



CosMx[®] Best Practices

Volume II

Protocol Updates: before we jump in to the second installment of Best Practices for CosMx™ SMI, we would like to pass along two protocol changes that have demonstrated increased workflow performance. These changes have been included into the CosMx SMI Slide Preparation Manuals and the CosMx SMI Instrument Manual available now in the [NanoString University Document Library](#).

- 1) In the slide preparation workflow, the volume of Cell Segmentation Mix 1 (CD298/B2M) added by the user is decreased when preparing the antibody mixes for either the RNA or protein FFPE assays.

TABLE 1: UPDATED RNA STAINING MIX PREPARATION

Cell Segmentation Mix 1 (CD298/B2M)	Marker Mix 1* (Optional PanCK/CD45)	Marker Mix 2* (Optional a la carte*)	Blocking Buffer	Total Volume
4 $\mu\text{L} \times n$	8 $\mu\text{L} \times n$	8 $\mu\text{L} \times n$	180 $\mu\text{L} \times n$	200 $\mu\text{L} \times n$

*If not adding PanCK/CD45 or optional a la carte markers, add Blocking Buffer in lieu of marker.

TABLE 2: UPDATED PROTEIN ANTIBODY MIX PREPARATION

Hs IO Protein Antibody Mix	CD298 / B2M Segmentation Marker	PanCK / CD45 Marker (optional)	A la carte marker (optional)	Diluted Custom Antibodies* (if applicable)	Buffer W (μL)	Total Volume (μL)
62.5 $\mu\text{L} \times n$	2.5 $\mu\text{L} \times n$	5 $\mu\text{L} \times n$	5 $\mu\text{L} \times n$	1.25 $\mu\text{L} \times n$	up to final volume of 125 $\mu\text{L} \times n$	125 $\mu\text{L} \times n$

*Volume is per antibody. NOTE: If using an antibody with a target concentration of 4 $\mu\text{g}/\text{mL}$, 2.5 $\mu\text{L} \times n$ must be used.

- 2) During instrument loading, volume of pyranose oxidase added by the user to instrument Buffer 4 is decreased. This workflow change occurs during instrument loading and is on page 46 of the CosMx SMI Instrument Manual (for software v1.2) or page 51 of the CosMx SMI Instrument Manual (for software v1.3). Please refer to the manual for exact details.

NanoString recommends implementing these two improvements to your workflow moving forward.

We are excited to share the second installment of **3 Tips for Successful CosMx™ SMI Single-cell Spatial Runs at 1000-plex**. Here we focus on evaluating tissue block quality, section quality, heterogeneity, and autofluorescence.

To review our first installment of tips, which focuses on data by tissue and disease type as well as tissue specific sample preparation considerations, please visit <https://nanosttring.com/blog/tips-for-successful-cosmx-smi-single-cell-spatial-runs-at-1000-plex/>.

Equipment and Materials	Source	Part Number
PBS - Phosphate-Buffered Saline (10X) pH 7.4, RNase-free	ThermoFisher Scientific® (or comparable)	Example: AM9625
TiYO Autofluorescence Quenching System	Bulldog Bio, Inc. (or comparable)	Example: TiYO

Tips When Generating Single-Cell Spatial Data: Sample Selection

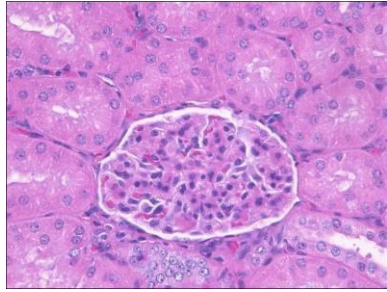
Tip 1: Evaluate Tissue Block Quality

Tissue block quality is a critical variable when generating single-cell spatial data. Two key factors when creating high-quality tissue blocks are **fixation** and **ischemic** time.

