Video Article

# Working with Human Tissues for Translational Cancer Research

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## Abstract

Medical research for human benefit is greatly impeded by the necessity for human tissues and subjects. However, upon obtaining consent for human specimens, precious samples must be handled with the greatest care in order to ensure integrity of organs, tissues, and cells to the highest degree. Unfortunately, tissue processing by definition requires extraction of tissues from the host, a change which can cause great cellular stress and have major repercussions on subsequent analyses. These stresses could result in the specimen being no longer representative of the site from which it was retrieved. Therefore, a strict protocol must be adhered to while processing these specimens to ensure representativeness. The desired assay(s) must also be taken into consideration in order to ensure that an optimal technique is used for sample processing. Outlined here is a protocol for tissue retrieval, processing and various analyses which may be performed on processed tissue in order to maximize downstream production from limited tissue samples.

## Video Link

The video component of this article can be found at https://www.jove.com/video/53189/

## Introduction

Medical research has benefited immensely from the study of both human cell lines and animal models. Cell lines allow for better control of components in a system, as well as use of cells from the proper organism. Working with human cells, while more representative, presents a drastically simplified snapshot of mechanisms which may be responsible for health and disease, because cell lines may not recapitulate the impact of other cells, stromal components, and organs which may affect responses[1]. On the other hand, animal models, although not entirely accurate in reproducing human disease and genetic heterogeneity, offer further insight by allowing for input of whole animal systems in most models[2,3].

Shortcomings aside, both methods have their benefits and are complementary. However, the main goal remains to study human cells and organs, in a full body, multi-organ system. Accordingly, translational research has taken great strides in facilitating the study of human organs and tissues extracted from patients, in order to better grasp the mechanisms underlying disease. Translational research offers the advantage of studying the result of treatments and diseases in the entire body and in appropriate cells, thereby providing the best of both worlds between cell and animal work[4].

Translational research is also not without flaws. Harvested specimens from human patients, upon removal from the host, are immediately subjected to ischemia and other external factors from which they would be otherwise protected. This may result in stress-induced molecular and genetic changes, and cause bias in subsequent studies. Therefore, much effort has been put into extracting and processing tissues through strict methods to best preserve organ and cell integrity for downstream studies[5]. Importantly, future applications must be considered closely when selecting methods of processing human samples, as certain methods may be detrimental to cellular components and signaling pathways while sparing others.

For any research involving human tissues, regulatory issues must be addressed. After the Tuskegee and Belmont reports, there was a growing acceptance of the fact that biomedical research was associated with inherent risks often resulting in unavoidable ethical dilemmas[6]. The need for national standards was recognized and the federal government created Institutional Review Boards (IRBs) as a means to uphold the principles of the Belmont Report (Respect for persons, Beneficence, Justice).

Any institution's IRB is a committee of doctors, researchers and community members who are responsible for protecting research participants. It requires that voluntary consent be obtained from all participants of biomedical research studies, and that this consent be documented in an

 

informed consent form. The informed consent process aims to provide adequate information to a participant, so that an informed decision may be made on whether or not to enroll in a study, or to continue participation. In this regard, the informed consent document must have good readability and should be written in language that can be easily comprehended ($6^{th}$ to $8^{th}$ grade reading level) by the target population. The possibility of coercion or undue influence must be minimized, and the subject must be given ample time to consider participation. The participant must also be allowed to exercise their right to withdraw consent at any stage during the course of the study without penalty. Voluntary informed consent is a mandatory pre-requisite for a subject's participation in research and it is also legally effective[7].

