Epidemiology and Genomics Research Program

Resources for Public Use: Standard Operating Procedures for Biospecimen Collection

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Fresh Frozen Tissue Protocol

I. Purpose

The goal is to describe the procedure for the collection, storage and distribution of colorectal cancer tissue in the Cancer Family Registry (CFR) frozen tissue biorepository. This unique resource provides approved investigators with access to optimally preserved tissue that is linked to relevant epidemiologic, genetic and clinical data.

II. Background

Emerging technologies are yielding breakthroughs in molecular profiling and functional screening methodologies

that are possible only with high quality tissue samples. The existing CFR resource of fixed paraffin-embedded tissue has limited utility for analysis of gene expression, protein structure and function, and other DNA mutation screening methodologies. The creation of a frozen tissue resource augments the investigative approaches that can be utilized by CFR investigators for the identification and characterization of hereditary and environmental contributions to colorectal cancer.

A number of CFR sites, including USC, Hawaii, and Cleveland, have received funding and IRB approval to participate in the fresh frozen tissue component of the renewal. Participating sites will collect tissue from 30 cases. Subjects will be followed in the same manner as existing CFR subjects with the exception that recruitment and informed consent will be obtained prior to the definitive surgical resection.

III. Objectives

a. Develop a hospital-based mechanism to prospectively identify and accrue colorectal cancer patients prior to definite surgical resection.

b. Prospectively collect a variety of specific fresh tissue samples from colorectal cancer resection specimens.

c. Link the repository of fresh frozen tissue to epidemiologic risk factor and genetic data.

d. Perform quality control experiments on samples in the frozen tissue repository.

IV. Subject Selection

Subjects potentially eligible for the frozen tissue sub-study are those between the ages of 18 and 84 with biopsy-confirmed adenocarcinoma of the colon or rectum who are scheduled for surgical resection and have surplus tissue available for harvesting. At most sites, eligible subjects will include 1) new subjects diagnosed with a primary adenocarcinoma of the colon or rectum and 2) existing CFR participants who are diagnosed with an initial primary (unaffected relatives or controls) or with a metachronous primary (probands and affected relatives). However, subjects who otherwise meet study criteria but have undergone radiation treatment prior to resection are excluded from the frozen tissue sub-study in order to preclude the possibility of iatrogenic DNA sequence changes. Additional exclusion criteria for the frozen tissue sub-study include subjects diagnosed with metastases of existing colorectal cancer, subjects not scheduled for definitive surgical resection, and subjects for whom surplus tissue is unavailable at the time of resection.

V. Study Design

a. Appropriate IRB approvals must be obtained prior to commencement of the frozen tissue sub-study.

b. Community physicians, particularly gastroenterologists and surgeons, should be notified of the study, and provided with a contact number for questions or information.

c. Existing CFR participants should be informed by newsletter or other means of the frozen tissue sub-study, as these subjects likely meet eligibility requirements if diagnosed with an initial or second primary colorectal cancer.

d. A means of expedient access to newly diagnosed biopsy cases meeting eligibility requirements must be established. At SEER sites, it may be possible to coordinate data collection and dissemination between the hospital pathology department, the hospital oncology registry, and the local SEER registry. Optimally, results of biopsies positive for adenocarcinoma of the colon or rectum will be provided daily via the hospital registry to SEER. The following information or a hard copy of the pathology report should be provided to the CRF site

as soon as possible after pathological confirmation of the biopsy.