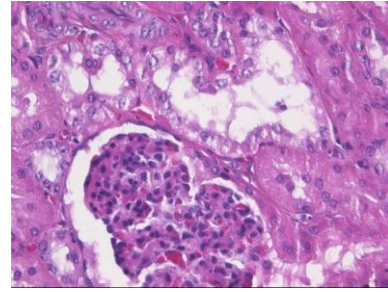
NanoString has validated protein and RNA tissues fixed with either 10% NBF or 4% PFA fixative. The use of other fixatives is generally not recommended. Because proper fixation is dependent on the diffusion of the fixative into the tissue, the recommended specimen thickness is 2-3 mm. At this thickness, most tissue types require a minimum fixation time of 24 hours at room temperature (up to 72 hours). Thicker tissue sections and tissue types such as bone, bloody or fatty tissues, HIV-containing tissues, and certain fetal tissues may require longer fixation times. Under-fixation of tissue can lead to degradation of the sample and poor morphological preservation. Over-fixation should also be avoided as it may result in non-specific background staining. The optimal fixation time for specimens may need to be empirically determined.

Ischemic time refers to the duration of time that a particular organ or tissue is deprived of adequate blood supply and consequently, oxygen and nutrients. While typically easier to control in mouse studies than human studies, ischemia is a critical factor in preventing tissue damage and RNA degradation. A shorter ischemic time is better for RNA quality.

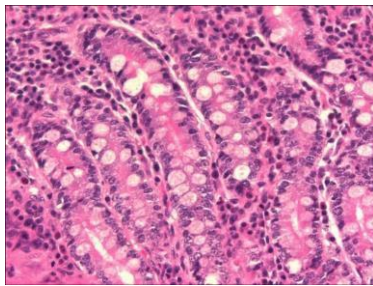
NanoString recommends performing H&E staining to assess tissue block quality. The H&E stain can be used to determine if the tissue was properly fixed (Figure 1: Leica Biosystems, [Process of Fixation and the Nature of Fixatives](#)) as well as to note any areas of poor tissue quality, such as inconsistent thickness, tears, folds, and chatter (Figure 2: Leica Biosystems, [H&E Basics Part 4: Troubleshooting H&E](#)). This assessment can also be used to aid in Field of View (FOV) placement for serial sections when run on the CosMx SMI instrument.



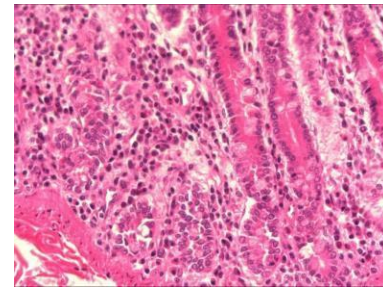
A: A paraffin section of kidney that has been fixed using neutral buffered formalin. This is an example of well-fixed tissue showing good nuclear and cytoplasmic morphology with minimal shrinkage showing clearly defined basement membranes and cell margins



B: A paraffin section of kidney that has been fixed using neutral buffered formalin. This is an example of poorly fixed tissue showing inferior nuclear and cytoplasmic morphology with excessive shrinkage and poorly defined cell margins. Note the vacuolation and fragmentation of both nucleus and cytoplasm of cells of the distal tubule and retraction of the glomerulus due to shrinkage.



C: A paraffin section of the mucosa of small intestine that has been fixed in neutral buffered formalin, a cross-linking fixative. Nuclear and cytoplasmic preservation is satisfactory, but some cellular shrinkage is present.



D: A paraffin section from the mucosa of small intestine that has been fixed in 95% ethanol, a denaturing fixative. While nuclear preservation is fair there is substantial shrinkage of cytoplasmic and extracellular elements. Compare the morphology demonstrated here with that shown in Figure 3, which was photographed at the same magnification.