As per the regulations for the protection of human subjects 21 CFR 50.25 (http://www.hhs.gov/ohrp/humansubjects/guidance/45cfr46.html[8]), study participants must be informed of the purpose of the research, procedures involved in the research, alternatives to participation, all foreseeable risks and discomforts (including not only physical injury but also possible psychological, social, or economic harm, discomfort, or inconvenience), benefits of the research to society and possibly to the individual, length of time the subject is expected to participate, person to contact for answers to questions or in the event of a research-related injury or emergency, statement indicating that participation is voluntary and that refusal to participate will not result in any consequences or any loss of benefits such as a compromise in regular clinical care that the participant is otherwise entitled to receive as a result of his/her diagnoses, and finally a statement regarding the subject's right to confidentiality and right to withdraw from the study at any time without any consequences. Tissue specimens from participants who withdraw consent during the course of the study should not be banked and must be immediately exhausted. Waiver of one or more elements of informed consent may be obtained from the IRB for some research projects that could not practically be done without an alteration to the required elements or for studies where required elements are not applicable. Great care must be taken to ensure data confidentiality. The Health Insurance Portability and Accountability Act (HIPAA) is a federal law that prevents using or disclosing “Protected Health Information” (PHI) without written consent from the participants. PHI includes but is not limited to health information transmitted or maintained in any form or medium and includes fields that could be used to identify an individual[9].

Through this work, we aim to outline the protocol that must be followed during tissue processing - including procurement, and storage and emphasize the importance of following these strict guidelines so as to minimize the biases that may result due to the stresses a tissue is subjected to upon extraction. We also endeavor to provide a brief overview of the downstream assays or analyses (**Figure 1**) that could be performed on such specimens so that readers may make a qualified judgment on what assay(s) best suits their needs.

## Protocol

Ethics statement: This protocol is approved by the University of Texas MD Anderson Cancer Center.

## 1. Specimen Procurement

1. Collect research tissue samples from surplus tumor and related normal tissue[10-12]. Store specimens on wet ice to avoid autolysis.
2. Send all tissues, medical devices and foreign bodies removed during a surgical procedure to the Department of Pathology for necessary examination in order to facilitate a pathologic diagnosis.
3. Handle tissue allocated for research in accordance with applicable research and tissue banking policies and procedures.
   NOTE: Biospecimen collection involves the coordination of pathologists, pathologists' assistants, histotechnologists and tissue procurement specialists.

## 2. Pathology Quality Assurance (QA)

1. Complete and verify patient records as an early QA measure and consult a pathologist to review diagnostic patient records (pathology reports, tissue slides, etc.) and tissue samples. Judge tissue samples on morphological characteristics of interest such as percentage of tumor, necrosis, tissue-tumor interface, etc. This is in accordance with biorepository best practices[10-12].
2. Following initial review, conduct periodic reviews of biorepository data and research samples during the entire length of stay of the biospecimen.
3. Link results and annotations from histopathological and other investigations to the samples and provide this data to the researchers through a proprietary biorepository software system.
   NOTE: QA processes ensure the integrity of both the clinical and technical aspects of biorepository operations.
4. Perform genetic and molecular tests to verify and characterize the samples for clinical use.
5. Collect and analyze data based on feedback from researchers who have been using the samples for laboratory experiments. Track samples using barcode technology. This electronic tracking system is also advocated in biorespository best practices[10-12].

## 3. Specimen/Tissue Processing

1. Always store fresh tissue specimens on wet ice, and process as quickly as possible, in order to limit changes induced by extraction of the tissue sample from its natural environment. Process normal tumor-matched and tumor tissue samples separately to avoid cross-contamination.
   NOTE: Based on research protocols, biospecimen processing can involve collection of the specimens in one or more types of media.
2. Before beginning the process, prepare a sterile Petri culture dish, a scalpel, as well as a needle or forceps for tissue processing. Ensure that tubes have been prepared, labeled, and laid out in order to limit time of sample processing (**Figure 2**).
3. All relevant tissue information, such as patient name, number, treatment, date of surgery, and other relevant information as may be appropriate for the study, should be made readily accessible in an institutional tissue database. Limited patient information should be held in the lab in paper or spreadsheet form.
   NOTE: Only collect biospecimen information relevant to the nature of the research viz., basic biomedical research, clinical research, translational research, etc. For disease-specific information, the biorepository staff should collect data deemed necessary by the current

literature and governing organizations to ensure proper diagnosis and meaningful research. This includes but is not limited to tumor grades and cancer staging information.

