an extension of this completed trial, Dr. Gabriel had implemented an on-going clinical trial (NCT02857374, Intravital Microscopy in Identifying Tumor Vessels in Patients with Stage IB-IIIC Melanoma Undergoing Sentinel Lymph Node Biopsy) that investigates the role of IVM in analyzing the vessels associated with sentinel lymph nodes. In addition, with members of the multidisciplinary team listed, above, we have also implemented a trial in HIVM at Mayo Clinic Florida that investigates HIVM in patients with peritoneal carcinomatosis, NCT03517852 Intravital Microscopy (IVM) in Patients with Peritoneal Carcinomatosis (PC). This proposed trial at Mayo Clinic Florida will further determine the usefulness of IVM in human subjects, now applying it to patients with intra-abdominal or retroperitoneal tumors as well as study the effects of fluid shifts on the tumor blood flow in real time.

In this clinical trial proposal, the following aims will be addressed:

Aim 1: Determine the feasibility of HIVM in characterizing the tumor-associated vessels in patients with deep space solid tumors and correlate these findings with patient outcomes. We hypothesize that HIVM will be feasible in deep space solid tumors and function as a predictive biomarker for response to systemic therapy.

Aim 2: Determine the effects of dynamic control on tumor vessel blood flow during surgical resection. We will investigate whether we can reproduce enhanced tumor blood flow, established in our previous animal models, in human subjects using our dynamic control protocol (consisting of a fluid bolus and vasopressors, routinely given during the course of surgery).

Objectives:

Primary objective(s): To determine the feasibility of performing HIVM in patients with deep space solid tumors during standard course of surgical resection. A successful intravital microscopic observation will include the ability to complete each of the following:

1. Identify and measure vessels associated with tumor
2. Determine vessel density per 10x field
3. Visualize vital dyes within the vessels (fluorescein)
4. Calculate the blood flow velocity of the vessels and tissue penetration of fluorescein as a marker of vessel permeability.
5. Obtain these vessel parameters after administration of an IV fluid bolus and/or vasopressor administration (phenylephrine or vasopressin) during the normal course of the surgical resection.

Secondary objective(s):

1. Compare the microscopic observation of the tumor-associated vessels with normal tissue (e.g. peritoneal surface) in each individual subject.
2. Correlate the microscopic observations of the tumor-associated vessels with pathologic grade of tumor.
3. To correlate the microscopic observation of the microvasculature with tumor-specific and overall survival.

Study Design: This is a non-randomized, single center, study of HIVM observation in subjects with deep space solid tumors undergoing surgical resection.

All subjects will meet the inclusion and exclusion criteria prior to participation in this study. Standard evaluation by the Preoperative Anesthesia clinic at Mayo Clinic Florida may include additional testing such as EKG, chest x-ray, and/or pulmonary function tests.

The study will be performed in two parts. Part 1 will enroll 10 patients with an expected feasibility rate of 70%. Whereas the feasibility rate in primary melanoma was 90%, the expected feasibility rate for deep space tumors may be lower as the site is in an anatomically deeper location. If Part 1 is not met (i.e. in three or more patients), the primary objective would not be considered technically feasible, and then the study will be stopped. If Part 1 is fulfilled, then the study will continue with an additional 40 patients enrolled (total of 50 patients). Accrual is expected to take 2.5 years.

Inclusion criteria:

1. Age $\geq$ 18 years of age.
2. ECOG Performance Status of $\leq$ 2.
3. Measurable tumor by direct visualization requiring surgical resection in the OR.

4. Tumor types of origin include gastric, pancreatic, hepatobiliary, colorectal, and sarcoma. Tumors may be primary or metastatic to solid or hollow intra-abdominal organs.
5. Subject must understand the investigational nature of this study and sign an Independent Ethics Committee/Institutional Review Board approved written informed consent.
6. Subject must have a skin prick test pre-operatively (at the time of the preoperative visit and after signed informed consent for entry into this clinical trial is given) to determine any sensitivity to fluorescein.

Exclusion criteria:

1. Uncontrolled intercurrent illness including, but not limited to, ongoing or active infection, symptomatic congestive heart failure, unstable angina pectoris, cardiac arrhythmia, or psychiatric illness/social situations.
2. Renal dysfunction as defined as a GFR < 45.
3. Liver dysfunction as defined by Child-Pugh score > 5, or LFT's 1.5x above normal range.
4. Any known allergy or prior reaction to fluorescein or a positive skin prick test to fluorescein.
5. Pregnant or nursing female subjects, determined preoperatively with a urine pregnancy test.
6. Unwilling or unable to follow protocol requirements.
7. Any condition which in the Investigators' opinion deems the patient unsuitable (e.g., abnormal EKG, including T wave inversion, elevated T waves, prolonged QRS interval, or conduction blocks) or that requires further work-up (including cardiac echo or stress test).
8. Any condition that excludes surgery as the standard of care (e.g. high disease burden where alternative treatments like systemic chemotherapy would be preferred).

Primary and Secondary Endpoints/Criteria for Evaluation:

Primary endpoint: A patient will be deemed a success if each of the following parameters were measured:

1. Identify tumor associated vessels and measure vessel diameters
2. Determine vessel density per 10x field
3. Visualize vital dyes within the tumor-associated vessels (fluorescein)
4. Calculate the blood flow velocity of the tumor-associated vessels and tissue penetration of fluorescein as a marker of vessel permeability.

Measurements will be obtained before and after any tumor vessel manipulation with either bolus fluids or vasopressors.

Secondary endpoints:

1. Post-operative comparison of the microvasculature of tumor with normal tissue (e.g. peritoneum) in each individual subject using vessel diameters, vessel density, detection of intravital dye and flow rates.
2. Post-operative correlation of the microvasculature with pathologic features of the tumor (i.e. tumor grade) at the time of the final pathology report (5-7 days after surgery).

3. Post-operative correlation of the microscopic observation of the tumor microvasculature tumor-specific and overall survival.

Subject Name: _____

Medical Record No.: _____

Title: Intravital Microscopy (IVM) in Human Solid Tumors

INCLUSION CRITERIA				
Y	**N**	**N/A**	**All answers must be "YES or "N/A" for subject enrollment.**	**Date**
☐	☐	☐	1. Age ≥ 18 years of age.	
☐	☐	☐	2. Have an ECOG Performance Status of ≤ 2. Refer to Appendix B.	
☐	☐	☐	3. Have measurable disease by direct visualization (visible lesion typically > 0.5 cm in maximal diameter).	
☐	☐	☐	4. Deep space tumor that meets indications for resection.	
☐	☐	☐	5. Subject must understand the investigational nature of this study and sign an Independent Ethics Committee/Institutional Review Board approved written informed consent form prior to receiving any study related procedure.	
☐	☐	☐	6. A negative skin-prick test to fluorescein.	

EXCLUSION CRITERIA				
Y	**N**	**N/A**	**All answers must be "NO" or "N/A" for subject enrollment**	**Date**
☐	☐	☐	1. Uncontrolled intercurrent illness including, but not limited to, ongoing or active infection, symptomatic congestive heart failure, unstable angina pectoris, cardiac arrhythmia, or psychiatric illness/social situations.	
☐	☐	☐	2. Renal dysfunction as defined as a GFR < 45.	
☐	☐	☐	3. Liver dysfunction as defined by Child-Pugh score > 5, or LFT's 1.5x above normal range.	
☐	☐	☐	4. Any known allergy or prior reaction to fluorescein or a positive skin prick test to fluorescein.	
☐	☐	☐	5. Pregnant or nursing female subjects, determined preoperatively with a urine pregnancy test.	
☐	☐	☐	6. Unwilling or unable to follow protocol requirements.	
☐	☐	☐	7. Any condition which in the Investigators' opinion deems the patient unsuitable (e.g., abnormal EKG).	
☐	☐	☐	8. Any condition that excludes surgical resection as the standard of care for the patient.	

