

**TITLE:**

Intravital Microscopy (IVM) in patients with peritoneal carcinomatosis

**Study Number:****Initial Date:**

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**SYNOPSIS: Intravital Microscopy (IVM) in patients with peritoneal carcinomatosis**

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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. This proposed trial at Mayo Clinic Florida will further determine the usefulness of IVM in human subjects, now applying it to patients with deeper sites of tumor, i.e. peritoneal carcinomatosis.

In these clinical trials, fluorescein was used to augment visualization of tumor-associated vessels. It is a commonly used and safe intravital dye. Similarly, indocyanine green (ICG) is a commonly used dye and will be also used in this study. Due to its heavier molecular weight compared to fluorescein, ICG allows additional analysis of vessel permeability. Although it is also considered a safe intravital dye, small studies report a higher risk of adverse events (AEs) associated with its use in patients with hepatic or renal dysfunction.

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

1. Determine the feasibility of HIVM in characterizing the tumor-associated microvasculature in patients with peritoneal carcinomatosis.
2. To correlate these findings to the HIVM observations at the primary site, extent of carcinomatosis, tumor response, and survival outcomes.

**Objectives:**

*Primary objective(s):* To determine the feasibility of performing HIVM in patients with peritoneal carcinomatosis during standard course of treatment (cytoreductive surgery with

hyperthermic intraperitoneal chemotherapy, or CRS-HIPEC). A successful intravital microscopic observation will include the ability to complete each of the following:

1. Identify and measure vessels associated with peritoneal tumor implants
2. Determine vessel density per 10x field
3. Visualize vital dyes within the vessels [fluorescein and indocyanine green (ICG)]
4. Calculate the blood flow velocity of the vessels and tissue penetration of fluorescein and ICG as markers of vessel permeability.

*Secondary objective(s):*

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 implants.
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 peritoneal carcinomatosis undergoing CRS-HIPEC.

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 the peritoneal surface 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 20 patients enrolled (total of 30 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 on the peritoneal surface by direct visualization requiring surgical resection in the OR.
4. Peritoneal carcinomatosis that meets indications for CRS-HIPEC. Sites of origin for tumor include appendix, colon, stomach, and primary peritoneal surface malignancies such as peritoneal mesothelioma.

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 ICG 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 CRS-HIPEC 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 implant associated vessels and measure vessel diameters
2. Determine vessel density per 10x field
3. Visualize vital dyes within the tumor-associated vessels (fluorescein and green ICG)
4. Calculate the blood flow velocity of the tumor-associated vessels and tissue penetration of fluorescein and ICG as markers of vessel permeability.

*Secondary endpoints:*

1. Post-operative comparison of the microvasculature of peritoneal carcinomatosis 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 implants (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 patients with peritoneal carcinomatosis**

<b>INCLUSION CRITERIA</b>				
<b>Y</b>	<b>N</b>	<b>N/A</b>	<b>All answers must be “YES or “N/A” for subject enrollment.</b>	<b>Date</b>
<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	1. Age $\geq$ 18 years of age.	
<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	2. Have an ECOG Performance Status of $\leq$ 2. Refer to Appendix C.	
<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	3. Have measurable disease in the peritoneum by direct visualization (visible lesion typically $> 0.5$ cm in maximal diameter).	
<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	4. Carcinomatosis that meets indications for CRS-HIPEC.	
<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	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.	
<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	6. A negative skin-prick test to fluorescein.	

<b>EXCLUSION CRITERIA</b>				
<b>Y</b>	<b>N</b>	<b>N/A</b>	<b>All answers must be “NO” or “N/A” for subject enrollment</b>	<b>Date</b>
<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	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.	
<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	2. Renal dysfunction as defined as a GFR $< 45$ .	
<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	3. Liver dysfunction as defined by Child-Pugh score $> 5$ , or LFT's 1.5x above normal range.	
<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	4. Any known allergy or prior reaction to fluorescein or ICG or a positive skin prick test to fluorescein.	
<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	5. Pregnant or nursing female subjects, determined preoperatively with a urine pregnancy test.	
<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	6. Unwilling or unable to follow protocol requirements.	
<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	7. Any condition which in the Investigators' opinion deems the patient unsuitable (e.g., abnormal EKG).	
<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	8. Any condition that excludes CRS-HIPEC 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 or ICG). 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] To date, there have been no studies of IVM in the setting of human peritoneal carcinomatosis.

