Preserving and Determining Tissue Quality for MERFISH Measurements Performed with MERSCOPE™

Fresh Frozen and Fixed Frozen Tissue Samples

ABSTRACT

MERFISH technology is based on single molecule fluorescence in situ hybridization (smFISH) and can measure both the copy number and spatial distribution of 100s to 1000s of RNAs simultaneously.[1] However, the RNA in fresh frozen and fixed frozen tissue samples are susceptible to degradation, easily lowering tissue quality. Therefore, preserving and determining the tissue quality of samples is vital for successful MERFISH measurements. To ensure that only high-quality tissue samples are imaged by the MERSCOPE™ Platform, Vizgen scientists carefully prepare tissue sections, measure sample RNA quality at multiple steps in the MERSCOPE workflow and verify sample preparation before running a full MERFISH experiment. Our quality control strategies are easily adoptable, and we highly recommend labs use these procedures as a guideline for developing in-house best practices.

Introduction

MERFISH technology is based on single molecule fluorescence in situ hybridization (smFISH) and can measure both the copy number and spatial distribution of 100s to 1000s of RNAs simultaneously.[1] . Quality and integrity is a prominent issue in gene expression analysis. Handling and preservation methods including formalin fixation, flash freezing, and chemical preservation are widely applied to keep high quality RNA within fresh tissue samples. However, the RNA in fresh frozen and fixed frozen tissue samples are susceptible to degradation, easily lowering the quality of tissue used for spatial analysis. Therefore, preserving and determining the tissue quality of samples is vital for successful MERFISH experiments.

The degradation of RNA is tightly controlled by cells to keep an organism functioning properly. One of the most common reasons for RNA degradation is RNA-degrading enzymes, commonly referred to as ribonucleases or RNases. These types of enzymes are found in all organisms, and many are highly conserved across species.[2] RNases easily react with the hydroxyl groups on the 2′ and 3′ positions of ribose molecules to efficiently break down RNA.[3] These enzymes are very conformationally and thermodynamically stable which makes them difficult to permanently inactivate.[4,5] Furthermore, RNases are found nearly everywhere, including on the skin, so lab equipment and glassware can be easily contaminated with these enzymes.[3] As a result, users need to properly handle and prepare tissue samples intended for MERFISH analysis to prevent RNA degradation and preserve RNA quality.

Establishing proper sample handling and quality control procedures such as the ones outlined in this document can assist laboratories with both preserving and determining tissue quality for MERFISH experiments performed on the MERSCOPE Platform.

How to Preserve Tissue for MERFISH Measurements

Tissue and RNA preservation starts with sample collection and includes cryosectioning and storage. Improper technique during these stages can greatly impact the success of a MERFISH experiment.

Sample Collection

RNases naturally begin to degrade RNA postmortem and after tissue excisions[6], so tissues should be harvested and either frozen or fixed and then frozen as soon as possible. Research has demonstrated that RNases can reactivate in thawed fresh frozen samples[7], so it is highly recommended to embed frozen tissue blocks with optimal cutting temperature (OCT) compound and kept at −80°C for long term storage. Furthermore, researchers should minimize the number of freeze-thaw cycles to prevent RNA degradation through the

cumulative action of transient RNase reactivation events at higher temperatures.

Depending on a tissue's origin in the body, different procedures may be applied to best preserve RNA in different samples. The need for standardization in tissue collection and handling has been emphasized by many regulatory, scientific, and commercial groups with interests in genomic expression testing. Choosing a suitable method for harvesting and preserving tissue can be challenging based on this lack of standardization in the available literature. Thus, Vizgen scientists have developed a simple guideline to help decide which method to use:

1) For samples that come from internal organs with no direct exposure to external elements, snap frozen sample preparation is the most practical and convenient method. Some tissue types suitable for this method include brain, liver, heart, retina, prostate, kidney, ovary, spinal cord, and solid tumors.
2) For samples that are exposed to the external environment (skin, gastrointestinal tract), contain more enzymatic activity (pancreas), or are exposed to media during the harvesting process (lung), the sample should be harvested in media containing RNase inhibitor if preparing fresh frozen – or, alternatively, prepared as fixed frozen samples.