Study participant meets all entry criteria: ☐ **Yes** ☐ **No**

Investigator Signature: _____ Date: _____

1 BACKGROUND

Intravital microscopy (IVM) is the microscopic observation of living tissue in real time. IVM has been successfully applied to the investigation of the tumor microvasculature of human primary melanoma.[3] It has been shown that tumor vessels lack the sequential hierarchy of normal vessels such that arterioles, capillaries, and venules typically cannot be discriminated within tumor tissues.[4, 5] This disorganization of aberrant tumor vessels was demonstrated in a previous feasibility clinical trial.[2] These tumor-associated vessels were characterized by irregular diameters, aberrant branching patterns, abnormal blood flow rates, and anastomotic structures. More specifically, the human tumor-associated vessel diameters observed in real time *in vivo* were larger than those predicted either by *in vivo* mouse models or by human immunohistochemistry (IHC) on tumor specimens analyzed *ex vivo*.[3] These characteristics could have profound influence on the delivery of agents (i.e., chemotherapy or cellular immunotherapy) to the tumor microenvironment.[6]

In order to obtain this data, human intravital microscopy (HIVM) was used first in melanoma primary tumors. Intravital microscopy (IVM) has also been used to directly examine the hemodynamic properties of tumor microvessels in preclinical mouse models.[1, 7-9] These studies were primarily based on the analysis of blood vessels and leukocytes labeled *in situ* with fluorescent tracking dyes (e.g., fluorescein). Recent mouse studies in B16 murine melanoma have used IVM to demonstrate that the abnormal, tortuous vascular structure in tumors represents a bottleneck to adoptive cell transfer immunotherapy because of poor trafficking of cytotoxic effector T cells at this site.[1] Others have used different forms of IVM to observe lymphatics or metastatic disease in real time in mouse models.[10, 11] There has also been reported real time imaging of human tumor and lymphatics using different techniques such as multiphoton imaging, high resolution ultrasound, or optical coherence tomography.[12, 13] With support from the 2018 Mayo Clinic Florida Focused Research Teams (FRT) Award Program, our group is actively investigating HIVM for patients with peritoneal carcinomatosis through NCT03517852: Intravital Microscopy (IVM) in Patients with Peritoneal Carcinomatosis (PC). However, there have been no studies of IVM in the setting of deep space solid tumors.

The detection of blood flow parameters (including vessel diameter, flow rates, vessel density, and fluorescent markers of tissue diffusion) in deep space solid tumors of patients may have utility in characterizing the clinical response of these patients to systemic therapies. In addition, because many of these patients require intraoperative fluid boluses and vasopressor support, both of which are known to alter blood flow dynamics, the use of real time HIVM may reveal the ability to control tumor-associated vessels. It is anticipated that this approach has the potential to generate a more complete picture of the tumor-associated locoregional vasculature. The investigation of tumor-associated vessels in real time may lead to a better understanding of factors influencing systemic drug efficacy. Reflective of the study's two specific aims, our approach has the strong potential to expand upon novel tumor imaging techniques and not only form a directly observed basis of the tumor vasculature as a barrier to systemic drug efficacy in humans, but also establish a rationale means to overcome this barrier through standard intraoperative practice.

1.1 DIAGNOSTIC INTERVENTION - FLUORESCEIN

Drug Information - Fluorescein is delivered intravenously during the course of this observation. Fluorescein is an FDA approved drug for visual angiography. The package insert is shown in Appendix A. A single dose consists of a 5 mL vial of 10 or 25% fluorescein and costs $3.90.

Fluorescein has a very rare incidence of anaphylactic reaction (1 in 222,000), but a skin-prick test may allow these patients to be recognized.[14] Therefore, any reported sensitivity to fluorescein will be considered an exclusion criteria as well as a positive skin-prick test performed during the routine preoperative visit (at least 1 week prior to surgery). The skin-prick test will be performed at the time of the preoperative visit if the patient decides to enter the clinical trial and signs the informed consent. Patients that are not eligible for the study or do not give consent to participate in the study will not undergo a skin-prick test.

The skin-prick test will be performed by the Principal Investigator using a sterile Duotip® lancet (Lincoln Medical, USA) in 2 separate areas of skin on the forearm. The skin-pricks will include 4 microliters of 10% fluorescein administered in areas of skin prepped with an alcohol pad. After 30 minutes, the appearance of a wheal greater than 3 mm in diameter is considered a positive test result. If a positive test is noted, the patient will no longer be eligible for this study. For comfort and relief of any skin irritation/pruritis, the patient will be offered an anti-histamine (fexofenadine [Allegra®] 30 mg po x 1) following the skin-prick test if necessary.

The package insert for fluorescein (which is a commonly used agent in the OR and readily available to the anesthesiologist in their PYXIS system and does not require delivery from pharmacy or an order to pharmacy) is attached in the Appendix section.

The fluorescein will be provided by the study. It will be ordered and sent to surgery for the patient at the time of the planned microscopic observation.

1.1.1 PRECLINICAL STUDIES WITH FLUORESCEIN

Murine tumors have been investigated with the use of intravital microscopy in an attempt to evaluate blood vessel characteristics, blood flow patterns, and interactions with immune cell subsets.[1, 7, 9] Fluorescein conjugated to Dextran has been employed for several intravital microscopy studies[1, 15] and allows for a more precise assessment of the tumor vasculature[1, 7].

Preclinical laboratory studies have established that fluorescein can be used to image the microvasculature in B16 mouse melanoma tumors in the similar doses used clinically. Fluorescein is not expected to have a direct intervention on tumor biology. The ability to study tumor microvasculature by using IVM does not require a window chamber, and human IVM techniques have provided highly reproducible and consistent measurements. There have been several published animal studies which have demonstrated IVM of the lymphatic system in addition to blood vessels.[10, 16]

1.1.2 CLINICAL STUDIES WITH FLUORESCEIN

This protocol intends to apply the commonly used intraoperative fluorescent agent, fluorescein, to further characterize blood vessels associated with deep space solid tumors. It is anticipated that fluorescein will be readily detectable in the microscopic field of observation following intravenous fluorescein injection.

Fluorescein is not expected to have a direct intervention on tumor biology. Fluorescein is routinely used during the course of surgery for a variety of indications (e.g. intraoperative assessment of bowel or organ perfusion, vessel angiography of the retina) and is deemed non-toxic with no long term side effects.

Clinically, fluorescein is most commonly used for ophthalmologic procedures including fluorescein angiography with intravenous injection. Fluorescein angiography is performed by injecting fluorescein sodium dye as a bolus into a peripheral vein. The normal adult dosage is 500 mg, and is typically packaged in doses of 5 mL of 10% or 2 mL of either 10 or 25% concentration. For pediatric patients, the dose is adjusted to 35 mg per 10 pounds of body weight.[17]

Upon entering the circulation, approximately 80% of the dye molecules bind to plasma proteins, which significantly reduces fluorescence because the free electrons that form this chemical bond are subsequently unavailable for excitation.[18] The remaining unbound or free fluorescein molecules fluoresce when excited with light of the appropriate wavelength. With a molecular weight of 376, fluorescein diffuses freely out of all capillaries except those of the central nervous system, including the retina.

The dye is metabolized by the kidneys and is eliminated through the urine within 24 to 36 hours of administration. During this period of metabolism and elimination, fluorescein has the potential to interfere with clinical laboratory tests that use fluorescence as a diagnostic marker.[19, 20] To avoid any false readings, it may be prudent to schedule clinical lab tests either before the angiogram, or postpone testing for 1 or 2 days to allow sufficient elimination of the dye. Side effects of intravenous fluorescein include discoloration of the urine for 24 to 36 hours and a slight yellow skin discoloration that fades within a few hours. Nursing mothers should be cautioned that fluorescein is also excreted in human milk,[21] for this reason, nursing mothers will be excluded from this study.

Use of fluorescein sodium may be contraindicated in patients with history of allergic hypersensitivity to fluorescein. Although generally considered safe for patients receiving dialysis, one manufacturer of fluorescein suggests using half the normal dose in dialyzed patients.[22] There are no known risks or adverse reactions associated with pregnancy, but most practitioners avoid performing fluorescein angiography in pregnant women, especially in their first trimester.[23-25]

Historically, adverse reactions occur in 5% - 10% of patients and range from mild to severe.[27-32] Anecdotal evidence suggests a lower incidence of reaction in recent years and the first large study conducted in over a decade seems to confirm that, reporting a frequency of adverse reaction of just over 1%.[33] Continued improvements in manufacturing processes and implementation of tighter pharmacopeial standards are credited with this reduction and may lead to lower rates of reaction in the future.[34]

Transient nausea and occasional vomiting are the most common reactions and require no treatment. These mild reactions typically occur 30 – 60 seconds after injection and last for about 1 to 2 minutes. Fortunately, they seldom compromise the diagnostic quality of the angiogram. The incidence of nausea and vomiting seems to be related to the volume of dye and rate of injection. A relatively slow rate of injection often reduces or eliminates this type of reaction but can adversely affect image quality and alter arm-to-retina circulation times. Premedication with promethazine hydrochloride or prochlorperazine may prevent or lessen the severity of nausea and vomiting in patients with a history of previous reactions to fluorescein, but is rarely needed and one study noted a higher frequency of these reactions in patients that had been premedicated.[30] Some patients report a strong taste sensation or hypersalivation following injection of fluorescein.