FIGURE 1: H&E STAINING TO DETERMINE BLOCK QUALITY

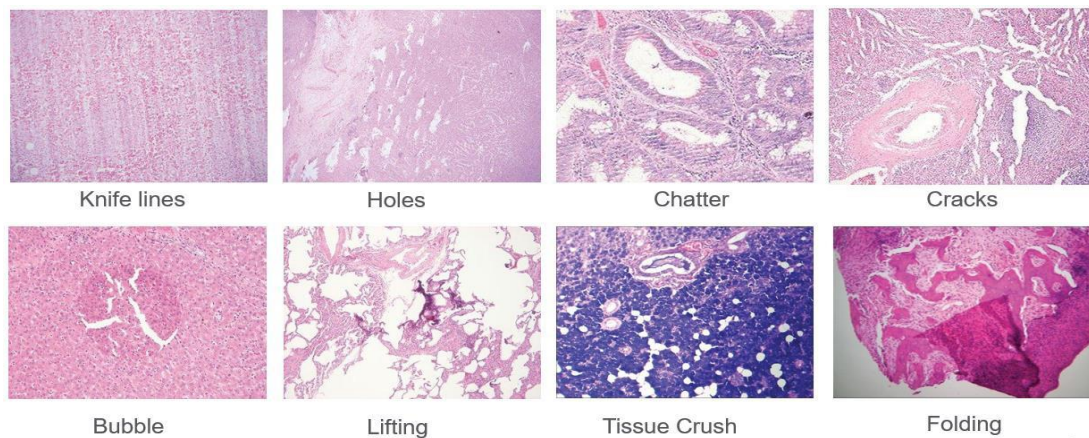


FIGURE 2: POOR TISSUE HANDLING OR SECTIONING QUALITY

Tip 2: Evaluate Tissue Section Quality

Tissue section quality plays a key role in signal generated from spatial assays. For CosMx RNA assays, it's recommended to use mounted sections within two weeks for best results. Before sectioning, plan the placement of the tissue in the center of the slide's scan area, indicated in green in Figure 3.

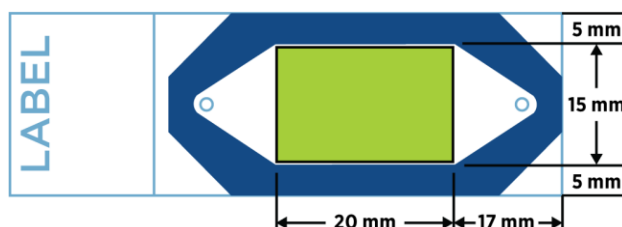


FIGURE 3: COSMX SMI TISSUE SCAN AREA

Successful completion of the downstream assay depends on technique during sectioning and slide preparation. Section adhesion and consistency of thickness have impact on downstream assay performance. For a more comprehensive guide on sectioning, please refer to [Sample Sectioning Tips and Tricks for CosMx SMI and GeoMx DSP Experiments](#) available on NanoString University.

Beginning with flat, well-mounted sections is important to maintain tissue integrity. Figure 4 shows the difference between high-quality and low-quality FFPE sections. The section shown on the left has few flaws and performed well during CosMx SMI workflow. The section on the right contains many tears and wrinkles, which can result in tissue detachment from the slide; this tissue section performed poorly during the sample preparation workflow.



FIGURE 4: GOOD (LEFT) AND BAD (RIGHT) SECTIONS MOUNTED ON A SLIDE

There are numerous potential artifacts during FFPE sectioning. Most solid organs should be placed on ice for 30 minutes or longer prior to sectioning. However, it's important to note that tissues, such as spleen and liver, being more delicate, should spend less than 30 minutes on ice. Sections should be cut at 5 μ m thickness on a calibrated microtome and mounted in the center of the scan area (Figure 3). It is essential to always discard the first few sections from the block face. For a comprehensive guide, consult [Leica Biosystems Introduction to Microtomy: Preparing & Sectioning Paraffin Embedded Tissue](#).

For frozen specimens, cryo-sections should be cut at 5-10 μ m thickness on a calibrated cryostat and mounted immediately to the center of the scan area (Figure 3). During sectioning, it is important to cut across the tissue with a smooth, consistent turn of the hand wheel. Knife edge temperature has a large impact on section quality. See Table 3 for knife edge temperature recommendations. For a more comprehensive guide, please consult [A Method for Preparation of Frozen Sections](#).