The detection of blood flow parameters (including vessel diameter, flow rates, vessel density, and fluorescent markers of tissue diffusion) in tumor implants of patients with peritoneal carcinomatosis may have utility in characterizing the locoregional spread of disease and in predicting drug uptake and clinical response in these tumor implants. The overall goal will be to characterize, for the first time, vasculature associated with peritoneal carcinomatosis in real-time. It is anticipated that this novel 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.

### 1.1 STUDY DISEASE – PERITONEAL CARCINOMATOSIS

Peritoneal carcinomatosis can occur in a variety of malignancies, including those of gastrointestinal (GI) origin, the genitourinary (GU) tract, and primary peritoneal mesothelioma. It is estimated to affect up to 13-17 % of patients with GI malignancies.[14, 15] Current treatments include systemic chemotherapy and regional therapies, namely as cytoreductive

surgery with hyperthermic peritoneal chemotherapy (CRS-HIPEC). This treatment modality can be associated with significant response and typically requires the expertise found in high-volume performing institutions (including Mayo Clinic Florida) in order to provide the infrastructure essential to performing this procedure. Significant oncologic benefits of CRS-HIPEC have been reported for certain malignancies.[16-19] The categorization of CRS-HIPEC has recently changed by the National Comprehensive Cancer Network (NCCN) from being an investigational approach to being a considered approach for the appropriate patient.[20] Despite these advances, current treatments for peritoneal carcinomatosis still generate heterogeneous responses, and many patients will not benefit from CRS-HIPEC or systemic chemotherapy, or may experience limited responses. As such, additional therapies for carcinomatosis are strongly needed. The relevance of this clinical trial in addressing this current need is the investigation of tumor vessels associated with carcinomatosis as a prognostic and ultimately therapeutic target for treatment.

## 1.2 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 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.[21] 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 3 separate areas of skin on the forearm. The skin-pricks will include 4 microliters of 10% fluorescein, histamine chloride (10 mg/ml) [positive control], and 50% saline solution [negative control] 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 in the context of the appropriate positive and negative control. 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.

### **1.2.1 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, 22] 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, 23]

### **1.2.2 CLINICAL STUDIES WITH FLUORESCEIN**

This protocol intends to apply the commonly used intraoperative fluorescent agent, fluorescein, to further characterize blood vessels associated with peritoneal carcinomatosis during the course of CRS-HIPEC. It is anticipated that fluorescein will be readily detectable in the microscopic field of observation following intravenous fluorescein injection. This is expected to be similar to a previous trial NCT01886235, which utilized fluorescein to study the microvasculature of melanoma primary tumors, and an on-going trial that uses fluorescein to visualize vessels associated with sentinel lymph nodes (NCT02857374).

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 25%. For pediatric patients, the dose is adjusted to 35 mg per 10 pounds of body weight.[24]

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.[25] 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.[26, 27] 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,[28] 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.[29] 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.[30-32]

Historically, adverse reactions occur in 5% - 10% of patients and range from mild to severe.[33-39] 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%. [40] 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.[41]

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.[37] 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.[42]

More severe reactions are rare, but include laryngeal edema, bronchospasm, anaphylaxis, tonic-clonic seizure, myocardial infarction, and cardiac arrest.[43-47] The overall risk of death from fluorescein angiography has been reported as 1 in 222,000.[37] 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.[48-50] 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.

### **1.3 RISKS AND/OR BENEFITS**

**Risks** – low risk of reaction to fluorescein as described above. Otherwise, no increased risk of surgical procedure anticipated. There are inherent risks to CRS. Studies have characterized the complications for which patients undergoing CRS-HIPEC are at risk, including anastomotic leak, ileus and obstruction, and neutropenia.[51-53] However, these risks are not expected to be increased due to the use of fluorescein.

**Benefits** – depending upon collected findings and correlation with clinical outcome, IVM may offer prognostic information and contribute to staging classifications for peritoneal carcinomatosis with the intent of potentially guiding treatment decisions in the future.