Moderate reactions occur less frequently, affecting less than 2% of patients that undergo angiography. Allergic reactions such as pruritus or urticaria can be treated with antihistamines, but any patient who experiences these symptoms should be observed carefully for the possible development of anaphylaxis. The advisability of performing angiograms in patients with a history of allergic reaction to fluorescein should be considered carefully, as allergic sensitization to the dye can increase with each subsequent use. Patients with previous history of mild allergic reaction to fluorescein can be pre-treated with an antihistamine, such as diphenhydramine, 30 - 40 minutes prior to any subsequent angiograms to limit allergic response, although this may not prevent serious reactions.[35]

More severe reactions are rare, but include laryngeal edema, bronchospasm, anaphylaxis, tonic-clonic seizure, myocardial infarction, and cardiac arrest.[36-40] The overall risk of death from fluorescein angiography has been reported as 1 in 222,000 due to anaphylaxis.[30] Although life-threatening reactions during angiography are rare, angiographic facilities and personnel should be properly equipped and prepared to manage serious reactions to the procedure. A resuscitative crash cart and appropriate agents to treat severe reactions should be readily available including epinephrine for intravenous or intramuscular use, soluble corticosteroids, aminophylline for intravenous use, oxygen, and airway instrumentation. It is generally recommended that a physician be present or available during angiographic procedures.

Extravasation of fluorescein dye during the injection can be a serious complication of angiography. With a pH of 8 to 9.8, fluorescein infiltration can be quite painful. If fluorescein dye extravasates, cold compresses should be placed on the affected area for 5 to 10 minutes, and the patient should be reassessed until edema, pain, and redness resolve. Serious complications are more likely to occur when large amounts of dye extravasate. Sloughing of the skin, localized

necrosis, subcutaneous granuloma, and toxic neuritis have been reported following extravasation of fluorescein.[41-43] To avoid these problems, continual observation of the injection site during the course of the injection and monitoring the patient for pain is recommended. Accidental arterial injections are rare, but can be quite painful. The dye remains concentrated and stains the effected extremity with little or no dye reaching the retinal vasculature. With proper technique, these complications of injection can usually be avoided.

1.2 RISKS AND/OR BENEFITS

Risks – low risk of allergic reaction to fluorescein as described above. Otherwise, no increased risk of surgical procedure anticipated.

Benefits – depending upon collected findings and correlation with clinical outcome, HIVM may offer prognostic information and potentially guide treatment decisions in the future.

2 RATIONALE

These studies will elucidate the hemodynamic properties of microvessels associated with deep space solid tumors. In order to model the effects of tumor-associated vessel function on patient outcomes, direct examination of the microvasculature in these patients is necessary to ascertain if the preclinical model systems have accurately recapitulated the clinical setting. Thus, the current proposed trial represents an opportunity to support the development of interventions to improve patient treatment by extending the application of IVM to the locoregional tumor microenvironment. In addition, by adding HIVM observations during the course of volume and pressor management during the course of surgery, further data regarding tumor vessel dynamics will obtained, which may offer a means to augment responses to systemic treatments.

3 OBJECTIVES

3.1 PRIMARY OBJECTIVE - PART I (10 PATIENTS):

To determine the feasibility of performing HIVM in patients with deep space solid tumors during the standard course of surgical treatment (resection). A successful intravital microscopic observation will include the ability to complete each of the following:

1. Identify and measure vessels associated with tumor
2. Determine vessel density per 10x field
3. Visualize vital dyes within the vessels (fluorescein)
4. Calculate the blood flow velocity of the vessels and tissue penetration of as a marker of vessel permeability.

3.2 SECONDARY OBJECTIVES - PART II (40 PATIENTS):

1. Compare the microscopic observation of the tumor-associated vessels with normal tissue (peritoneal surface) in each individual subject.
2. Correlate the microscopic observations of the tumor-associated vessels with pathologic grade of tumor.
3. To correlate the microscopic observation of the microvasculature with tumor-specific and overall survival.

3.3 PRIMARY AND SECONDARY ENDPOINTS:

Primary endpoint: A patient will be deemed a success if each of the following parameters were measured:

1. Identify tumor associated vessels and measure vessel diameters
2. Determine vessel density per 10x field
3. Visualize vital dyes within the tumor-associated vessels (fluorescein)
4. Calculate the blood flow velocity of the tumor-associated vessels and tissue penetration of fluorescein as a marker of vessel permeability.

Measurements will be obtained before and after any tumor vessel manipulation with either bolus fluids or vasopressors.

Secondary endpoints:

1. Post-operative comparison of the microvasculature of tumor with normal tissue (e.g. peritoneum) in each individual subject using vessel diameters, vessel density, detection of intravital dye and flow rates.
2. Post-operative correlation of the microvasculature with pathologic features of the tumor (i.e. tumor grade) at the time of the final pathology report (5-7 days after surgery).
3. Post-operative correlation of the microscopic observation of the tumor microvasculature tumor-specific and overall survival.

4 METHODOLOGY

4.1 Study Design

This is an open-label, non-randomized, single center, study of IVM observation in conjunction with fluorescein in subjects with deep space solid tumors undergoing surgical resection. The first part is a Pilot study of feasibility.

Subjects will be treated on an inpatient basis. All subjects will meet the inclusion and exclusion criteria summarized in **Section 5.1** and **Section 5.2** prior to participation in this study. Standard evaluation by the Preoperative Anesthesia clinic may include additional testing such as EKG, chest x-ray, and/or pulmonary function tests, but this is performed based upon their established

guidelines. If any of these tests preclude a surgery in the operating room, then the patient is excluded from this study.

4.2 STUDY METHODS

During the course of treatment for deep space solid tumors while the patient is under anesthesia, surgical resection will be performed. Approach to surgery may include open, laparoscopic, or robotic, as the HIVM microscope can be used through any of these approaches.

The IVM technology utilized at Mayo Clinic currently consists of a high resolution confocal endomicroscope (Appendix C). This apparatus has the ability to provide single cell resolution and high quality images of the microvasculature. While the typical application of this device is to investigate GI mucosal surfaces (i.e. esophagus or colon), it can be readily and easily applied to any surface. It can be sterilized via standard STERRAD techniques or used with a sterile sleeve in order to interface with the peritoneal surface, as currently used in NCT03517852: Intravital Microscopy (IVM) in Patients with Peritoneal Carcinomatosis (PC).

The microscope will be moved into position next to the OR table by the operating surgeon after the tumor is exposed. The microscope will be covered with a standard sterile drape similar to that used for cords associated with other intraoperative devices (NeoProbe or intraoperative ultrasound). The only exposed area will be the microscopic objective located at the tip of the endoscope, which will be made sterile along with the entire probe. This microscopic objective will come into close contact with the peritoneal surface for microscopic observation. Once the microscopic objective is in proper position, the epifluorescent light source will be turned on and digital video recording will commence. All video images will be captured to the hardware directly attached to the microscope (Appendix C). All data will be backed up on a password-protected hard drive in the Principal Investigator's laboratory.

1-2 mL of 10 or 25% fluorescein will be injected intravenously and observation will continue until loss of fluorescence over 2-4 areas of both grossly normal and tumor tissue. IVM is continuously performed at the time the fluorescent agent is injected and video is recorded in real-time. The fluorescein is almost immediately visible within the microscopic field, and the vessels are quickly outlined in great detail. The fluorescence lasts for a few minutes (2-3 minutes) and then either fades or begins to permeate through the tissue. The extravasation of dye will be determined by visualizing fluorescence outside of the defined vessels for a total time of 5 minutes. The distance from the vessels will be measured, recorded, and expressed as a function of time (extravasation rate) in an analysis performed off-line from the digitally recorded video. On average, the total obervation time per field is 1-2 minutes.

During the course of the surgery, if fluid boluses and/or vasopressors (e.g.vasopressin and/or phenylephrine) are required and administered to maintain blood pressure during the resection, another round of IVM observations will be performed with a second dose of 1-2 mL of fluorescein. Communication with anesthesia will indicate when fluid boluses and/or vasopressors are administered, in order to coordinate the observation.