4. Plan all desired subsequent experiments in advance, to decide on the size of tissue sections required for each type of analysis, as well as the location of excisions. Ideally, although impossible, all analyses should be representative of the entire tissue section in order to account for tissue heterogeneity and limit bias of downstream analyses. Lay out all required items in advance as depicted in **Figure 2**.
5. Place the tissue specimen in the open Petri culture dish, flat against the surface.
   NOTE: Allow only authorized biorepository staff to handle identified samples and to review identified health data. Investigators should access only those patient identifiers as allowed by the signed informed consents and as regulated by the IRB. All specimens must be adequately labeled and should include at minimum, a unique tissue identification number and tissue type. Do not include any PHI in the labels, in accordance with HIPAA regulations.
6. Inspect the sample from all angles, to gain a better understanding of its structure and dimensions. Pin the sample to the bottom of the dish with the needle, and proceed to bisect the tissue along its largest and best representative axis.
7. Cut out a small piece of tissue and place it in a sterile 1.5 ml microcentrifuge tube containing 1ml of RNA-stabilizing solution. This sample can be placed on ice for the remainder of the process and stored at 4 °C until needed.
   NOTE: When working with RNA, gloves should always be worn, and RNAse-free filter tips should be used to limit chances of degradation and RNA contamination.
8. Lay out an open and pre-labeled biopsy cassette in the lid of the Petri culture dish. Cut out a slice of tissue no thicker than 3 to 5 mm and transfer it to the cassette. Place the cassette in 10% Neutral Buffered Formalin (NBF) for a minimum of 72 hr. Transfer the cassette in 70% ethanol for long-term storage before paraffin-embedding.
   NOTE: Labeling of cassettes should be carried out in pencil or with recommended markers as use of xylene during processing will otherwise erase labels.
9. Cut out a piece of tissue and place it in a tissue mold. Cover the tissue section in optimal cutting temperature (OCT) compound, and freeze it immediately at -20 °C for cryosectioning. This tissue section may be stored at -20 °C until ready for cryosectioning.
10. Cut out one or many pieces of tissue and transfer them to a 50 ml tube containing MACS buffer (1x phosphate buffered saline (PBS), 0.5% fetal bovine serum (FBS), 2 mM Ethylenediaminetetraacetic acid (EDTA)) for subsequent flow cytometry analysis. For flow cytometry, larger samples may be preferable as cells may be lost during tumor disassociation, cell fixation and permeabilization when required. Generally, this section may be taken at the end, once all other pieces have been processed. Leftover representative tumor sample may be used for flow cytometry. Optionally, this tube may be stored at 4 °C O/N or used for flow cytometric staining immediately following digestion into a cell suspension (see Step 4 - Flow Cytometric Staining).
11. Cut remainder of tissue into pieces no larger than a maximum size of 0.5 mm x 0.5 mm and transfer them to cryogenic vials for immediate freezing in liquid nitrogen. These snap-frozen samples may be used for future analyses and collaborative purposes.
    NOTE: Extra care should be taken when working with liquid nitrogen as direct contact can cause severe injury. Eyes should be protected with safety glasses with side shields or with a face shield. Cryogenic gloves should also be worn to protect from liquid nitrogen burns caused by splashing and direct contact. Gloves should fit properly but be loose enough to be removed quickly should liquid nitrogen splash into them.