### **1.4 DIAGNOSTIC INTERVENTION – INDOCYANINE GREEN (ICG)**

Drug Information – Indocyanine green (ICG or IC-Green) is the second intravenous (IV) dye to be administered in this clinical trial. The ICG will be administered after the fluorescein has been observed to washout from the tumor associated vasculature, which can be directly monitored during HIVM. Following this, the ICG will be injected. ICG injection after fluorescein injection is favored due to the characteristics of these dyes, with ICG being heavier and more albumin

bound than fluorescein. ICG would be expected to stay within the intravascular compartment longer than fluorescein with estimates of up to 20-30 minutes. It is therefore a more optimal utilization of time to administer the dyes in this order, because the reverse order (ICG prior to fluorescein) may result in an increased waiting time for the ICG to dissipate from the circulation.

ICG was approved by the FDA in November 2007 for use in the study of cardiac output determination, hepatic function and liver blood flow measurement, and ophthalmic angiography.[54, 55] However, it has been used in many different clinical applications, including those listed above, for over 50 years.

It is supplied by Akorn, Inc. under the brand name IC-GREEN®. In this study, a single unit for a given patient consists of a 25 mg vial to which 5 ml of aqueous solvent (sterile water) is added, thereby reconstituting the drug to 5 mg/ml. Further dilutions are achieved using sterile saline, making the final solution slightly hypo-osmolar.

Administered dosages vary for different investigative purposes. Based on hepatic function studies, the dose to be administered is calculated for 0.5 mg/kg of body weight.[55] The total dose of ICG should not exceed 2 mg/kg. The dose used for the purposes of vessel evaluation have been reported to be much lower (e.g. 0.5 mg - 5 mg per patient).[56] Administration of 5 mg is the recommended dose for cardiac dilution studies for adults.[55] Therefore, it is anticipated that the dose used for this evaluation is well below the maximum recommended dose. The dose of 5 mg ICG would be an appropriate amount for this study.

The drug is supplied as a kit containing six 25 mg vials and six 10 ml Aqueous Solvent ampules. The cost of a single kit is \$508.76.

ICG is an iodide-containing, water soluble tricarbocyanine dye that fluoresces when exposed to infrared light (800 nm).[57] Compared to Fluorescein, the molecular weight of ICG is over double at 774.98 g/mol (Fluorescein molecular weight is 332.31 g/mol). The package insert and the Material Safety Data Sheet are shown in the Appendix B.

Following IV injection, ICG is rapidly bound to plasma proteins of which albumin is the main carrier (95%).[55, 58] The drug undergoes no significant extrahepatic or enterohepatic circulation; it is taken up mainly by hepatic parenchymal cells and excreted into bile.[55, 59] It has a plasma half-life of approximately 3-4 minutes.[55, 58] Due to its high binding to albumin and significantly greater molecular weight than Fluorescein, ICG is expected to persist within the tissues for a longer period of time resulting from a slower washout. This characteristic would have several advantages to the study of HIVM in tumor associated vessels, including increased imaging times, more extensive characterization of blood vessel architecture, and more accurate calculation of blood flow velocities.

The ICG will be provided by the study. It will be ordered and sent to the operating room during surgery at the time of the planned HIVM observation. The dose to be administered will be calculated prior to the surgery based on the patient's most accurate and current weight. It will be

injected through the IV by a physician member of the surgical or anesthesia team. Surgeons and anesthesiologists have experience with the IV administration of ICG during other procedures, such as injection during plastic surgery reconstructive procedures to assess tissue viability.

Similar to Fluorescein, ICG has a rare incidence of anaphylactic reaction (0.05 – 0.15%). It does contain an iodide component, which accounts for the allergy potential of ICG. Therefore, patients with a known allergy to iodine or contrast dye will be excluded from the study. Patients who have received iodine based contrast computerized tomography (CT) scans with IV iodine contrast dyes would be unlikely to have an iodine allergy, particularly if multiple scans have been performed. During the staging of patients with peritoneal carcinomatosis, routine use of imaging is routinely recommended to investigate the degree of metastasis. Thus, it is extremely unlikely that a patient who has received IV iodine contrast dye during a CT scan will have an allergic reaction to ICG.

