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## Subcellular fractionation from fresh and frozen gastrointestinal specimens

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Vineeta Bajaj, Ph.D.  
Review Editor  
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Dear Dr Bajaj,

We are resubmitting our manuscript entitled "Subcellular fractionation from fresh and frozen gastrointestinal specimens" for publication in JoVE. In this manuscript, we describe a protocol for a simple cellular fractionation method for the subcellular separation of cytoplasmic and nuclear proteins in human fresh and frozen intestinal biopsies. We have attached the modified video and manuscript together with a document addressing editorial comments. I hope you will find everything in order, but please let me know if you need anything else.

I look forward to hearing from you.

Sincerely,

Ainara Castellanos-Rubio

**TITLE:**

Subcellular Fractionation from Fresh and Frozen Gastrointestinal Specimens

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**KEYWORDS:**

Cellular fractionation, nucleus, cytoplasm, intestinal biopsy, protein, fresh tissue, frozen tissue

**SUMMARY:**

Here, we present a protocol to perform a simple cellular fractionation for the subcellular separation of cytoplasmic and nuclear proteins in human fresh and frozen intestinal biopsies.

**ABSTRACT:**

The purpose of this protocol is to fractionate human intestinal tissue obtained by endoscopy into nuclear and cytoplasmic compartments for the localization analysis of specific proteins or protein complexes in different tissue states (*i.e.*, healthy vs. disease). This method is useful for the fractionation of both fresh and frozen intestinal tissue samples; it is easily accessible for all laboratories and not time consuming.

**INTRODUCTION:**

Proteins participate in almost all biological functions within a cell and any variation in their structure, quantity or location can lead to a pathogenic scenario. Tissue sample fractionation methods are a useful approach to reduce the complexity of disease related protein analyses. Some studies use protein localization information or enrich a protein from a specific cellular compartment, so a protocol for reproducible fractionation of intact proteins is useful to answer certain biological questions. Determining the subcellular localization of proteins and monitoring

their compartmental redistribution or interactions at basal and disease conditions will help identify disease related functional differences<sup>1,2</sup>. Thus, this method aims to reproducibly fractionate intestinal tissue biopsies, both fresh and frozen, into cytoplasmic and nuclear subcellular compartments.

Intestinal biopsy samples are routinely obtained during endoscopy procedures<sup>3</sup> (**Figure 1**) and can be effectively used for protein quantification or immunoprecipitation studies. Since tissue samples from intestinal epithelial will contain proteins that may be directly involved in the disease pathogenesis<sup>4</sup>, intestinal biopsy samples are a very valuable source for the successful identification of intestinal disease-specific proteins. Stored frozen, patient tissue samples together with the clinical information are useful resources for protein analysis, and a simple and reproducible sample preparation is a key issue to provide cell compartmental information using limiting amounts of frozen tissue<sup>5</sup>.

There are several commercially available kits for the separation of cellular fractions, but these are more expensive and generally more time consuming than the protocol presented here. Protocols based on multiple-step gradient centrifugation and continuous gradients have also been previously used for the fractionation of diverse cellular compartments in different tissues<sup>6,7</sup>. However, maintaining the consistency of continuous gradients is often a difficult task. Similar protocols based on the sequential lysis of membranes have been previously described but they generally need more than two buffers and the hands on time is longer<sup>8</sup>.

When fractionating tissue samples, bear in mind that tissue samples present cell-cell and cell-matrix interactions that are both not present in cultured cells and important when proceeding to protein extraction. The proper breakdown between cells and extracellular matrix without affecting the protein quality will be a critical factor in the intestinal tissue protein isolation<sup>9</sup>. In this protocol, cell-cell and cell-matrix contacts are first broken, releasing the cells and allowing the buffer to reach the individual cells. To avoid protein-compartment mixing prior to fractionation, the breakdown of the contacts must occur without altering the integrity of the cells or the nuclear membranes.