TABLE 3: KNIFE EDGE TEMPERATURE FOR CRYO SECTIONING

Knife Temperature	Tissues
-10°C to -15°C	Adrenals, bone marrow, brain, cartilage, spleen, bloody tissue, testicular tissue
-15°C to -17°C	Bladder
-15°C to -18°C	Breast (less fatty), cervix, intestine, liver, thyroid
-15°C to -20°C	Kidney, lung, pancreas, prostate, ovary, rectal, skin without fat, uterus
-18°C to -22°C	Heart and vessel
-20°C to -25°C	Muscular
-25°C to -30°C	Skin with fat, breast (fatty)

Tip 3: Consider Tissue Heterogeneity and Autofluorescence

As mentioned earlier, we recommend H&E staining with a serial section prior to beginning a CosMx SMI experiment. This serial section can be used to both evaluate tissue block quality and guide for FOV placement, an important part of experimental planning. By taking the time to properly consider and plan FOV placement, we can avoid selecting low-quality tissue areas and reduce instrument run time.

Consider the scientific question when selecting tissue areas. For example, the focus of the study below (Figure 5) was to study cancer, muscle, and normal tissue but not connective tissue. FOVs were placed answer the scientific question of the study. In Figure 5, the left image shows the H&E section that was used to guide FOV placement for the serial section on the right that is run using the CosMx SMI 1K RNA assay. Also overlaid on the image on the right are the RNA counts per FOV. RNA counts will vary based on tissue morphology. Relatively lower RNA counts are typically observed in connective tissue, fatty tissue, and mucus.

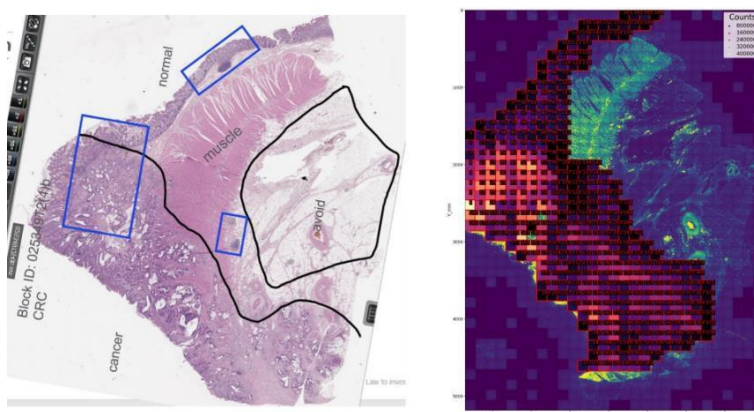


FIGURE 5: SERIAL SECTIONS H&E AND COSMX SMI EXAMPLE

Avoid placing FOVs over areas of tissue necrosis, peeling, folds, wrinkles, and tears when possible (Figure 6).

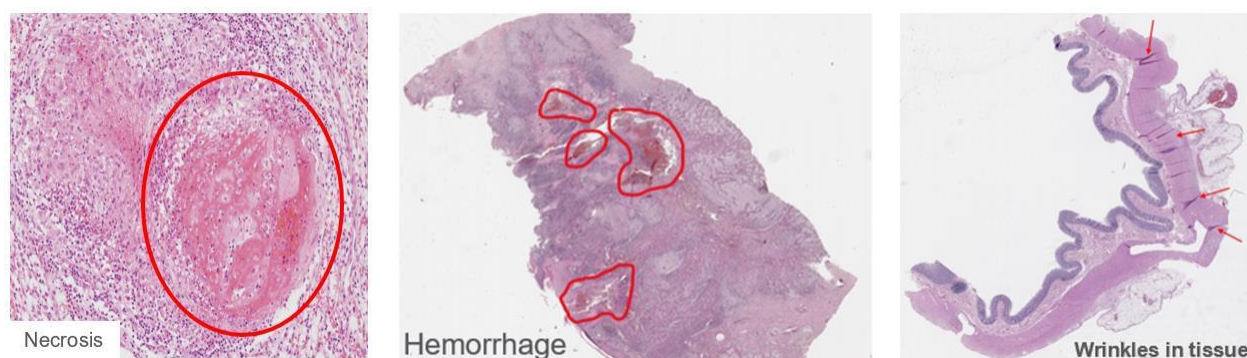


FIGURE 6: AREAS TO AVOID DURING FOV PLACEMENT

Tissue autofluorescence is another variable to consider. High tissue autofluorescence can result in higher background and impact cell segmentation. Selecting the correct pre-bleaching profile (Table 4) during run set-up will minimize autofluorescence and to improve data quality. If a tissue type of interest is not listed in the table below, start with the default condition (Configuration C) and adjust as needed. For reference, pre-bleaching times associated with each configuration are listed below Table 4.