#### **1.4.1 PRECLINICAL STUDIES WITH ICG**

Similar to animal studies with Fluorescein, ICG has been used to study blood flow in several different models.[60-62] ICG has been successfully used in these studies to characterize the vasculature associated with the tumor microenvironment.

#### **1.4.2 CLINICAL STUDIES WITH ICG**

Similar to Fluorescein, ICG is not expected to have a direct intervention on tumor biology. ICG has been used during the course of surgery and other procedures for widespread indications and is FDA approved. In general, it is well tolerated, non-toxic, and has no long term side effects.

Clinically, ICG is most commonly used in the assessment of liver function,[63-65] ophthalmic angiography,[66-68] and the analysis of cardiac function with increasingly novel applications in cardiac studies.[69, 70] It is typically administered by a member of the anesthesia team during cases or procedures in the operating suite under general anesthesia. In addition to these applications, ICG has been used in a variety of other studies in a safe manner. ICG has been used to map sentinel lymph nodes (SLN) in several malignancies,[71] including melanoma,[56, 72, 73] breast,[74-76] and colon cancer.[77] These studies have not reported any major adverse reactions or toxicities resulting from exposure to ICG. While the doses of ICG used in these studies varies, the recommended dose per the manufacturer is calculated on the basis of 0.5 mg/kg of body weight.[55]

Other applications of ICG have been studied in the field of plastics surgery. ICG has been given in patients to determine the perfusion and viability of perforator flaps.[78, 79] Similar to the studies in malignancy and SLN, the fluorescent properties of ICG have been utilized and shown to be effective in other studies. ICG has also been used to identify and target esophageal varices.[80] Further, during neurovascular operations, ICG has been readily administered by

anesthesiologists during intraoperative videography of aneurysm clippings and carotid bypass procedures.[81, 82]

As discussed in the previous section, the use of ICG is contraindicated in patients with a history of allergic hypersensitivity to iodine. There have been rare but reported cases of anaphylaxis and death due to allergic reactions during procedures using ICG.[25, 83, 84] Therefore, patients with a history iodine allergy will be excluded from our clinical trial. Other adverse reactions have been reported with the use of ICG in patients with allergic reactions.[85]

A higher risk of adverse reactions has been reported in small studies in patients with liver disease, chronic kidney disease, and uremia.[84] Although the manufacturer does list these conditions as contraindications, patients with uremia or liver disease will be identified through a thorough history and physical, and screened with a basic metabolic panel (which includes a serum creatinine and blood urea nitrogen or BUN) and liver function tests (LFT's). These patients will be informed of the potential increased risk for side effects and may be excluded from the study.

The safety of ICG has been assessed in pregnancy, although there exist no strong level of evidence (i.e. randomized controlled trials) to determine conclusively the safety of ICG in pregnant patients. As such, it is labeled by the FDA as pregnancy category C. There do exist retrospective series and case reports that have safely utilized ICG in the pregnant patient. ICG has been used in the study of ophthalmic angiography during preeclampsia.[86] A survey of ophthalmologists who use ICG in the study of retinal vessels showed that 89% of the surveyed ophthalmologists have encountered at least one pregnant patient who required either ICG or Fluorescein angiography, and of these 24% withheld ICG angiography.[87] Regarding nursing mothers, it is unknown where ICG is excreted in human milk and can have any adverse effects in the newborn or infant period. Due to the limited data, pregnant and nursing mothers will be excluded from the trial in a similar manner with regard to the use of Fluorescein. A routine day of surgery pregnancy test is performed for pre-menopausal patients.

Historically, the rate of adverse reactions to ICG are low. A study of over 1900 cases of ICG video angiography cases in the ophthalmologic setting reported the incidence of mild, moderate and severe reactions to be 0.15%, 0.2% and 0.05%, respectively.[84] ICG was administered IV in these cases. The death rate after ICG injection has been cited as 1 in 333,333.[84]

Transient nausea and vomiting, and local discomfort, are the most common reactions and require no treatment. These mild reactions typically occur shortly after injection and are transient.[84]

Moderate reactions such as pruritus or urticaria can be treated with antihistamines, but any patient who experiences these symptoms will be observed carefully for the possible development of anaphylaxis. These symptoms have been successfully treated with antihistamines, and were of short duration.[84] In the event of severe reactions, assessment and treatment will be implemented similar to protocol used in Fluorescein adverse reactions.