Due to the enrichment of various proteases in the intestinal mucosa<sup>10</sup>, it is important to control the protein degradation during extraction. To minimize the potential protein degradation, multiple protease inhibitor must be included in the protein extraction buffers. Additionally, if extracts are going to be used for functional assays, it is essential to avoid the denaturation of proteins or proteolysis as this will cause a loss of protein activity<sup>11</sup>. For this purpose, the protocol is performed at 4 °C and fresh phenylmethylsulfonyl fluoride (PMSF) is added to the buffers at a final concentration of 0.1 mM just prior to use to further inhibit proteolysis.

The first step in cell fractionation is tissue disruption and cell lysis. As mentioned previously, the objective is to disaggregate the cells and break them open with minimum damage. Intestinal tissue samples must be homogenized, and the cells lysed to achieve maximum breakage of the cell membrane. In this protocol, we use a motorized pestle mixer to break the intestinal tissue which is homogenized within seconds by means of high speed vortexing action. After

homogenization, tissue cells are incubated in a hypotonic buffer that will burst the cell membrane but will keep the nuclei intact, followed by addition of a non-denaturing detergent (nonyl phenoxypolyethoxylethanol) and a short vortex to separate nuclei from the cytoplasmic fraction. After the removal of the cytoplasm, the cell nuclei membrane is burst into a hypertonic buffer with shaking.

The method presented here is an appropriate method for the subcellular fractionation of fresh and frozen intestinal biopsies that have been obtained during endoscopic procedures and range between 2 and 10 mg in weight. The protocol is easy and reproducible and could be performed using basic laboratory equipment and reagents in under an hour.

## **PROTOCOL:**

This study was approved by the Cruces University Hospital Ethics Board and analyses were performed after informed consent was obtained from all subjects or their parents.

### **1. Biopsy Collection**

Note: Biopsy specimens from the distal duodenum of patients are obtained during routine diagnosis endoscopy managing biopsies that range between 2 and 10 mg in weight. Biopsied tissue is a complex tissue composed of different cell types including epithelial, immune and endothelial cells. Clinical gastroenterologists perform the endoscopy following established clinical guidelines.

1.1. Place the biopsy on a sterile filter paper just after endoscopy.

1.2. Use forceps to transfer the biopsies in 1.5 mL tubes, soak them with PBS, and place it into a cryotube.

1.3. Keep tubes on ice until they arrive at the laboratory.

1.3.1. Start processing of fresh biopsies immediately.

1.4. For frozen biopsies, flash freeze the biopsy containing tubes and store in liquid nitrogen until use.

Note: It is important to keep the sample frozen until the cold supplemented buffer 1 is added.

### **2. Buffer Preparation**

Note: Before starting, supplement the buffers as follows.

2.1. Add 1 mM DTT (final concentration) and protease and phosphatase inhibitor cocktail (1x final concentration) to the hypotonic buffer 1 and hypertonic buffer 2 (**Table 1**).

2.1.1. Prepare 1.5 mL of buffer 1 and 150  $\mu$ L of buffer 2 per sample. DTT will prevent the oxidation of the samples.

2.2. Prepare nuclei wash buffer by adding 1% detergent (nonyl phenoxypolyethoxylethanol) to a final concentration of 0.1% to the already supplemented buffer 1. Prepare 1 mL of nuclei wash buffer per sample.

### **3. Biopsy Homogenization**

#### **3.1. Fresh Biopsies**

3.1.1. Place the disposable pestle in the motorized mixer. Ensure that the pestle reaches the bottom of the tube.

3.1.2. Take the biopsy out from the cryotube and place it in a 1.5 mL tube with a pipette tip.

3.1.3. Add 200  $\mu$ L of supplemented buffer 1 to the 1.5 mL tube.

3.1.4. Homogenize the biopsy with the pestle until the tissue is completely disrupted and keep it on ice.

3.1.5. Once all the biopsies are homogenized proceed with step 4.

Note: Disposable pestles must be changed between biopsies.

#### **3.2. Frozen Biopsies**

CAUTION: Handle liquid nitrogen carefully; contact of liquid nitrogen with the skin or eyes may cause serious freezing injury. Protect hands and eyes always when working with liquid nitrogen.