At the completion of the entire observation period, the microscope will be removed and sterilized between uses. This would complete the observation period, which is anticipated to take 10 – 12 minutes total time. The observation will take place during the course of standard surgery and is not anticipated to delay or lengthen the duration of the procedure. In fact, during this observation period, other portions of the surgical procedure will be performed, including pathologic biopsy assessment, margin assessment, intra-abdominal irrigation, drain placement and securement, implantation of hemostatic agents (Surgicel, Tissell spray, GelFoam, etc), or in the case of more than one tumor or satellite tumors, additional dissection of the remaining tumors. The patient will recover in the PACU per standard protocol. The patient will be monitored for any allergic reactions and/or side effects to fluorescein administration and treated appropriately as described in previous sections.

Technical difficulties with the microscope apparatus (e.g. malfunction of the software or structural damage to any of the microscope components) or unforeseen events during the course of surgery that are unrelated to the study intervention but that result in the termination of the surgery (e.g. adverse reaction to anesthetic prior to administration of fluorescein or hemodynamic instability from a complication of the surgery) will not be considered failures of the primary objective. In these circumstances, the subject will be considered to be off-study and be replaced with a subsequent patient. If vasopressors are not administered during the course of the surgery, the patient data will still be included in the study as it addresses Aim 1.

Off-line analysis of digitally recorded live video will be performed using parameters and statistical methods that have been developed in our preclinical imaging studies.[1] Lumenal cross-sectional diameter (D) of vessels and velocity (V) of dye labeled cells will be measured in off-line observations. Wall shear rate (γ) will be calculated as $8 (V \div D)$.[44] Vessel density will be determined by measuring the calculated blood vessel area as a percent of the total visual field area. If visible, the uptake of fluorescein will be measured as a diffusion rate (distance from tumor vessel over time) and as a percent of the total tumor field observed (percent of visual field expressed as surface area with dye detected). Blood flow velocity will be determined by the equation $Q = (\text{RBC velocity}/1.6) \times (d/2) \times \text{pi}$. Software is available from Mauna Kea, which can analyze and calculate these data.

These data will be calculated and recorded in a spread sheet that will also be saved onto a password-protected hard drive in the Principal Investigator's laboratory. Additionally, all data will be de-identified and provided to the Biostatistician. Generated data and the raw video images will be stored for review at any time to satisfy any audit of the collected data. This saved data will readily allow the determination of a successful observation including the identification of tumor vessels, the associated vessel measurements, vessel density, and dye enhanced imaging acquisition.

TARGET ACCRUAL AND STUDY DURATION

A maximum of 50 subjects [Part I (10 patients) and Part II (40 patients)] will be enrolled. The number of subjects required is a function of the expected feasibility. Accrual is expected to take up to 2.5 years.

5 SUBJECT SELECTION

Patients who are scheduled to undergo surgical resection in the operating room who can provide informed consent, have a good performance status (ECOG ≤ 2), and have no known allergy or prior reaction to fluorescein, are eligible.

5.1 INCLUSION CRITERIA

1. Age ≥ 18 years of age.
2. Have an ECOG Performance Status of ≤ 2. Refer to Appendix B.
3. Have measurable tumor by direct visualization (visible lesion typically > 0.5 cm in maximal diameter).
4. Deep space tumor that meets indications for resection.
5. Subject must understand the investigational nature of this study and sign an Independent Ethics Committee/Institutional Review Board approved written informed consent form prior to receiving any study related procedure.
6. A negative skin-prick test to fluorescein.

5.2 EXCLUSION CRITERIA

1. Uncontrolled intercurrent illness including, but not limited to, ongoing or active infection, symptomatic congestive heart failure, unstable angina pectoris, cardiac arrhythmia, or psychiatric illness/social situations.
2. Renal dysfunction as defined as a GFR < 45.
3. Liver dysfunction as defined by Child-Pugh score > 5, or LFT's 1.5x above normal range.
4. Any known allergy or prior reaction to fluorescein or a positive skin prick test to fluorescein.
5. Pregnant or nursing female subjects, determined preoperatively with a urine pregnancy test.

6. Unwilling or unable to follow protocol requirements.
7. Any condition which in the Investigators' opinion deems the patient unsuitable (e.g., abnormal EKG).
8. Any condition that excludes surgical resection as the standard of care for the patient.

5.3 INCLUSION OF WOMEN AND MINORITIES

Both men and women and members of all races and ethnic groups are eligible for this study.

5.4 SUBJECT WITHDRAWAL

Any time prior to the planned surgical procedure, the patient may withdraw from this observational study for any reason without prejudice.

6 STUDY PROCEDURES

6.1 BASELINE EVALUATIONS

Standard presurgical assessment with labs and studies as determined by the preoperative Anesthesia clinic based upon their established guidelines.

The following will be performed within 1-4 weeks prior to the planned microscopic observation:

- Informed consent: Must be completed prior to receiving any study-related procedures.
- Medical history
- Physical examination, including vital signs (i.e., temperature, heart rate, respiratory rate, blood pressure, body weight, and height).
- If determined to be eligible for the study and after giving informed consent, a skin-prick test to determine sensitivity and risk of anaphylaxis to fluorescein will be performed by the Principal Investigator. For testing allergic response to fluorescein, a sterile Duotip® lancet (Lincoln Medical, USA) is used in 2 separate areas of skin on the forearm. The skin-pricks will include approximately 4 microliters of 10% or 25% fluorescein administered in areas of skin prepped with an alcohol pad. After 30 minutes, the appearance of a wheal greater than 3 mm in diameter is considered a positive test result. If a positive test is noted, the patient will no longer be eligible for this study. For comfort and relief of any skin irritation/pruritis, the patient will be offered an anti-histamine (fexofenadine [Allegra®] 30 mg po x 1) following the skin-prick test if necessary.
- If indicated by anesthesia pre-op assessment (standard operating procedure for pre-op clinic) - Hematology [i.e., CBC (with or without differential, auto or manual), ANC, and platelets].

- A complete metabolic panel (CMP): chloride, Co2, potassium, sodium, BUN, glucose, calcium, creatinine, total protein, albumin, total bilirubin, alkaline phosphatase, AST, ALT, A/G ratio, BUN/creatinine ratio, osmol (Calc), anion gap).
- If indicated by anesthesia pre-op assessment (standard operating procedure for the pre-op clinic) – 12-lead electrocardiogram, chest x-ray, and/or pulmonary function tests if indicated. These tests are not strictly required to support eligibility for this study, but if indicated and performed, may exclude the patient from receiving a general anesthetic and therefore, a wide excision performed in the operating room (thereby excluding the patient from this study).
- Pregnancy test (urine) in females of childbearing potential. Females who have undergone surgical sterilization or who have been postmenopausal for at least 2 years are not considered to be of childbearing potential. The standard operating procedure for all females of childbearing potential is to obtain a urine HCG (or a serum HCG if urine unable to be obtained). If the pregnancy test is positive, the patient is excluded from the study.

6.2 EVALUATIONS PERFORMED AT END OF TREATMENT

- Standard Post Anesthesia Care Unit (PACU) protocols for patient recovery from surgery. Patients will be continuously monitored for any reaction to fluorescein both intraoperatively and during the recovery period. Adverse events will be recorded throughout the time of entry into the operating room until the post-operative follow up. If the patient is transported directly to the Intensive Care Unit (ICU), these protocols will also be performed.

6.3 POST-TREATMENT FOLLOW-UP EVALUATIONS

- Standard follow-up and safety evaluations from surgery includes a post-op visit at 2-3 weeks and scheduled follow-up based upon final staging of the patient's cancer. Follow-up will be based upon current NCCN guidelines.

Study Calendar

	Pre-operative Visit	Surgery	Post-operative Visit
History and Physical	X		
Informed consent	X		
Fluorescein skin prick test	X		
Tests as medically indicated	X		

Surgical resection with IVM		X	
Post-surgical monitoring in recovery and inpatient stay		X	
Review of pathology			X
Routine cancer surveillance			X

- At the completion of the surgery, if gross tumor is entirely debulked (R0 resection), then patients are considered disease free. Treatment response will be based upon the presence of a recurrence of tumor at either the primary site, locoregional recurrence (peritoneum), or metastatic sites.
- The time to recurrence will determine the standard length of clinical follow up. However, for this study, a 10-year limit will be placed on clinical data collection during the standard clinical follow up. After a patient is enrolled, the duration of data collection will end at 10 years from the time of microscopic observation (surgery). These data (time of recurrence and/or survival) will be correlated with findings from the one-time, initial IVM observation for the defined 10-year period of data collection.
- The surveillance data (no evidence of disease, disease recurrence, death) will be stored in a secured database and matched to the microscopic observation data. These data will serve as the basis for statistical comparisons of microscopic findings and clinical outcomes to be performed by our study statistician as described in section 9.