Severe reactions include anaphylactic reactions. The dose of ICG has not been well correlated to the severity of symptoms. These symptoms include severe generalized pruritis, diaphoresis, weakness, agitation, and syncope.[84, 88] Signs include hypotension and hemodynamic instability. In extreme cases, death may result. 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 will 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.

Local toxicity has been described for ICG, but largely in the setting of topical application during ophthalmologic procedures.[89, 90] ICG has widespread use as a vital stain during macular surgery. It is hypothesized that the local irritant effects combined with the hypo-osmolar tonicity over prolonged exposure times (over 10 minutes) may contribute to local toxicities to the macula, potentially resulting in decreased visual acuity, optic disc changes, and visual field deficits.[91] However, in our application of ICG for HIVM, local effects should be minimized through IV injection and dilution as it enters the blood stream. These toxicities have not been reported in studies where ICG has been used intravenously for liver and cardiac function. Mild discomfort has been associated with extravasation.[84] Care will be taken to minimize injection within the soft tissue and limit extravasation of ICG.

## 1.5 RISKS AND/OR BENEFITS

**Risks** – similar to the section on fluorescein, low risk of reaction to ICG as described above. Otherwise, no increased risk to the surgical procedure anticipated.

**Benefits** – depending upon collected findings and correlation with clinical outcome, IVM may offer prognostic information and improve the staging classification of peritoneal tumors with the intent of potentially guiding treatment decisions in the future.

## 2 RATIONALE

These studies will elucidate the hemodynamic properties of microvessels associated with peritoneal carcinomatosis. 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.

### 3 OBJECTIVES

#### 3.1 PRIMARY OBJECTIVE - PART I (10 PATIENTS):

To determine the feasibility of performing HIVM in patients with peritoneal carcinomatosis during standard course of treatment (cytoreductive surgery with hyperthermic intraperitoneal chemotherapy, or CRS-HIPEC). A successful intravital microscopic observation will include the ability to complete each of the following:

1. Identify and measure vessels associated with peritoneal tumor implants
2. Determine vessel density per 10x field
3. Visualize vital dyes within the vessels [fluorescein and indocyanine green (ICG)]
4. Calculate the blood flow velocity of the vessels and tissue penetration of fluorescein and ICG as markers of vessel permeability.

#### 3.2 SECONDARY OBJECTIVES - PART II (20 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 implants.
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 implant associated vessels and measure vessel diameters
2. Determine vessel density per 10x field
3. Visualize vital dyes within the tumor-associated vessels (fluorescein and green ICG)
4. Calculate the blood flow velocity of the tumor-associated vessels and tissue penetration of fluorescein and ICG as markers of vessel permeability.

*Secondary endpoints:*

1. Post-operative comparison of the microvasculature of peritoneal carcinomatosis 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 implants (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 and ICG in subjects with peritoneal carcinomatosis undergoing CRS-HIPEC. 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 wide excision in the operating room, then the patient is excluded from this study.

### 4.2 STUDY METHODS

During the course of treatment for peritoneal carcinomatosis while the patient is under anesthesia, CRS-HIPEC will be performed. Surgical exposure is performed through a midline incision and the assistance of retractors as shown in the technical schema (Appendix D).

The IVM technology utilized at Mayo Clinic currently consists of a high resolution confocal endomicroscope (Appendix E). 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 or used with a sterile sleeve in order to interface with the peritoneal surface.

The microscope will be moved into position next to the OR table by the operating surgeon. 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. 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 E). All data will be backed up on a password-protected hard drive in the Principal Investigator's laboratory.

Following several observations over various portions of the peritoneal surface, 1 ampule (5 mL) 25% fluorescein will be injected intravenously and observation will continue until loss of

fluorescence. 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.