TABLE 4: PRE-BLEACHING PROFILES

Tissue Type	RNA	RNA	Protein
	Normal	Malignant	
Brain	Configuration B	Configuration B	Configuration C
Skin	Configuration C	Configuration C	Configuration C
Lung	Configuration C	Configuration C	Configuration C
Breast	Configuration C	Configuration C	Configuration C
Liver	Configuration B	Configuration C	Configuration C
Colorectal	Configuration C	Configuration C	Configuration C
Tonsil	Configuration C	Configuration C	Configuration C
Pancreas	Configuration C	Configuration C	Configuration C
Kidney	Configuration B	Configuration B	Configuration C
Fresh Frozen	Configuration C	Configuration C	Configuration C
CPA	Configuration A	Configuration A	Configuration A

Configuration A: 30 seconds; Configuration B: 90 seconds; Configuration C: 60 seconds

When increasing pre-bleaching time(ex: choosing Configuration B over C for tissues with higher autofluorescence), please note that the instrument will run for a longer duration (Table 5). For details on operating the CosMx SMI instrument, please refer to the [CosMx SMI Instrument User Manual](#).

TABLE 5: PRE-BLEACHING CONFIGURATION IMPACT ON TURN-AROUND-TIME

Pre-bleaching Configuration Impact on TAT/Throughput	Configuration A 30 seconds	Configuration C 60 seconds	Configuration B 90 seconds
Pre-bleach duration 2 flow-cells 766 Total FOVs	6.4 hours	12.8 hours (+6.4-hour increase)	19.2 hours (+12.8-hour increase)

*6.4 hour increase from 30 seconds to 60 seconds. Increase will scale linearly with Total number of FOVs.

When placing FOVs, it is also important to consider tissue and cell-specific fluorescence. Table 6 highlights some of the various tissue and cell types to be aware of during FOV selection.

TABLE 6: TISSUE/CELL SPECIFIC AUTOFLUORESCENCE CONSIDERATIONS

Tissue/Cell Type	Special Considerations
Blood Cells	Various components of blood, including red blood cells (erythrocytes) and white blood cells (leukocytes), can exhibit autofluorescence, primarily due to their hemoglobin content and other intracellular molecules.
Liver Tissue	Hepatocytes in the liver can produce autofluorescence, attributed to their content of flavin adenine dinucleotide (FAD) and nicotinamide adenine dinucleotide (NADH).
Nervous Tissue	Neurons and glial cells in the nervous system may display autofluorescence because of their mitochondria and other cellular components.
Lung Tissue	Lung tissue can be auto fluorescent, particularly due to the presence of collagen, elastin, and various proteins within the alveoli.
Kidney Tissue	Certain structures within the kidney, such as the glomerulus and renal tubules, may exhibit autofluorescence due to their constituent molecules.
Bone Tissue	Bone tissue, including bone matrix and osteocytes, can generate autofluorescence because of the presence of collagen and minerals like calcium.
Pancreatic Islets	Islets of Langerhans in the pancreas can show autofluorescence, primarily due to the presence of insulin-containing beta cells.
Intestinal Epithelium	The epithelial lining of the intestine can produce autofluorescence, potentially linked to the presence of mucins.

Placenta	Contains more red blood cells - see Blood Cells above.
Lipofuscins	Prominent in neurons, glial cells, and cardiac muscle cells, but found in a wide range of cell types, and predominately in post-mitotic cells. Lipofuscin has an enigmatic chemistry and stains positive for proteins, carbohydrates, and lipids, appearing brown in color. It usually occurs as small, punctate intracellular structures that are strongly fluorescent under any excitation ranging from 360 nm to 647 nm.
Elastin and Collagen	Typically, from blood vessel walls, elastic and collagen contain naturally fluorescent molecules that can be intensely fluorescent over a range of excitation wavelengths.

Thank you for choosing CosMx SMI. If you have any further questions or need assistance, contact Bruker Spatial Biology Support Support.Spatial@Bruker.com.