6.4 PATHOLOGY

Standard pathologic evaluation (no deviation from current standard practice of pathology department) will be performed.

7 SAFETY EVALUATION

7.1 ADVERSE EVENTS

7.1.1 DEFINITION

An adverse event or adverse experience (AE) is any untoward medical occurrence associated with the use of a drug in humans, whether or not considered drug related. Therefore, an AE can be **ANY** unfavorable and unintended sign (including an abnormal laboratory finding), symptom,

or disease temporally associated with the use of a medicinal (investigational) product, whether or not considered related to the medicinal (investigational) product (attribution of 'unrelated', 'unlikely', 'possible', 'probable', or 'definite').

An AE is considered "unexpected" if it is not listed in the investigator brochure or is not listed at the specificity or severity that has been observed; or if an investigator brochure is not required or available, is not consistent with the risk information described in the general investigational plan in other study-related documents. "Unexpected" also refers to adverse events that are mentioned in the investigator brochure (or other study-related documents) as occurring with a class of drugs or as anticipated from the pharmacological properties of the drug, but are not specifically mentioned as occurring with the particular study drug.

Adverse events will be recorded in this study for the purpose of safety monitoring and adverse events are anticipated to be absent or unlikely.

7.1.2 GRADING AND RELATIONSHIP TO DRUG

The descriptions and grading scales found in the CTEP Version 4 of the NCI Common Terminology Criteria for Adverse Events (CTCAE) will be utilized for AE reporting. CTEP Version 4 of the CTCAE is identified and located at: http://ctep.cancer.gov/protocolDevelopment/electronic_applications/ctc.htm. AEs not covered by specific criteria should be reported with common medical terminology and graded according to definitions provided in the CTCAE Version 4.

AEs NOT listed in the NCI-CTCAE, the severity of AEs will be graded as follows:

- **Grade 1 – Mild:** Event barely noticeable to subject; no limitation in activity, no minimal medical intervention/therapy required.
- **Grade 2 – Moderate:** Event of a sufficient severity to make participant uncomfortable. ADL limited; no or minimal medical intervention/therapy required.
- **Grade 3 – Severe:** Event causes significant discomfort/limitation in activity. May be such severity that the subject cannot continue on study. Severity may cause cessation of treatment. Treatment for symptoms may be given and/or subject hospitalized.
- **Grade 4 - Life Threatening:** Event places the participant at immediate risk of death from the reaction as it occurred. Significant medical intervention/therapy required; hospitalization or hospice care probably.
- **Grade 5 - Death**.

The relationship of event to study drug will be documented by the Investigator as follows:

- **Unrelated:** The event is clearly related to other factors such as the subject's clinical state, other therapeutic interventions or concomitant drugs administered to the subject.

- **Unlikely:** The event is doubtfully related to investigational agent(s). The event was most likely related to other factors such as the subject's clinical state, other therapeutic interventions, or concomitant drugs.
- **Possible:** The event follows a reasonable temporal sequence from the time of drug administration, but could have been produced by other factors such as the subject's clinical state, other therapeutic interventions or concomitant drugs.
- **Probable:** The event follows a reasonable temporal sequence from the time of drug administration, and follows a known response pattern to the study drug. The event cannot be reasonably explained by other factors such as the subject's clinical state, therapeutic interventions or concomitant drugs.
- **Definite:** The event follows a reasonable temporal sequence from the time of drug administration, follows a known response pattern to the study drug, cannot be reasonably explained by other factors such as the subject's condition, therapeutic interventions or concomitant drugs; AND occurs immediately following study drug administration, improves upon stopping the drug, or reappears on re-exposure.

A suspected adverse reaction is any AE for which there is a reasonable possibility that the drug caused the adverse event. Reasonable possibility means there is evidence to suggest a causal relationship between the drug and the adverse event, such as:

- A single occurrence of an event that is uncommon and known to be strongly associated with drug exposure (e.g., angioedema, hepatic injury, Stevens-Johnson Syndrome).
- One or more occurrences of an event that is not commonly associated with drug exposure, but is otherwise uncommon in the population exposed to the drug.
- An aggregate analysis of specific events observed in a clinical research study that indicates those events occur more frequently in the drug treatment group than in a concurrent or historical control group.

An AE for which there is a reasonable possibility that the drug caused the adverse event includes those assessed as having a Possible, Probable, or Definite causality assessment.

7.1.3 REPORTING ADVERSE EVENTS

Table 1. Guidelines for Routine Adverse Event Reporting for Pilot Studies (Regardless of Expectedness)

Attribution	Grade 1	Grade 2	Grade 3	Grade 4	Grade 5
Unrelated	X	X	X	X	X
Unlikely	X	X	X	X	X
Possible	X	X	X	X	X
Probable	X	X	X	X	X
Definite	X	X	X	X	X

All subjects are evaluated at baseline and any abnormalities are documented.

- Worsening abnormalities and/or worsening of a pre-existing condition (e.g., diabetes, migraine that has increased in severity, frequency or duration of the condition, or an association with significantly worse outcome) can become an AE or serious adverse event and must be documented and followed.
- A persistent AE is one that extends continuously, without resolution between treatment cycles/courses. Reporting of persistent AEs is as follows:
    a) Routine reporting: The AE must be reported only once unless the grade becomes more severe in a subsequent cycle/course. If the grade becomes more severe the AE must be reported again with the new grade.
    b) Expedited Reporting: the AE must be reported only once unless the grade becomes more severe in the same or a subsequent cycle/course.
- A recurrent AE is one that occurs and resolves during a cycle/course of therapy and then reoccurs in a later cycle/course. Reporting of recurrent AEs is as follows:
    a) Routine Reporting: An AE that resolves and then recurs during a subsequent cycle/course must be reported by the routine procedures.
    b) Expedited Reporting: An AE that resolves and then recurs during a subsequent cycle/course does not require reporting unless the grade increases and hospitalization is associated with the recurring AE.
- At each evaluation, the Investigator will determine whether any AEs have occurred by evaluating the subject's signs, symptoms, and current laboratory and/or other test results.
- All AEs (expected or unexpected), including worsening of a pre-existing conditions (increased severity, frequency or duration of the condition and/or associated with significantly worse outcomes) which occur during the specified collection period, whether observed by the Investigator or by the subject, and whether or not thought to be related to study drug, will be documented in detail and followed until resolution, stabilization, death, or the start of new treatment.
- Whenever possible record a diagnosis for signs and symptoms of a common syndrome (i.e., cough, sneezing, runny nose would be reported as an upper respiratory infection).

All AEs for Pilot studies must be entered on the Adverse Event eCRF page, using the pTrax database.

7.2 SERIOUS ADVERSE EVENTS

7.2.1 DEFINITION

A serious adverse event (SAE) is any adverse event (experience) that in the opinion of either the investigator or sponsor results in **ANY** of the following:

- Death.
- A life-threatening adverse event (experience). Any AE that places a subject or subject, in the view of the Investigator or sponsor, at immediate risk of death from the reaction as it occurred. It does **NOT** include an AE that, had it occurred in a more severe form, might have caused death.
- Inpatient hospitalization or prolongation of existing hospitalization (for > 24 hours).
- A persistent or significant incapacity or substantial disruption of the ability to conduct normal life functions.
- A congenital anomaly or birth defect.
- Important Medical Event (IME) that, based upon medical judgment, may jeopardize the subject or subject and may require medical or surgical intervention to prevent one of the outcomes listed above.

Elective hospital admission (including planned treatment for progressive disease or a planned protocol procedure), admission for socioeconomic reasons, and death due to tumor progression will not be considered an SAE.

All SAEs will be collected from the date of subject or subject signing consent until 30 days after the last dose of study drug and/or procedure.

7.2.2 REPORTING SERIOUS ADVERSE EVENTS

AE may be identified as an Unanticipated Problem by the Investigator. Please refer to **Section 7.3.2** for details on reporting Unanticipated Problems.

The SAE Form should be completed with all available information, including a brief narrative describing the adverse event and any other relevant information. If applicable, the narrative must also include identification of similar reports and an analysis of the significance of the AE.

7.2.3 FOLLOW-UP FOR SERIOUS ADVERSE EVENTS

All SAEs will be followed until resolution, stabilization, death, or the start of new treatment.