Following the dissipation of the fluorescein from the visual field, 5 mg of ICG will be injected intraveneously. The observation will repeat in a similar manner, now with the use of ICG. Tumor-associated vasculature will be observed in order to record the similar vessel characteristics. ICG is expected to persist within the vasculature for an extended period of time, thereby allowing further characterization of the field. It is not necessary to wait for the ICG to dissipate from the field as this may take up to 20-30 minutes due to its high level of albumin binding.

At the completion of the observation period with ICG, the microscope will be removed and sterilized between uses. This would complete the observation period, which is anticipated to take 15 - 20 minutes total time. The observation will take place during the course of standard surgery and is not anticipated to delay or significantly lengthen the duration of the procedure. For example, other portions of the procedure will be continued during the observation period (e.g., setting up the circuit required for HIPEC). The tumor implants will be removed and undergo standard pathology evaluation. The patient will recover in the PACU per standard protocol.

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 intravital dye 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.

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 ( $\gamma$ ) will be calculated as  $8(V \div D)$ .[92] 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 or ICG 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  $Q = (RBC \text{ velocity}/1.6) \times (d/2) \times \pi$ .

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 30 subjects [Part I (10 patients) and Part II (20 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 CRS-HIPEC in the operating room who can provide informed consent, have a good performance status (ECOG  $\leq 2$ ), and have no known allergy or prior reaction to fluorescein, ICG or iodine, are eligible.

### **5.1 INCLUSION CRITERIA**

1. Age  $\geq 18$  years of age.
2. Have an ECOG Performance Status of  $\leq 2$ . Refer to Appendix C.
3. Have measurable disease in the peritoneum by direct visualization (visible lesion typically  $> 0.5$  cm in maximal diameter).
4. Carcinomatosis that meets indications for CRS-HIPEC.
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 ICG 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 CRS-HIPEC 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 week 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 and a patch test for ICG will be

performed by the Principal Investigator. For testing allergic response to fluorescein, a sterile Duotip® lancet (Lincoln Medical, USA) is used in 3 separate areas of skin on the forearm. The skin-pricks will include 4 microliters of 10% fluorescein, histamine chloride (10 mg/ml) [positive control], and 50% saline solution [negative control] 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 in the context of the appropriate positive and negative control. 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 patient will also be screened for a history of allergic reaction to iodine or iodinated contrast dyes.

- 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 either dye 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		
CRS-HIPEC with intra-op 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 or ablated, 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](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
<b>Unrelated</b>	X	X	X	X	X
<b>Unlikely</b>	X	X	X	X	X
<b>Possible</b>	X	X	X	X	X
<b>Probable</b>	X	X	X	X	X
<b>Definite</b>	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 CRS EXPeRT 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 Mayo Clinic Data and Safety Monitoring Board will assess the progress of the study, the safety data, and critical efficacy endpoints. The DSMB will review the study annually and will make recommendations that include but not limited to; (a) continuation of the study, (b) modifications to the design (c) or termination of the study.

## 9 STATISTICAL METHODOLOGY

Initially ten patients will be treated to determine the feasibility of HIVM for peritoneal carcinomatosis. 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 peritoneal carcinomatosis. Criteria include the ability to complete each of the following:

1. Identify and measure vessels associated with peritoneal tumor implants
2. Determine vessel density per 10x field
3. Visualize vital dyes within the vessels [fluorescein and indocyanine green (ICG)]
4. Calculate the blood flow velocity of the vessels and tissue penetration of fluorescein and ICG as markers 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 20 patients will be accrued to this study in order to address the following:

1. Post-operative comparison of the microvasculature of peritoneal carcinomatosis 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 implants (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 intravital dye 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 peritoneal tumor implants 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 implant site) and tumor implant itself will be depicted with graphics, correlations, and model results.

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 CRS-HIPEC to death due to recurrence. Overall survival and disease specific survival will be generated using standard Kaplan-Meier methodology.

A maximum of 30 subjects will be enrolled in this study (initially 10 to determine feasibility and then 20 additional subjects if deemed feasible). The accrual rate is estimated at one subject per month, and the estimated study duration is 30 months.