## 4. Flow Cytometric Staining

1. **Tissue Homogenization**
   1. Prepare digestion medium with DMEM (40 ml), DNase I (50 U/ml) and Collagenase I (2 mg/ml). Place fresh or O/N-stored tumor in MACS buffer in the lid of a Petri dish, and submerge with digestion medium.
      NOTE: Different digestion methods exist, each with advantages (*i.e.*, cell surface protein expression, viability). Ensure you have the proper method for your desired downstream application.
   2. Pin tumor to the bottom of the dish with a needle, and cut from the outside to center with a scalpel, until the tumor has a paste-like texture. Transfer tumor mix to a 50 ml tube, seal and place in a plastic bag to avoid leaks. Transfer tube to an incubator, and rotate at 225 RPM, 37 °C, 1 hr.
   3. Filter tumor cell suspension through a 70µm cell strainer. Centrifuge cell suspension at 250 x g, 4 °C, 5 min. Resuspend cell pellet in 1 ml FACS buffer (500 ml PBS, 1 ml 0.5 M stock EDTA, 10 ml FCS or FBS).
   4. Count cells using a hemocytometer and re-suspend at a concentration of $1x10^7$ cells/ml. Transfer 100 µl per well ($10^6$ cells) in a 96 well round bottom plate for staining.

2. **Viability and Antibody Staining**
   1. Add 200 µl of viability stain solution to each well and mix. Incubate for 30 minutes, at 4 °C, in the dark. Spin down cells at 250 x g, 4 °C, 5 min, and aspirate supernatant.
   2. Add antibody mixture at recommended/titrated concentration (as per manufacturer) for surface antigens in 200 µl of FACS buffer. Incubate cells at 4 °C, 30 min, in the dark. Spin down cells at 250 x g, 4 °C, 5 min, and aspirate supernatant.
   3. Wash with 200 µl of FACS buffer, spin down cells at 250 x g, 4 °C, 5 min, and aspirate supernatant. Resuspend cells in 200 µl of FACS buffer and proceed to flow cytometer for acquisition and analysis.
      NOTE : If performing intracellular flow cytometry staining, perform fixation and permeabilization steps and intracellular staining between steps 4.2.2 and 4.2.3. If performing cytokine staining, cells should be pre-activated preceding staining as recommended, and an inhibitor of intracellular protein transport and secretion such as monensin should be used during activation to ensure retention of soluble factors. Fixed cells may be stored short-term (one week) at 4 °C in the dark prior to acquisition on a flow cytometer.

## Representative Results

The results displayed below depict flow cytometry gating strategy for broad immune phenotyping on a processed melanoma tumor. The day of staining, tissue sample was digested and homogenized with collagenase I and DNase I incubation for 60 min, at 37 °C under 225 RPM rotation. Following digestion, cell suspension was filtered through a 70 µm cell strainer to eliminate debris and cells were stained with fluorescence-labeled flow cytometry antibodies for immune cell phenotyping. Shown below (**Figure 3**) is the gating strategy and staining for multiple markers on tissue stored O/N at 4 °C in MACS buffer. As shown, specimens processed as described and stored O/N in MACS buffer at 4 °C show extremely limited mortality. Furthermore, this figure demonstrates that processing tissue as described above allows multi-color broad phenotyping of immune cell subsets in melanoma tumors.