7.3 UNANTICIPATED PROBLEMS

7.3.1 DEFINITION

An Unanticipated Problem (UP) is any incident, experience, or outcome that meets all of the following criteria:

- Unexpected (in terms of nature, severity, or frequency) given:

  - The research procedures that are described in the study-related documents, including study deviations, as well as issues related to compromise of subject privacy or confidentiality of data.
  - The characteristics of the subject population being studied.
- Related or possibly related to participation in the research (possibly related means there is a reasonable possibility that the incident, experience, or outcome may have been caused by the procedures involved in the research).
- Suggests that the research places subjects or others at a greater risk of harm (including physical, psychological, economic, or social harm) than was previously known or recognized and if in relation to an AE is also deemed **Serious** per **Section 7.2**.

7.3.2 REPORTING UNANTICIPATED PROBLEMS

SAEs that are serious, unexpected and for which there is a reasonable possibility that the study drug caused the adverse event are reported on the Unanticipated Problem Form and submitted to CRS Compliance Office within 24 hours of becoming aware of the Unanticipated Problem.

Within 5 business days, an Unanticipated Problem Form should be completed with all available information, including a brief narrative describing the unanticipated problem and any other relevant information.

Within 5 business days of becoming aware of new information about the Unanticipated Problem, submit this updated information to CRS Compliance with an updated UP Form.

8 DATA AND SAFETY MONITORING

The Data Safety Monitoring Plan is attached in the IRB. The study members will meet monthly to ensure that standard procedures are being followed.

9 STATISTICAL METHODOLOGY

Initially ten patients will be treated to determine the feasibility of HIVM for deep space tumors. This first part of the study is a pilot study of feasibility. If seven or more patients are deemed a success by meeting all of the criteria below, then HIVM will be deemed feasible in the study of deep space tumors. Criteria include the ability to complete each of the following:

1. Identify and measure vessels associated with tumor
2. Determine vessel density per 10x field
3. Visualize vital dyes within the vessels (fluorescein)
4. Calculate the blood flow velocity of the vessels and tissue penetration of fluorescein as a marker of vessel permeability.

If the true success rate is 60%, the probability of deeming HIVM feasible is 63%; if the true success rate is 70%, then the probability of deeming HIVM feasible is 88%; and if the true success rate is 90%, then the probability of deeming HIVM feasible increases to 99%.

If HIVM is deemed feasible, then an additional 40 patients will be accrued to this study in order to address the following:

1. Post-operative comparison of the microvasculature of tumor with normal tissue (peritoneum) in each individual subject using vessel diameters, vessel density, detection of intravital dye and flow rates.
2. Post-operative correlation of the microvasculature with pathologic features of the tumor (i.e. tumor grade) at the time of the final pathology report (5-7 days after surgery).
3. Post-operative correlation of the microscopic observation of the tumor microvasculature tumor-specific and overall survival.

An abnormal HIVM observation is defined by any of the following criteria:

1. Aberrant vessel structure compared to normal tissue, which includes tortuosity of the vessel wall or a closed loop formation.
2. Aberrant vessel branching patterns compared to normal tissue, which includes erratic and disorganized branching points.
3. Blood vessel density that is less than or greater than one standard deviation of the blood vessel density observed in normal tissue.
4. Inability to detect flow or fluorescein within a vessel.
5. Average blood flow rate through observed vessel that is less than one standard deviation of the average blood flow rate through a normal vessel.

A matched analysis will be performed for observations of microvasculature between the tumor and normal tissue. For dichotomous variable comparisons between vessel structure (normal vs aberrant), vessel branching patterns (normal vs aberrant), or detection of intravital dye (present vs absent), a McNemar's test will be performed. For continuous variable comparisons between blood vessel density and average blood flow rate, a paired t test will be performed.

Associations between characteristics for the baseline (normal peritoneal microvasculature observed surrounding the tumor) and tumor itself will be depicted with graphics, correlations, and model results.

Similar analyses will be performed to compare baseline observations with those performed after fluid bolus and/or vasopressor administration.

Adverse event and complication rates will be described using upper one-sided 95% Clopper Pearson confidence limits.

Overall survival will be defined as the duration of time from diagnosis to time of death. Disease specific survival will be defined as the time from surgery to death due to recurrence. Overall survival and disease specific survival will be generated using standard Kaplan-Meier methodology.

A maximum of 50 subjects will be enrolled in this study (initially 10 to determine feasibility and then 40 additional subjects if deemed feasible). The accrual rate is estimated at 1-2 subjects per month, and the estimated study duration is 30 months (2.5 years).

9.1 RANDOMIZATION

This is a nonrandomized study. No randomization scheme will be generated. Subjects will be assigned to a treatment group based on their date of qualification for participation.

9.2 DEMOGRAPHICS AND BASELINE CHARACTERISTICS

Patients of all racial and socioeconomic demographics having met the criteria for study entry will be eligible for this protocol. Descriptive statistics (as appropriate: n, percent, mean, median, min and max) will be used to summarize demographic and baseline characteristics.

10 ETHICAL AND REGULATORY STANDARDS

10.1 ETHICAL PRINCIPLES

This study will not be initiated until the protocol and informed consent document(s) have been reviewed and approved by a properly constituted Institutional Review Board (IRB) or Independent Ethics Committee (IEC). Each subject (or legal guardian) shall read, understand, and sign an instrument of informed consent prior to performance of any study-specific procedure. It is the responsibility of the investigator to ensure that the subject is made aware of the investigational nature of the treatment and that informed consent is given.

The Investigator is responsible for the retention of the subject log and subject records; although personal information may be reviewed by authorized persons, that information will be treated as strictly confidential and will not be made publicly available. The investigator is also responsible for obtaining subject authorization to access medical records and other applicable study specific information according to Health Insurance Portability and Accountability Act regulations (where applicable).

This study will be conducted in compliance with all applicable laws and regulations of the state and/or country and institution where the subject is treated, in accordance with the Declaration of Helsinki, Good Clinical Practice, and according to the guidelines in this protocol, including attached appendices.

10.2 INFORMED CONSENT

The Investigator is responsible for obtaining written consent from each subject in accordance with ICH-GCP guidelines using the approved informed consent form, before any study specific procedures (including screening procedures) are performed. The informed consent form acknowledges all information that must be given to the subject according to ICH-GCP, including the purpose and nature of the study, the expected efficacy and possible side effects of the treatment(s), and specifying that refusal to participate will not influence further options for therapy. Any additional information that is applicable to the study must also be included. Additional national or institutionally mandated requirements for informed consent must also be adhered to. The subject should also be made aware that by signing the consent form, processing of sensitive clinical trial data and transfer to other countries for further processing is allowed.

The Investigator shall provide a copy of the signed consent form to the subject and the signed original shall be maintained in the Investigator File. A copy of the signed consent form must be filed in the subject file. At any stage, the subject may withdraw from the study and such a decision will not affect any further treatment options.

11 STUDY RESPONSIBILITIES

11.1 DATA COLLECTION

Data entry into the database is to be completed in a timely fashion (approximately within 28 days) after the subject's clinic visit. If an AE is considered serious it is captured on both the Adverse Event page and the Serious Adverse Event Form, which is handled in an expedited fashion.

Data management activities will be performed using PTrax. PTrax is a suite of software tools that enables the collection, cleaning and viewing of clinical trial data. CRS data management will design the study-specific database and facilitate its development by the PTrax Information Technology team. Once the database design is approved by the Investigator, Statistician, and Clinical Research Coordinator, the database will be put into production and data entry can begin. Data can be entered and changed only by those with the rights to do so into the eCRFs. PTrax is compliant with all relevant technical aspects of relevant GCP guidelines.

- The system can generate accurate copies of stored data and audit trail information in human readable form.
- System access is limited to authorized individuals through the controlled assignment of unique ID and password combinations.
- The system is designed to periodically force users to change their passwords and verifies that user ID and password combinations remain unique.
- The system automatically generates a permanent time-stamped audit trail of all user interactions.

When data entry is complete, data management will review the data and will query any missing, incomplete, or invalid data points for resolution by the Clinical Research Coordinator and Investigator. Once all queries have been resolved, the data can be released to the statistician for analysis.

11.2 MAINTENANCE OF STUDY DOCUMENTS

Essential documents should be retained for 6 years from the study termination date. These documents could be retained for a longer period, however, if required by the applicable local regulatory requirements or by an agreement with Mayo Clinic. If, for any reason, the Investigator desires to no longer maintain the study records, they may be transferred to another institution, another investigator, or to Mayo Clinic upon written agreement between the Investigator and Mayo Clinic.

12 ADMINISTRATIVE RULES

12.1 REVISIONS TO THE PROTOCOL

Mayo Clinic may make such changes to the protocol as it deems necessary for safety reasons or as may be required by the U.S. FDA or other regulatory agencies. Revisions will be submitted to the IRB/ERC for written approval before implementation.