## 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 or the subject's legally authorized representative 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 EXPeRT. EXPeRT 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 EXPeRT 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. EXPeRT 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:**

<b>ACTIVE:</b>	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);
<b>PRESERVATIVE:</b>	None;
<b>INACTIVES:</b>	Sodium Hydroxide and/or Hydrochloric Acid may be used to adjust pH (8.3 to 9.8), and Water for Injection.
<b>STORAGE:</b>	Store at 20° to 25°C (68° to 77°F). Do not freeze.

NDC #	CARDINAL	AMERISOURCEBERGEN	MCKESSON	MORRIS DICKSON
17478-253-10	2119436	861-005	2772069	084699
17478-250-20	2119444	089-829	1257831	TBA



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P343 Rev. 10/13

Version 2.0, 15 March 2018



## Appendix B: Indocyanine Green Drug Package Insert.

# See what you're missing with

**IC-GREEN®**  
(indocyanine green  
for injection, USP)

- **IC-GREEN®** is indicated for ophthalmic angiography, determining cardiac output, hepatic function and liver blood flow
- **IC-GREEN®** is a water soluble, tricarbocyanine dye with a peak spectral absorption at 800 nm
- **IC-GREEN®** contains sodium iodide and should be used with caution in patients who have a history of allergy to iodides
- Available direct or through your authorized wholesaler or distributor



## IC-Green® (indocyanine green for injection, USP)

RSS Barcoded    UPC Barcoded

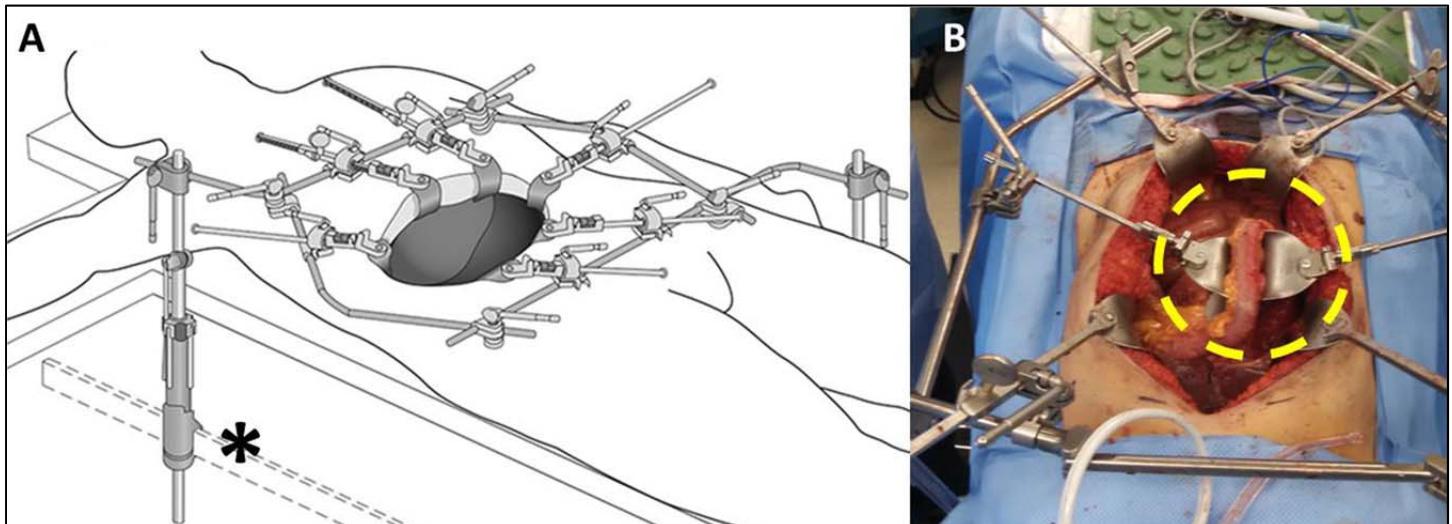
NDC #	DESCRIPTION	SIZE	UNIT OF SALE		
17478-701-02	25 mg Sterile Indocyanine Green Single-dose Vials and Sterile Aqueous Solvent Ampules	25 mg Vial and 10 mL Ampule (Solvent)	6		
<b>EACH mL CONTAINS:</b>					
ACTIVE:	Lyophilized green powder containing 25 mg of Indocyanine Green, Aqueous Solvent consisting of Sterile Water for Injection;				
PRESERVATIVE:	None;				
INACTIVES:	Contains not more than 5.0% Sodium Iodide.				
STORAGE:	Store at 20° to 25°C (68° to 77°F) [see USP Controlled Room Temperature].				
NDC #	CARDINAL	AMERISOURCE	BERGEN	MCKESSON	MORRIS DICKSON
17478-701-02	2311801	2158897	520-916	2738532	584219