a. Name and birth date of patient
b. Name(s) of relevant physicians
c. Date of biopsy
d. Histology of biopsy
e. Site of biopsy
f. Pathology number and medical number
g. Race of patient (when possible)

e. The CFR case management coordinator will screen cases to determine if patients are current Family Registry participants, or if new patients meet site-specific eligibility requirements. It is imperative that cases be screened as quickly as possible after the biopsy date, as the period for successful recruitment for new subjects consists of the narrow window between date of biopsy and date of definitive resection.
f. The physician(s) should be contacted to confirm eligibility, and to seek permission to contact patients. The date and hospital of resection and name of the attending surgeon needs to be obtained. Patients not scheduled for resection are disqualified for this sub-study.
g. Patients are contacted in a discreet and compassionate manner to explain the study and invite participation. For new patients who verbally agree, a concerted effort must be made to obtain signed consent at a time and place convenient for the subject, such as the subject's home or at a medical appointment. The informed consent must be signed prior to the date of surgical resection. Patients who are current participants in the Family Registry may not need to sign a new consent form (see your IRB).
h. The surgeon(s) is notified of his/her patient's willingness to participate in the study, and the date, time and location of the surgery is re-confirmed.
i. The pathology department at the hospital where the resection is scheduled is contacted, and arrangements for collection of the tissue are made. In most cases, the pathologist on call will collect the surgical specimen and provide tissue samples to the CFR technician. The tissue is then transported to the CFR for appropriate storage.

VI. Preparation for Tissue Collection

a. The day prior to the surgical procedure, contact the pathology department to finalize tissue collection arrangements. *Remind the OR and the pathology department that the resection specimen must **NOT** be placed in formalin prior to transport to the pathology receiving room.*
b. Ensure that supplies required for the collection procedure are available. Supplies can be stored in a tissue collection kit such as an insulated carrier or fishing tackle box.
    a. Liquid nitrogen in approved LN2 transport carrier
    b. Safety glasses or face shield
    c. Freezer gloves
    d. Disposable sterile latex or nitrile surgical gloves
    e. Surgical mask
    f. Clean laboratory coat
    g. Clean protective shoes
    h. Sterile cryovials, labeled and unlabeled extras
    i. Racks for cryovials (one for tumor and one for normal)

j. Sterile disposable scalpels or scalpel blades (#10, 21, 22, 60 or single edge razor blade)
k. Sterile disposable forceps (Fisher, #NC 9812170)
l. Sterile 21 G needles
m. Sterile disposable towels or drapes
n. Sterile dishes or plates, such as Petri or cell culture dish
o. Sterile saline (PBS)
p. Sterile gauze
q. Marking pen or pencil
r. OCT cryo-compound (Fisher, #NC 9159334)
s. Tissue cryomold for OCT compound (Fisher, #NC 9511236)
t. Biopsy nylon bag (Fisher, #15-182-116)
u. RNA-preserving reagent, such as RNA later
v. 10% Buffered formalin (Allegiance, #C4320-105 or Richard-Allan Scientific #56401)
w. Chipped ice
x. Flat container for chipped ice, such as an autoclavable pyrex cake dish
y. Sharps container for disposal of biohazardous sharps

c. Label cryovials with the subject identifier, date, and specimen type.
d. Pre-label sterile culture dishes with the following: Tumor – central, Tumor – margin, Normal mucosa – distant, Normal mucosa-perilesional. Have additional dishes and marking pens available during the procedure in the event that additional tissue is collected or if a dish is inadvertently contaminated. These dishes will be used to hold specimens that need to be bisected or trimmed prior to transfer to storage vials.
e. Make a copy of the patient's signed informed consent to bring to the OR.

VII. Tissue Collection

a. Tissue for the study is collected from surplus tumor and uninvolved tissue that is removed during the surgical resection and would otherwise be discarded. Respect the fact that the determination of surplus tissue is at the sole discretion of the anatomic pathologist and/or attending surgeon.
b. When surplus tissue is available, tumor tissue should be collected from the following sites:
    a. Central portion of the tumor
    b. Leading edge of the tumor (tumor margin)

    Normal tissue, in terms of priority, should be collected from the following sites:
    a. Distant, grossly uninvolved colorectal mucosa
    b. Perilesional grossly uninvolved colorectal mucosa
    c. Surrounding stroma
    d. Adjacent mucosal precursor
    e. All polyps
c. In cases where surplus tissue is marginal, the minimum to be harvested includes one specimen from the central tumor area, and one specimen from grossly uninvolved colorectal mucosa.
d. Request that the resection specimen be placed into a sterile basin in the operating theater, covered with a sterile towel, and transported as quickly as possible to the pathology receiving room.