12.2 TERMINATION OF THE STUDY

It is agreed that, for reasonable cause, either the Investigators or the Sponsor, Mayo Clinic may terminate this study, provided a written notice is submitted within the time period provided for in the Clinical Trial Agreement. In addition, Mayo Clinic may terminate the study at any time upon immediate notice if it believes termination is necessary for the safety of subjects enrolled in the study.

12.3 CONFIDENTIALITY

Any data, specimens, forms, reports, video recordings, and other records that leave the site will be identified only by a participant identification number (Participant ID, PID) to maintain confidentiality. All records will be kept in a limited access environment. All computer entry and networking programs will be done using PIDs only. Information will not be released without written authorization of the participant.

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14 APPENDICES

Appendix A: Fluorescein Drug Package Insert.

- AK-FLUOR® (fluorescein injection, USP) is a sterile solution for use intravenously as a diagnostic aid
- Indicated in diagnostic fluorescein angiography or angioscopy of the retina and iris vasculature
- Meets strict USP quality standards
- AK-FLUOR® is contraindicated in patients with known hypersensitivity to fluorescein sodium or any other ingredients in this product
- Rare cases of death due to anaphylaxis have been reported [see *Warnings and Precautions* (5.1) and *Adverse Reactions* (6.2)]
- Available direct or through your authorized wholesaler or distributor

AK-FLUOR® (fluorescein injection, USP) PRESERVATIVE FREE RSS Barcoded UPC Barcoded

NDC #	DESCRIPTION	SIZE	UNIT OF SALE	ORANGE BOOK CODE
17478-253-10	10% Single-dose Vial	5 mL	12	AP
17478-250-20	25% Single-dose Vial	2 mL	12	AP

EACH mL CONTAINS:	
ACTIVE:	17478-253-10: Fluorescein Sodium (equivalent to Fluorescein 10% w/v, 100 mg/mL); 17478-250-20: Fluorescein Sodium (equivalent to Fluorescein 25% w/v, 250 mg/mL);
PRESERVATIVE:	None;
INACTIVES:	Sodium Hydroxide and/or Hydrochloric Acid may be used to adjust pH (8.3 to 9.8), and Water for Injection.
STORAGE:	Store at 20° to 25°C (68° to 77°F). Do not freeze.

NDC #	CARDINAL	AMERISOURCEBERGEN	MCKESSON	MORRIS DICKSON
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17478-250-20	2119444	089-829	1257831	TBA

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HIGHLIGHTS OF PRESCRIBING INFORMATION

These highlights do not include all the information needed to use AK-FLUOR® 10% and AK-FLUOR® 25% safely and effectively. See full prescribing information for the products in AK-FLUOR® 10% and AK-FLUOR® 25%.

AK-FLUOR® (fluorescein injection, USP) 10%
AK-FLUOR® (fluorescein injection, USP) 25%
Intravenous Injection

Initial US Approval: 1976

----------INDICATIONS AND USAGE----------

AK-FLUOR® is indicated in diagnostic fluorescein angiography or angioscopy of the retina and iris vasculature. (1)

----------DOSAGE AND ADMINISTRATION----------

- The normal adult dose of AK-FLUOR® 10% is 5 mL (500 mg) and of AK-FLUOR® 25% is 2 mL (500 mg) via intravenous administration. (2.1)
- For children, the dose should be calculated on the basis of 35 mg for each ten pounds of body weight (7.7 mg/kg body weight). (2.2)
- Do not mix or dilute with other solutions or drugs. (2.2)

----------DOSAGE FORMS AND STRENGTHS----------

- AK-FLUOR® (fluorescein injection, USP) 10%, 100 mg/mL in a 5 mL single dose vial (3)
- AK-FLUOR® (fluorescein injection, USP) 25%, 250 mg/mL in a 2 mL single dose vial (3)

----------CONTRAINDICATIONS----------

- Hypersensitivity to any component of this product. (4.1)

----------WARNINGS AND PRECAUTIONS----------

- Respiratory reactions.(5.1)
- Severe local tissue damage. (5.2)

----------ADVERSE REACTIONS----------

The most common adverse reactions include skin discoloration, urine discoloration, nausea, vomiting, and gastrointestinal distress.

To report SUSPECTED ADVERSE REACTIONS, contact Akorn at 1-800-932-5676 or FDA at 1-800-FDA-1088 or www.fda.gov/medwatch.

----------USE IN SPECIFIC POPULATIONS----------

Caution should be exercised when fluorescein sodium is administered to a nursing woman. (8.3)

See 17 for PATIENT COUNSELING INFORMATION.

Revised: 07/2008

FULL PRESCRIBING INFORMATION:
CONTENTS*

*** Sections or subsections omitted from the Full Prescribing Information are not listed.**

FULL PRESCRIBING INFORMATION:

1 INDICATIONS AND USAGE

AK-FLUOR® 10% (100 mg/mL) and 25% (250 mg/mL) is indicated in diagnostic fluorescein angiography or angioscopy of the retina and iris vasculature.

2 DOSAGE AND ADMINISTRATION

2.1 Dosing

Adult Dose

The recommended dosage of AK-FLUOR® 10% (100 mg/mL) and of 25% (250 mg/mL) is 500 mg via intravenous administration.

Pediatric Dose

For children, the dose is 7.7 mg/kg (actual body weight) up to a maximum of 500 mg, via intravenous infusion calculated on the basis of 35 mg for each 10 lbs. (4.54 kg) of body weight.

2.2 Preparation for Administration

Parenteral drug products should be inspected visually for particulate matter and discoloration prior to administration. Do not mix or dilute with other solutions or drugs.

2.3 Administration

Inject the dose (over 5-10 seconds is normally recommended) into the antecubital vein, after taking precautions to avoid extravasation. A syringe, filled with AK-FLUOR®, may be attached to transparent tubing and a 23 gauge butterfly needle for injection. Insert the needle and draw the patient's blood to the hub of the syringe so that a small air bubble separates the patient's blood in the tubing from the fluorescein. With the room lights on, slowly inject the blood back into the vein while watching the skin over the needle tip. If the needle has extravasated, the patient's blood will be seen to bulge the skin and the injection should be stopped before any fluorescein is injected. When assured that extravasation has not occurred, the room light may be turned off and the fluorescein injection completed. Luminescence usually appears in the retina and choroidal vessels in 7 to 14 seconds and can be observed by standard viewing equipment.

Reduction in dose from 500 mg to 200 mg of AK-FLUOR® 10% may be appropriate in cases when a highly sensitive imaging system e.g., scanning laser ophthalmoscope is used.

3 DOSAGE FORMS AND STRENGTHS

AK-FLUOR® (fluorescein injection, USP) 10%, 100 mg/mL in a 5 mL single use vial.

AK-FLUOR® (fluorescein injection, USP) 25%, 250 mg/mL in a 2 mL single use vial.

4 CONTRAINDICATIONS

4.1 Hypersensitivity

AK-FLUOR® is contraindicated in patients with known hypersensitivity to fluorescein sodium or any other ingredients in this product. Rare cases of death due to anaphylaxis have been reported [see *Warnings and Precautions* (5.1) and *Adverse Reactions* (6.2)].

5 WARNINGS AND PRECAUTIONS

5.1 Respiratory Reactions

Caution should be exercised in patients with a history of allergy or bronchial asthma. An emergency tray should always be available.

If a potential allergy is suspected, an intradermal skin test may be performed prior to intravenous administration, i.e., 0.05 mL injected intradermally to be evaluated 30 to 60 minutes following injection. Given the sensitivity and specificity of skin testing, a negative skin test is not proof that a patient is not allergic to fluorescein.

5.2 Severe local tissue damage

Extravasation during injection can result in severe local tissue damage due to high pH of fluorescein solution. The following complications resulting from extravasation of fluorescein have been noted to occur: Sloughing of the skin, superficial phlebitis, subcutaneous granuloma, and toxic neuritis along the median nerve in the antecubital area. Complications resulting from extravasation can cause severe pain in the arm for up to several hours. When extravasation occurs, the injection should be discontinued and conservative measures to treat damaged tissue and to relieve pain should be implemented. [see *Administration* (2.3) and *Adverse Reactions* (6.6)].

6 ADVERSE REACTIONS

6.1 Skin and urine discoloration

The most common reaction is discoloration of the skin and urine. Skin will attain a temporary yellowish discoloration. Urine attains a bright yellow color. Discoloration of the skin usually fades in 6 to 12 hours and usually fades in urine in 24 to 36 hours.