3.1.1. Take some liquid nitrogen in a box to store the cryotubes retrieved from the nitrogen tank.

3.1.2. Find the biopsy in the nitrogen tank and place the cryotube in the box with the liquid nitrogen. Repeat this step for all the biopsies.

3.1.3. Transfer the biopsy from the cryotube to a 1.5 mL tube.

Note: Biopsies will be attached to the frozen tube. Push the biopsy with a sterile pipette tip so it will detach from the tube.

3.1.4. Add 200  $\mu$ L of supplemented buffer 1.

3.1.5. Homogenize the tissue using the pestle and mortar until the tissue is completely disrupted, maintaining the temperature at 4 °C throughout all the procedure.

Note: Do not let biopsies thaw before adding the buffer.

#### **4. Cytoplasm Isolation**

4.1. Incubate the homogenized biopsy on ice for 10 min.

4.2. Add 10 µL of 1% detergent (nonyl phenoxypolyethoxylethanol) to each sample to a final concentration of 0.05%.

Note: Cells will swell and burst by osmotic lysis as the mild detergent will disrupt the cell membrane.

4.3. Incubate on ice for 5 minutes.

4.4. Vortex briefly and centrifuge at 400 x g at 4 °C for 2 min.

4.5. After vortexing, remove the supernatant and transfer it to a new clean 1.5 mL tube ; this will be the cytoplasmic fraction. Do not discard the pellet.

#### **5. Nuclear Isolation**

5.1. Resuspend the pellet from the previous step with 200 µL of nuclei wash buffer.

5.2. Centrifuge the tube for 2 min at 400 x g and 4 °C.

5.3. Discard the supernatant and repeat the washing procedure two more times.

Note: The washes will remove cytoplasmic contamination in the nuclear fraction.

5.4. After the third wash, resuspend the pellet in 100 µL of buffer 2.

5.5. Shake the sample vigorously at 4 °C for 30 min. Alternatively, incubate the sample on ice and vortex every 5 min.

5.6. After shaking, centrifuge the sample for 10 min at top speed (> 12,000 x g) and 4 °C.

5.7. After vortexing, transfer the supernatant into a new clean 1.5. mL tube. This will be the nuclear fraction.

#### **6. Protein Quantification**

Note: Quantify the proteins with a bicinchoninic acid (BCA) protein assay kit. The concentration of proteins in the fractions will range between 0.5 and 5  $\mu\text{g}/\mu\text{L}$ , being lower in the nuclear fraction (see **Table 2** for protein concentrations resultant from 2 mg biopsies).

6.1. Prepare a standard curve with the following bovine serum albumin (BSA) amounts ( $\mu\text{g}$ )<sup>12</sup>: 0-0.5-1-2-3-4-5-6.

6.2. Prepare the reagents needed to use 100  $\mu\text{L}$  of final BCA mix per sample.

6.3. Pipette 2  $\mu\text{L}$  of each protein fraction in a 96 well plate, add the BCA mix and follow the manufacturer's instructions for the reading.