e. In most cases, the pathologist or pathology technician will do the initial sectioning of the surgical specimen. Ask that the surgical resection specimen be placed onto a sterile tray during the procedure, and sectioned under sterile conditions. Placing the tray on ice will delay autolysis. Separate blades should be used for sectioning tumor tissue and normal tissue. Ask the pathologist to use utmost care to avoid cross-contamination of tissue. The CFR technician should provide sterile supplies such as blades, forceps, and dishes/plates for use by the pathologist, as well as ice chips for keeping the specimens cold during processing.

f. The maximum sample size for each specimen to be frozen is 0.5 cm x 0.5 cm. Sample sizes larger than this may take longer to flash freeze in LN2, resulting in diminished sample integrity. Larger specimens can be further sectioned under sterile conditions by the CFR technician (appendix 1 (http://epi.grants.cancer.govbio_fresh_tissue_app.html#ap1)).

g. Place the tissue samples provided by the pathologist into a sterile culture dish or other sterile dish that has been placed on ice for further sectioning. Specimens must be kept sterile, hence avoid contact of specimens with ice. *Always place tumor and normal tissue samples in separate dishes*.

h. See appendix 1 (http://epi.grants.cancer.govbio_fresh_tissue_app.html#ap1) for tissue sectioning procedure.

VIII. **Specimen Processing and Storage**

a. Tissue processing should be done in the pathology receiving room next to the operating theatre if possible. Expediting the processing is essential in order to ensure tissue integrity, which is the primary goal of the fresh frozen tissue sub-study. Ideally, the tissue should be processed within a few minutes after the surgical resection.

b. Tissue samples can be processed by a number of different methodologies, including flash freezing in liquid nitrogen, embedding the sample in OCT medium for subsequent frozen tissue sectioning, immersion and refrigeration in an RNA-preserving reagent followed by flash freezing 24 hours later, or immersion in a commercial fixative to provide for paraffin-embedded samples.

c. Flash freezing in liquid nitrogen provides excellent specimen integrity and a wide array of options for tissue analysis. The specimen is placed into a sterile 1-2 ml cryovial, which is then tightly capped and submerged in liquid nitrogen for "flash freezing". The vial is then transferred from the temporary LN2 transport container to a liquid nitrogen storage tank for long-term storage.

d. Embedding in OCT embedding compound followed by flash freezing not only preserves DNA, RNA, and protein integrity, but also enables sectioning of the frozen tissue. In this method, the OCT compound is poured into a small plastic tray, the tissue sample is submerged in the compound, and the tray is placed on dry ice or in the vapor phase of liquid nitrogen for freezing. After hardening of the OCT, the tray is transferred to a liquid nitrogen storage tank or -80 C freezer for storage. The advantage of this method is that the frozen OCT containing the specimen can be placed into a cryo-microtome chuck and sectioned. Frozen sections can be mounted on microscope slides and stained to characterize the tissue sample and determine the tumor/normal ratio, and if need be microdissected to isolate specific cell populations.

e. Preserving the tissue in formalin enables the embedding of specimens into paraffin blocks. The advantage of this method is that sections obtained from the block can be stained with hematoxylin and eosin (H&E) for optimal tumor characterization. In addition, sections can be used for IHC and other immunohistochemical analyses.

f. **When surplus tissue is limited, the priority for specimen collection is:**

Priority	Specimen Collection
First priority:	Fresh frozen sample
Second priority:	Formalin fixation