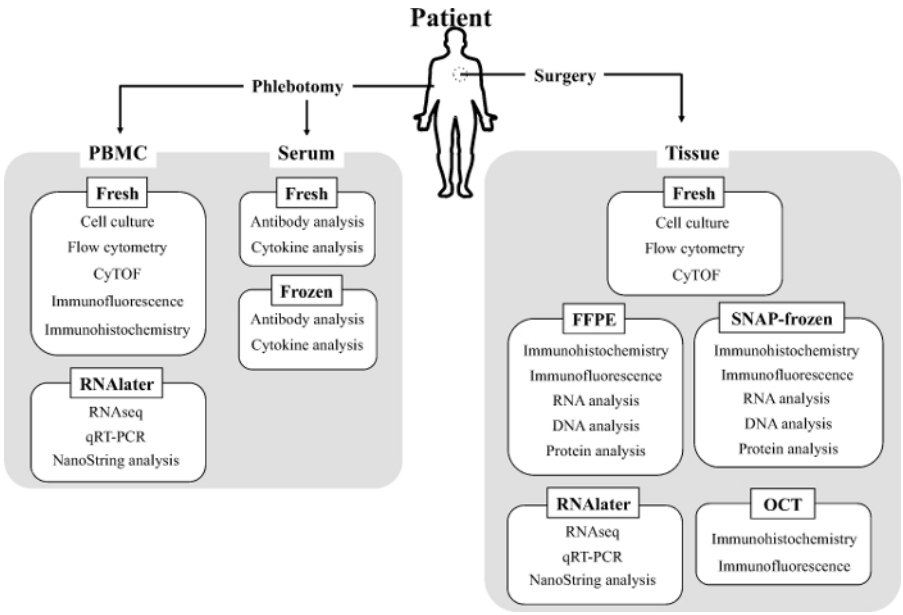


**Figure 1. Analyses which may be carried out from human blood and tissue samples.** Overview of assays which may be performed following processing. Please click here to view a larger version of this figure.

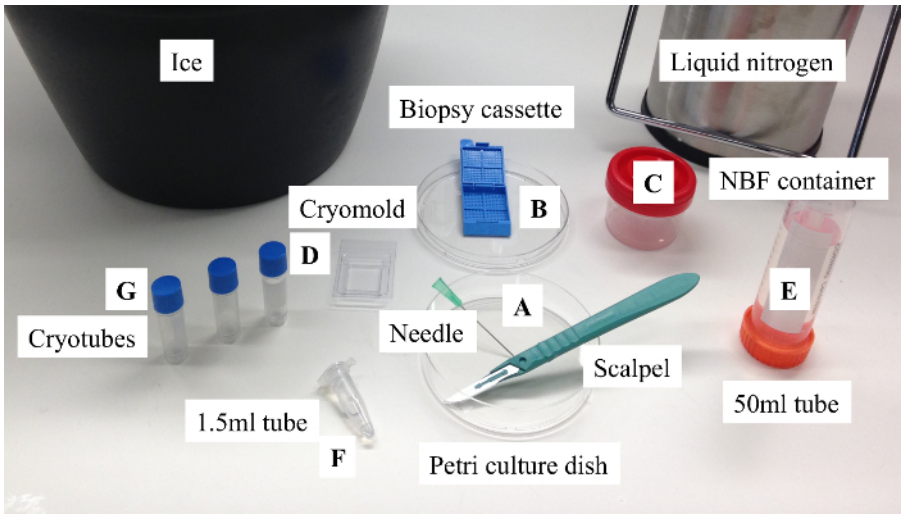


**Figure 2. Items required for proper tissue processing and storage.** Example of setup for tissue processing. Tissue may be dissected in the Petri culture dish (**A**) with the needle and scalpel. Representative pieces of tissue should then be transferred to the biopsy cassette (**B**) which should then be transferred to a NBF container (**C**) for three day fixation at RT. Another piece of tissue should be placed in a cryomold (**D**) and covered in OCT solution before being frozen at -20 °C. A large piece of tissue should be transferred to a 50 ml tube (**E**) for flow cytometry staining and may be stored at 4 °C in MACS buffer O/N. A small piece of tissue should be transferred to a 1.5 ml tube with RNA-stabilizing solution (**F**) and stored at 4 °C until RNA extraction. Finally, leftover tissue should be cut into pieces and transferred to cryotubes (**G**) for snap-freezing in liquid nitrogen and long-term storage. Please click here to view a larger version of this figure.