### **REPRESENTATIVE RESULTS:**

**Figure 1** shows a hematoxylin eosin staining of an intestinal biopsy section with intact villi and crypt structures.

Representative results of nuclear and cytoplasmic fractionation using this protocol are shown in **Figures 2** and **3**. In the experiment shown in **Figure 2**, a fresh (Fh) and a frozen (Fz) biopsy were simultaneously fractionated following the protocol presented here and a western blot was performed using 20  $\mu\text{g}$  of each sample. Protein concentrations in each fraction and biopsy are shown in **Table 2**, and the protein concentrations are not very different between the fresh and the frozen tissue. The yield of protein extracted from the 2 mg biopsies is around 3-4  $\mu\text{g}/\mu\text{L}$  in the cytoplasmic fraction and approximately 1.5  $\mu\text{g}/\mu\text{L}$  in the nuclear fraction. To check the purity of the fractions, HSP90 and tubulin were used as a cytoplasmic controls and HDAC1 and H3 as a nuclear controls<sup>13</sup>. It can be observed that HSP90 and tubulin are primary located in the cytoplasm and HDAC1 and H3 are located in the nucleus with very minimal mixing between the two fractions. To assess whether the protocol affects cell death, we also blotted for the presence of caspase 3. From **Figure 2**, both samples present a very minimal staining for caspase 3 confirming that the procedure does not affect the cell death pathway even after flash freezing the biopsies. It was also analyzed if protein modifications are maintained after the procedure, so proteins can be used for downstream functional assays. PhosphoSTAT1 was used for staining as the frozen biopsy used in this fractionation came from an individual with an intestinal inflammatory condition; this is not true of the fresh biopsy. As expected, phosphoSTAT1 is located in the cytoplasm of the frozen biopsy, confirming that the protein modifications are maintained after fractionation.

The reproducibility of the method using frozen biopsies is shown in **Figure 3**. A western blot was performed after fractionation of three independent frozen biopsies, and the fractions are clean with minimal compartment protein mixing (**Figure 3A**): HSP90 located in the cytoplasmic fraction and HDCA1 in the nuclear fraction. Additionally, the XPO1 protein that is present in both nuclear and cytoplasmic fractions<sup>14</sup> was also blotted. It can be observed that the levels of XPO1 quantified by ImageJ are stable in each fraction (**Figure 3B**), confirming reproducibility of the results using different biopsies.



## FIGURE AND TABLE LEGENDS:

**Figure 1: Light micrograph of an intestinal epithelial section, stained with hematoxylin eosin, from a biopsy acquired by endoscopic procedure.**

**Figure 2: Representative western blot of a subcellular fractionation performed in a fresh (Fh) and a frozen (Fz) biopsy.** HSP90 was used as a cytoplasmic control and HDAC1 as a nuclear control. The localization of XPO1 was also assessed.

**Figure 3: Representative western blot of a subcellular fractionation performed in three frozen biopsies.** HSP90 was used as a cytoplasmic control and HDAC1 as a nuclear control. The localization of XPO1 was also assessed.

**Table 1: Buffer composition.**

**Table 2: Protein concentration of each of the fractions in a fresh and frozen 2 mg biopsy used in Figure 2; concentration is presented in  $\mu\text{g}/\mu\text{L}$ .**

## DISCUSSION:

The protocol described here is used for the nuclear and cytoplasmic fractionation of intestinal biopsies. The purified proteins are not denatured and can be used not only in western blot analysis as shown in **Figures 2** and **3** but also in assays requiring native-folded proteins such as immunoprecipitation, electrophoretic mobility shift assay (EMSA) or native polyacrylamide gel electrophoresis (PAGE).

The described method relies on mechanical homogenization of the tissue together with osmotic lysis to burst the cell membrane without affecting the nuclear membrane. The result is a clean fractionation of both the cytoplasmic and nuclear proteins to be used for downstream analyses. It is important to control the degradation of the proteins during the process by the addition of protein and phosphatase inhibitors. Moreover, if extracted proteins are to be used for downstream functional assays, it is also important to avoid denaturation and proteolysis of the samples. The processes should be performed at 4 °C and PMSF should be also added to the buffers.

The procedure described in this protocol is simple and affordable, and the sample processing time is minimal. Problems such as long processing times and the use of costly fractionation kits are not constraints in this protocol.

Fresh and frozen biopsies can both be used in this protocol, with frozen biopsies giving quite clean and reproducible fractionation results as seen in **Figures 2** and **3**. If there is compartment contamination, the cell lysis time can be reduced to avoid the release of nuclear to the cytoplasm and washing steps can be added to avoid cytoplasmic contamination in the nuclear compartment.

The possibility of using frozen tissues for the independent analysis for nuclear and cytoplasmic protein fractions can increase our knowledge about intestinal inflammatory diseases in which frozen biopsies are stored. Moreover, this technique could be adapted to other kinds of human tissues acquired by biopsies. The volume of buffers used must be modified depending on the size of the biopsy.