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NOT FOR PRESCRIBING PURPOSES. PLEASE REFER TO PACKAGE INSERT FOR FULL PRESCRIBING INFORMATION.

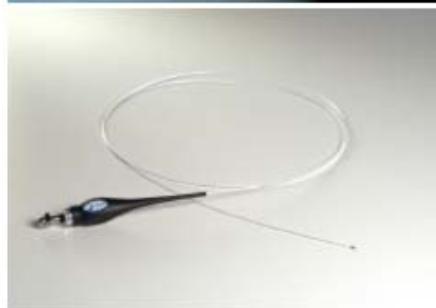
## Appendix C. ECOG Performance Status Scores.

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 D. Technical Schema of exposure for IVM of peritoneal carcinomatosis.**

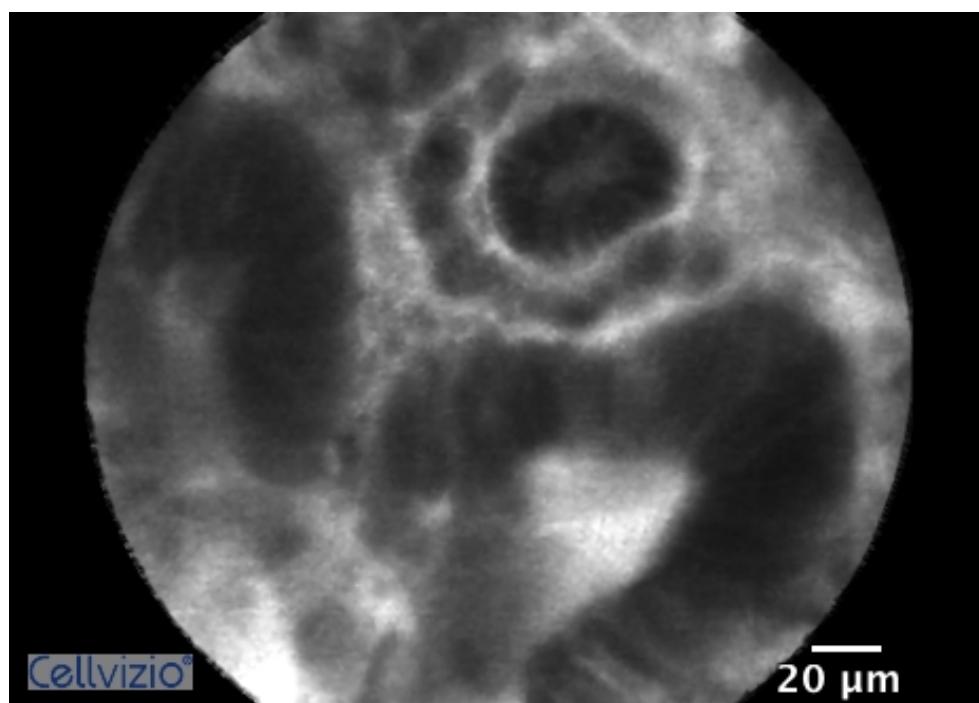
A. Depiction of retraction and exposure of the peritoneal cavity during cytoreductive surgery with hyperthermic intraperitoneal chemotherapy (CRS-HIPEC).

B. Tumor implants can be present along the surface of the peritoneum itself or on organs within the abdominal compartment. In this case, the small bowel also has tumor implants. This can also be observed with IVM.

**Appendix E. 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.

A



B. Example of images of colonic mucosa generated using the IVM endomicroscope. High quality resolution can be achieved at the single cell level. This photo does not show the addition of intravital dyes (fluorescein or ICG). These dyes will be used for imaging of peritoneal tumor implants.

B