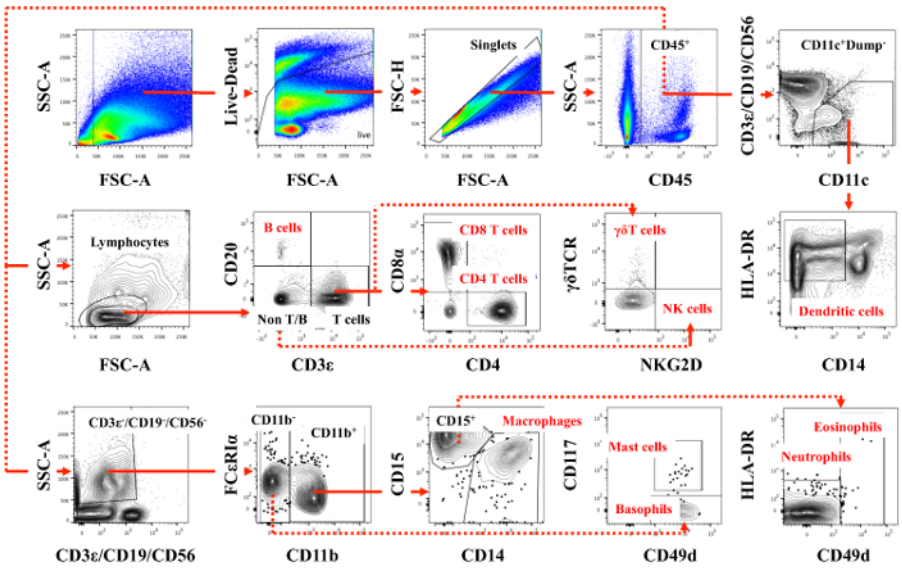


**Figure 3. Flow cytometric gating strategy for broad immune phenotyping.** Example of broad immune phenotyping gating strategy by flow cytometry on melanoma tumor stored O/N at 4 °C in MACS buffer[13]. Please click here to view a larger version of this figure.

## Discussion

Ultimately, the methods of processing must be dictated by the desired hypothesis and anticipated technical assays to be performed (**Figure 1**). The following section serves as an overview to describe potential assays that may be performed on such tissue sections. It is by no means an exhaustive overview, but is intended to help guide sample processing methods and choice of downstream assays by describing techniques and listing their strengths and weaknesses.

Flow cytometry staining may be performed on freshly-dissected tissue sections, or fragments stored O/N in MACS buffer at 4 °C. Flow cytometry facilitates highly specific phenotyping and analysis of cell populations extracted from tissue samples, and allows staining of up to 17 cell surface markers and intracellular proteins, cytokines and proteases in a single cell. Furthermore, certain cytometers allow single cell magnification, in order to determine localization of proteins within the cell rather than solely positivity and intensity of signals. Finally, mass cytometry allows for the quantification of more than 35 proteins in the same sample, with the downside being that cells must be lysed and therefore may not be further cultured, sorted, or analyzed as a whole[14]. This technique allows analysis of many markers and proteins as well as detailed characterization at a single cell level. However, flow cytometry requires tissue homogenization and loss of tissue architecture and must be performed on fresh tissue to minimize changes in cell phenotype. Importantly, delayed storage of tissue intended for flow cytometry may impact cell content, as some cell subsets may be more vulnerable to ischemia. Phosphoproteins may also be poorly detected by flow cytometry if processing kinetics are suboptimal[15].

Formalin-fixation and paraffin-embedding (FFPE) of tissue sections may be the best choice in case of uncertainty regarding future analyses, because it allows high flexibility in the types of assays which may be performed. Traditionally, FFPE sections are sectioned on a microtome into 5 µm sections for immunohistochemistry (IHC) or immunofluorescence (IF) staining. However, FFPE tissues are well-conserved, and may therefore be used for multiple purposes. In fact, cutting thicker sections from FFPE blocks allows both DNA and RNA extraction with commercially-available kits, as well as reverse-phase protein array (RPPA) analysis[16-18]. Accordingly, processing tissue samples into FFPE may offer the advantage of added flexibility, as well as the advantage of maintaining tissue structure for immunostaining and therefore providing a more accurate *in vivo*-like depiction of tissue structures and cell populations. FFPE allows for tissue sections to be conserved for long periods of time. This technique does, however, have some disadvantages. Thin sections used for FFPE do not account for tissue heterogeneity from sequential cuts and tissue sections begin to oxidize once exposed to ambient air thereby impairing RNA and DNA quality. Finally, due to crosslinking, co-staining of multiple proteins by IHC on FFPE sections requires optimization.