6.2 Gastrointestinal Reaction

The next most common adverse reaction is nausea. Vomiting, and gastrointestinal distress have also occurred. A strong taste may develop after injection.

6.3 Hypersensitivity Reactions

Symptoms and signs of hypersensitivity have occurred. Generalized hives and itching, bronchospasm and anaphylaxis have been reported. [see *Contraindications* (4.1) and *Warnings and Precautions* (5.1)]

6.4 Cardiopulmonary Reactions

Syncope and hypotension may occur. Cardiac arrest, basilar artery ischemia, severe shock and death may occur rarely. [see *Warnings and Precautions* (5.1)]

6.5 Neurologic Reactions

Headache may occur. Convulsions may rarely occur following injection.

6.6 Thrombophlebitis

Thrombophlebitis at the injection site has been reported. Extravasation of the solution at the injection site causes intense pain at the site and a dull aching pain in the injected arm. [see *Administration* (2.3) and *Warnings and Precautions* (5.2)].

8 USE IN SPECIFIC POPULATIONS

8.1 Pregnancy

Pregnancy Category C. Adequate animal reproduction studies have not been conducted with fluorescein sodium. It is also not known whether fluorescein sodium can cause fetal harm when administered to a pregnant woman. Fluorescein sodium should be given to a pregnant woman only if clearly needed.

8.3 Nursing Mothers

Fluorescein sodium has been demonstrated to be excreted in human milk. Caution should be exercised when fluorescein sodium is administered to a nursing woman.

8.4 Pediatric Use

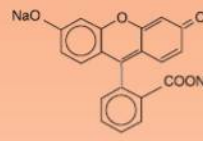
Pediatric patients have been included in clinical studies. No overall differences in safety or effectiveness have been observed between pediatric and adult patients.

8.5 Geriatric Use

No overall differences in safety or effectiveness have been observed between elderly and other adult patients.

11 DESCRIPTION

AK-FLUOR® (fluorescein injection, USP) is a sterile solution f
intravenously as a diagnostic aid. It is a dark reddish orange solutio
a pH of 8.3 to 9.8 and an osmolality of 572 to 858 mOsm/kg for th
and 1800 to 2200 mOsm/kg for the 25%. Its chemical na
spiro[isobenzofuran-1 (3H),9'-[9H]xanthene]-3-one,3'6'-dihy
disodium salt. The active ingredient is represented by the ch
structure:



MW = 376.27

AK-FLUOR® 10% contains:
Active: fluorescein sodium (equivalent to fluorescein 10% w/
mg/mL)
Inactives: Sodium Hydroxide and/or Hydrochloric Acid may be u
adjust pH (8.3 to 9.8), and Water for Injection.

AK-FLUOR® 25% contains:
Active: fluorescein sodium (equivalent to fluorescein 25% w/
mg/mL)
Inactives: Sodium Hydroxide and/or Hydrochloric Acid may be u
adjust pH (8.3 to 9.8), and Water for Injection.

12 CLINICAL PHARMACOLOGY

12.1 Mechanism of Action

Fluorescein sodium responds to electromagnetic radiation an
between the wavelengths of 465 to 490 nm and fluoresces, i.e.,
light at wavelengths of 520 to 530 nm. Thus, the hydrocarbon is
by blue light and emits light that appears yellowish green. Foll
intravenous injection of fluorescein sodium in an aqueous solutio
unbound fraction of the fluorescein can be excited with a blue ligh
from a fundus camera as it circulates through the ocular vascu
and the yellowish green fluorescence of the dye is captured
camera. In the fundus, the fluorescence of the dye demarcates the
and/or choroidal vasculature under observation, distinguishing
adjacent areas/structures.

12.3 Pharmacokinetics

Distribution.
Within 7 to 14 seconds after IV administration into the antecubit
fluorescein usually appears in the central retinal artery of the eye.
a few minutes of IV administration of fluorescein sodium, a yell
discoloration of the skin occurs, which begins to fade 6 to 12 hour
dosing. Various estimates of volume of distribution indicat
fluorescein distributes into interstitial space (0.5 L/kg).

Metabolism.
Fluorescein is metabolized to fluorescein monoglucuronide. A
administration of fluorescein sodium (14 mg/kg) to 7 healthy su
approximately 80% of fluorescein in plasma was convert
glucuronide conjugate after a period of 1 hour post dose.

Excretion.
Fluorescein and its metabolite are mainly eliminated via renal exc
After IV administration, the urine remains slightly fluorescent fo
36 hours. A renal clearance of 1.75 mL/min/kg and a hepatic cle
(due to conjugation) of 1.50 mL/min/kg have been estimate
systemic clearance of fluorescein was essentially complete by 48
hours after administration of 500 mg fluorescein.

13 NONCLINICAL TOXICOLOGY

13.1 Carcinogenesis, Mutagenesis, Impairment of Fertility

There have been no long-term studies done using fluorescein in a
to evaluate carcinogenic potential.

16 HOW SUPPLIED/STORAGE AND HANDLING

AK-FLUOR® (fluorescein injection, USP) 10% is supplied in a sing
5 mL glass vial with a gray bromobutyl serum siliconized stopp
orange flip-off cap. It contains a sterile dark reddish orange solu
fluorescein sodium.

(NDC 17478-253-10) 5 mL, single dose vials in a package of 12.

AK-FLUOR® (fluorescein injection, USP) 25% is supplied in a sing
2 mL glass vial with a gray bromobutyl serum siliconized stopp
orange flip-off cap. It contains a sterile dark reddish orange solu
fluorescein sodium.

(NDC 17478-250-20) 2 mL, single dose vials in a package of 12.

AK-FLUOR® should be stored at 20° to 25°C (68° to 77°F). Do not

17 PATIENT COUNSELING INFORMATION

After administration of fluorescein sodium, skin will attain a tem
yellowish discoloration. Urine attains a bright yellow color. Discol
of the skin usually fades in 6 to 12 hours and usually fades in urin
to 36 hours. [see *Warnings and Precautions* (6.1)].

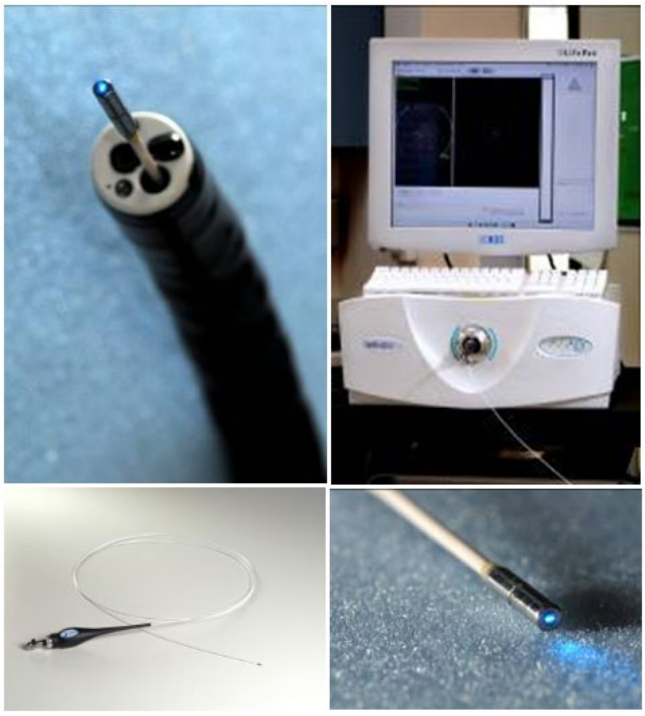
AKORN
Manufactured by: Akorn, Inc.
Lake Forest, IL 60045

FL00N Rev. 0

Appendix B. ECOG Performance Status Scores.

Description	Status
Fully active, able to carry on all pre-disease performance without restriction.	0
Restricted in physically strenuous activity but ambulatory and able to carry out work of a light or sedentary nature, e.g., light housework, office work.	1
Ambulatory and capable of all self-care but unable to carry out any work activities.	2
Capable of only limited self-care, confined to bed or chair more than 50% of waking hours.	3
Completely disabled. Cannot carry on any self-care. Totally confined to bed or chair.	4
Dead	5

Appendix C. Intravital endomicroscope used for performing the human IVM observation.



A. Images depicting the intravital endomicroscope to be used in this study. The fiberoptic objective is deployed at the tip of the endoscope. Bottom insets show a complete view (left) and zoomed in view (right) of the microscope. The hardware is directly attached to the endomicroscope apparatus and records images/videos for post-hoc analysis.

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