The protocol described here is designed to generate reproducible tissue fractions; however, some intrinsic variability within the sample, including the size and the status of the tissue can lead to deviations in the protein amount and distribution managed in each fraction.

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#### DISCLOSURES:

The authors have nothing to disclose.

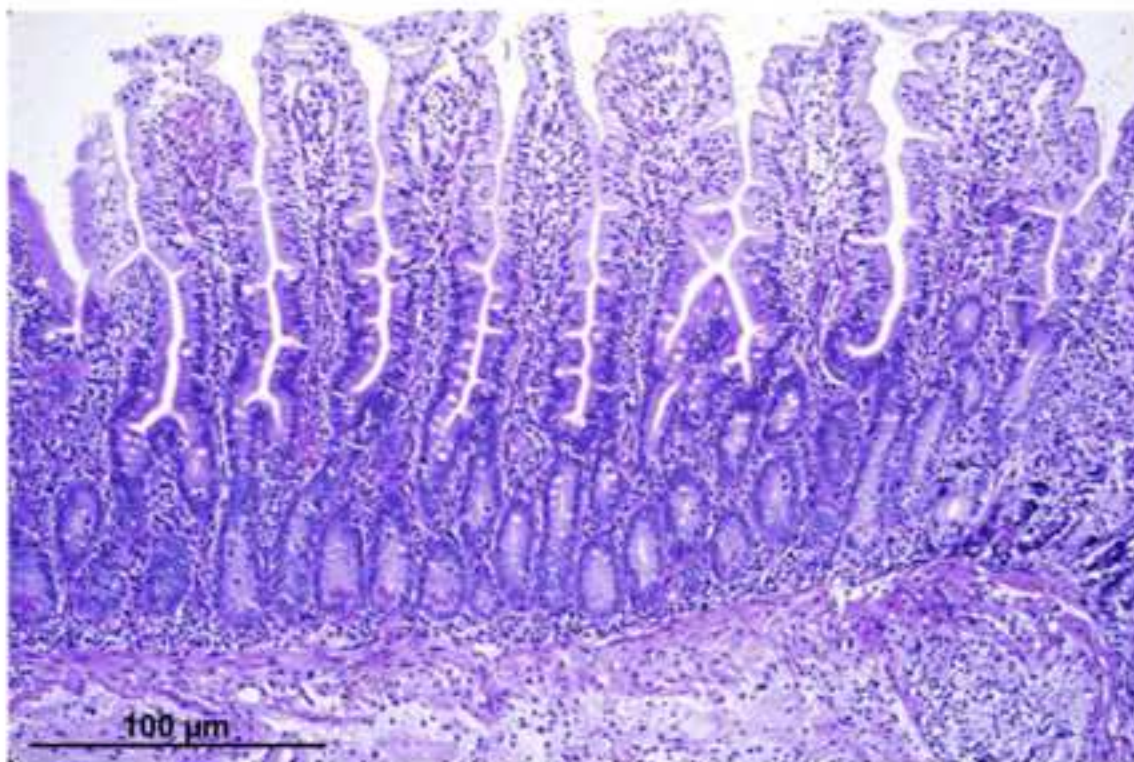
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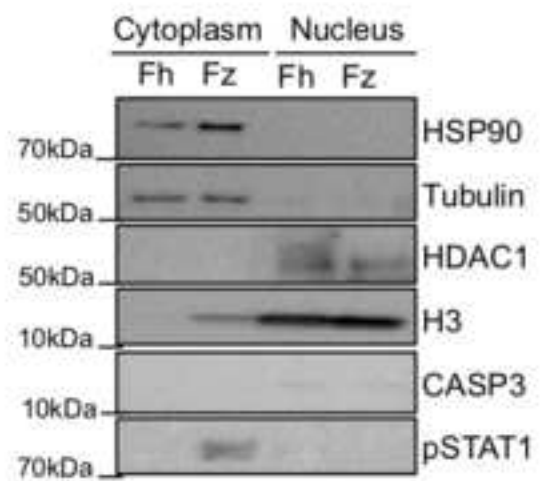
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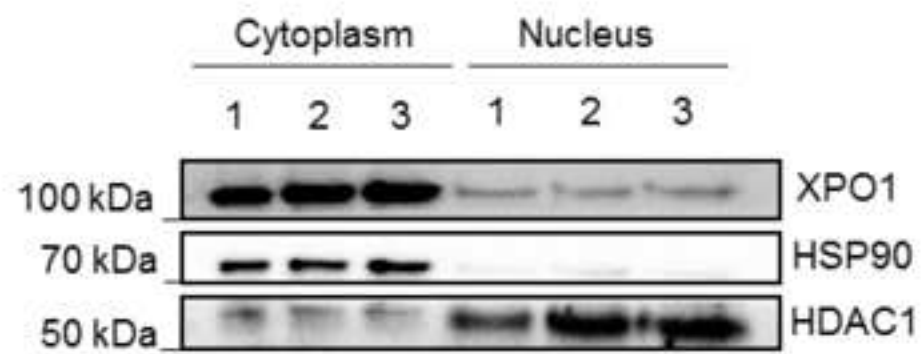
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Figure 1