Third priority:	OCT embedded sample
Fourth priority:	RNA stabilizing reagent

IX. Specimen Tracking

a. Following processing and storage of specimens, information must be manually entered into the laboratory fresh frozen tissue log. The following data must be recorded:
    a. Patient's name
    b. Patient FR identification number
    c. Patient age
    d. Patient gender
    e. Patient race
    f. Hospital at which procedure was performed
    g. Date of surgery
    h. Medical Record number
    i. Pathology number - biopsy
    j. Pathology number - resection (procedure from which frozen tissue was obtained)
    k. Name of surgeon
    l. Histological type
    m. Site of tumor
    n. Types of tissue collected (tumor - normal)
    o. Amount of tissue collected
    p. Sample preparation (flash frozen, OCT, fixative etc.,)
    q. Cryovial/specimen label identifiers
    r. Storage location of each sample
b. Information from the log is entered into the CFR tracking system. The laboratory log is stored in a locked cabinet. A sample log is included in the appendix 2.

X. Safety Precautions

a. Universal precautions is a method of infection control in which all human blood and body fluids are treated as if they are infectious for Hepatitis Viruses, Human Immunodeficiency virus, and other known and unknown infectious agents.

b. Hepatitis B and Hepatitis C viruses may be transmitted through blood and other body fluids, and is associated with acute hepatitis, chronic liver disease, and hepatocellular carcinoma in humans. The Centers for Disease Control estimates that approximately 12,000 health care workers become infected with Hepatitis B each year. Of these, 3,000 will become chronically ill, 600 will require hospitalization, and 200 will die. The probability of seroconversion after needlestick exposure is estimated at 7%. Untreated virus can persist for up to one week at room temperature. All Family Registry staff who work with human tissue must provide evidence of Hepatitis B vaccination.

c. Human Immunodeficiency virus (HIV) is a retrovirus that affects a subset of lymphocytes, causing severe

immunodeficiency. Infection increases the risk of developing malignancies, infection by opportunistic organisms, and death. The probability of seroconversion after needlestick exposure is estimated at 0.5%. Infectivity of untreated virus persists for up to one week at room temperature.

d. Other potentially infectious agents, both known and unknown, pose hazards to those working with human tissue. Included are tuberculosis, HTLV1, Coccidiomycosis, Creutzfeldt-Jacob disease, amongst others.

e. Personal protective equipment (PPE) must be used at all times while working with human tissue. These include disposable latex or nitrile gloves, face shield, protective splash-resistant laboratory coat (disposable preferred), and covered protective shoes.

f. Gloves should be immediately removed and replaced in the event that they become torn or perforated. Gloves must be removed prior to leaving the work area, and disposed of in an appropriate waste disposal container. Hands must be washed in a "clean" sink after removal of gloves.

g. Face shields, goggles and masks should be worn whenever a potential for exposure to splashes, spray, splatter, droplets, aerosols of blood or tissue fluid, or other potentially infectious materials may be generated, and if there is a potential for eye, nose or mouth contamination. They should be worn at all times while handling tissue in the pathology receiving room and in the processing/storage laboratories.

h. Protective lab coats, preferably disposable types, must be donned while working with tissue. Contaminated clothing must be removed prior to leaving the work area, and appropriately laundered or discarded. See your institutional guidelines for additional information.

i. Shoes must cover the entire foot, and be fabricated from a material that is puncture-resistant and fluid-resistant.

j. All waste must be disposed of prior to leaving the work area. Biohazardous sharps must be properly disposed of in an approved "sharps" container. All other non-sharp waste must be disposed of in an approved orange or red biohazardous waste disposal bag. Do not leave waste for the pathology receiving room staff to dispose of.

k. After completion of work with human tissue, all work surfaces must be disinfected with a product that has been demonstrated to be effective against bacteria, viruses, pseudomonas, tuberculosis and fungi. Refer to the product literature for appropriate use.

l. Refer to your institutional and OSHA guidelines for additional information on bloodborne pathogens, laboratory safety, chemical safety, and biohazardous waste disposal.

m. Any injuries or exposure to human tissue or potentially infectious biologic agents must be reported promptly as specified in your institutional safety guidelines.