When microscopy through IHC or IF is desired, use of an OCT compound is preferable. OCT-stored sections must be cut with a cryotome before being placed onto slides and used for IHC and IF. OCT preserves antigenicity and minimizes background making it the optimal technique for microscopy. OCT also allows immediate freezing and cutting compared to FFPE which requires several days in NBF. Despite these benefits, OCT is generally less stable and less flexible than FFPE because only IHC and IF may be performed from OCT-embedded tissue sections.

With regards to RNA analysis, samples stored at 4 °C in RNA-stabilizing solution harden over time, allowing better RNA extraction through commercially-available extraction kits and TRIzol, among others. RNA extraction permits downstream analyses such as reverse-transcription polymerase chain reaction (RT-PCR) to quantify expression of transcribed genes, RNA sequencing analysis, and broad spectrum analysis of modulated genes by microarray, or nanoString analysis. Accordingly, RNA isolation from tissues can be extremely high-yield, by providing extensive information on gene expression and pathways affected by therapies and diseases. Importantly, however, RNA expression may vary drastically based on tissue structure and sampling size, so extra care should be taken to avoid biasing analyses due to sampling location. Furthermore, the nature of RNA suggests that dynamic changes in expression levels may occur, so extra care should also be taken to ensure proper kinetics are followed to study genes and pathways of interest[19,20]; processing should be limited following extraction from RNA-stabilizing solution to ensure stability. Accordingly, storage of complementary DNA (cDNA) of increased stability is preferred to RNA (if in line with

downstream analysis)[21]. Finally, because RNA may often be amplified in downstream analyses, minute fractions of contaminating cells may result in large biases in obtained results.

DNA analysis may be carried out on previously snap-frozen tissue samples, or on FFPE tissue sections. These methods allow subsequent whole exome or whole genome sequencing, two techniques which have shown immense promise through identification of mutations and polymorphisms linked to patient response and prognosis in multiple disease contexts. DNA extraction may further allow identification of T-cell and B-cell receptor (TCR and BCR) sequences present in samples, in order to acquire knowledge pertaining to antigens and immune response in therapy and disease[20,21]. Compared to RNA, DNA is highly stable once extracted, however it does take into account transcriptional and translational regulation of genes as well as post-translational modifications and regulation[22].

Protein may be extracted directly from snap-frozen tissue sections, or from FFPE samples in order to perform Western blot analyses, co-immunoprecipitation to identify protein interactions and networks, as well as RPPA, a high-yield technique allowing relative quantification of hundreds of proteins in a single sample[23]. However, this technique allows identification of known proteins only through its requirement of specific antibodies. Importantly, further post-translational modification analysis, such as protein phosphorylation, requires rapid processing of tissue due to instability[15].

Snap-freezing of samples is simple and requires limited resources. It also allows long-term storage of samples when downstream analyses have not yet been defined, except in the case of flow cytometry which must be performed on fresh samples. Snap-frozen samples may also easily be shared for collaborative purposes.

Ultimately, the amount of data which may be extracted from a single piece of tissue is endless. There is no universal right answer to which assays should be performed in any given study. Each investigator must therefore ensure that he/she takes into account all hypotheses, desired experiments as well as available resources before recruiting patients and processing tissue, as this may dramatically affect the design of studies and the answers which are obtained.

## Disclosures

The authors have no conflicts of interest to disclose.

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