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Buffer composition		
Buffer	Components	Notes
Hypotonic buffer 1	10 mM HEPES pH 7.9	1.5 ml buffer/sample
	10 mM KCl	
	0.1 mM EDTA	
Hypertonic buffer 2	20 mM HEPES pH 7.9	150 µl buffer/sample
	400 mM NaCl	
	0.1 mM EDTA	

*\*Note: Buffers can be stored at 4°C for a month.*

*\*\*Note: Buffers have to be supplemented with proteinase and phosphatase inhibitors and DTT before use*

	Fresh	Frozen
Cytoplasm	2,975,387	4,612,796
Nucleus	1,516,216	1,385,509



Name of Material/ Equipment	Company	Catalog Number	Comments/Description
HEPES	Sigma Aldrich	H4034-1kg	
KCl	Sigma Aldrich	P9333-1kg	
EDTA	Sigma Aldrich	E9884-100G	
NaCl	Sigma Aldrich	5588886-1kg	
DTT	Sigma Aldrich	10197777001	
PMSF	Thermo Scientific	36978	
Proteinase and phosphatase inhibitors	Thermo Scientific	A32959	
NP-40	Sigma Aldrich	CA-630	Detergent
BCA assay	Thermo Scientific	23227	Protein quantification kit
Disposable plastic pestles	Sigma Aldrich	Z359947-100EA	
Dounce homogeneizer	VWR	431-0100	
Microcentrifuge	Eppendorf	5415-R	
Shacker	IKA	MS3 basic	





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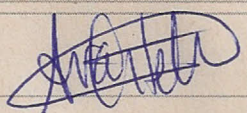
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## Answer to Editorial comments:

1. The editor has formatted the manuscript as per the journal's style. Please retain the same.

We have kept the manuscript in the style formatted by the editor.

2. Please address specific comments marked in the manuscript.

Specific comments marked in the manuscript have been addressed following editor's notes.

3. Please refer the supplemental table in the manuscript protocol and include the supplemental table legend in the figure/table legend section.

Table 1 has been referred to in the protocol and has been added as a supplementary table.

## Video

1. 6:47: There is a background noise of somebody talking. Please edit.

We were unable to edit the background noise in 6:47, it is in the same soundtrack and can not be removed.

2. Representative result: Please include a result showing how much protein was obtained from fresh and frozen samples with this protocol from 2 and 10 mg of the biopsies used for the study.

The table with the protein concentration results has been added to Representative Results. In this case the biopsies used in our laboratory are normally in the 2 mg range because they came from pediatric patients, so we do not have results for bigger pieces right now.

3. 9:15: please edit the cut noise just before the start of the interview.

Cut noise in 9:15 has been deleted.