In general, Vizgen scientists have observed that fixed frozen tissue has fewer RNase contamination issues, as compared to fresh frozen tissue. If working with a sample that is prone to RNA degradation, it is worth considering fixed frozen tissue preparation to harvest and process the sample. However, due to the dehydration caused by the sucrose gradient exchange in the fixed frozen tissue preparation protocol, tissue slices may become more difficult to affix to the slide during sectioning. Therefore, users may have to balance RNA quality and tissue adhesion requirements when choosing the best method to use for the sample of interest.

Recommended options for preparing fresh frozen and fixed frozen tissue blocks for MERFISH imaging are provided in Vizgen's 91600002 MERSCOPE Fresh and Fixed Frozen Tissue Sample Preparation User Guide.

Cryosectioning Fresh and Fixed Frozen Tissue

Tissue blocks should be carefully cut with a cryostat into 10µm thick tissue slices. Fresh frozen tissue slices should be fixed in 4% Paraformaldehyde (PFA) and later permeabilized with 70% ethanol. Fixed frozen tissue sections can be placed directly into 70% ethanol without the need of further fixation after sectioning. Since RNase is not active in 70% ethanol, the samples can be stored in 70% ethanol at 4 °C for up to one month.

MERSCOPE™ Slides have a defined imageable area that all users should understand and utilize to guide the placement of tissue on these slides. Within the 40mm diameter MERSCOPE Slide, there is a 2X1.5cm rectangular area for placing tissue sections. Users have the flexibility to place one or more tissue slices within this area. However, any tissue outside of the usable area will not be imageable. As such, end users and histologists should follow these imaging area guidelines closely to ensure the tissue slice is sectioned properly and placed in the correct, imageable area on the MERSCOPE Slide. This is a simple but critical way that users can ensure consistent success of their MERSCOPE runs.

To facilitate the precise capture of multiple tissue sections within a given imageable area, MERSCOPE supports imaging of multiple discrete regions, up to a total area size of 1cm^2. Users can draw one or multiple regions of interest within the imageable area as long as the total size of the selected regions is below 1cm^2 (Figure 1). This option gives users the flexibility to potentially image multiple samples on the same MERSCOPE slide, if the tissue area for each sample is small.

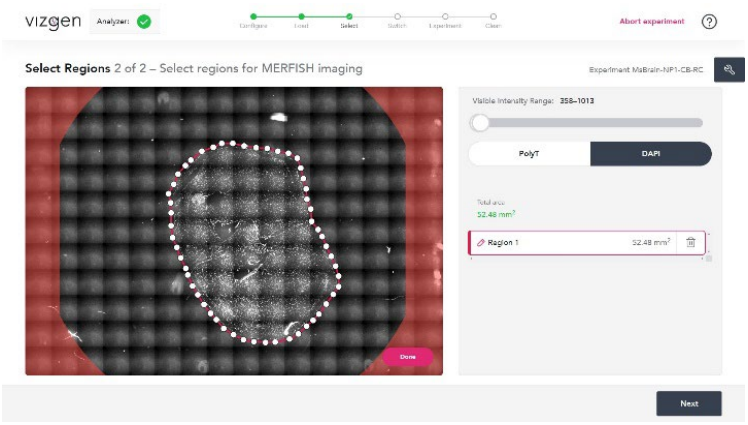

Figure 1. Selecting regions of interest for MERFISH imaging on the MERSCOPE Platform. The MERSCOPE Instrument acquires a low-resolution mosaic. Then boundaries are drawn on the mosaic to define the region of interest for the experiment. Up to 10 regions can be selected with a total area of up to 100 mm^2 (1 cm^2). A 52.48 mm^2 area was drawn around this mouse brain tissue section loaded into the MERSCOPE Instrument.

How to Measure the RNA Quality of Tissue Samples

Proper sample preparation and handling plays a major factor in determining the reproducibility and ultimate quality of the final MERFISH data. The time from death or tissue excision to tissue sectioning is not always known for human tissue samples, and disease state samples can exhibit increased RNA degradation. Furthermore, differences in sample origin, procurement, shipping conditions, and the tissue processing workflows of various institutions all add to the variability observed in RNA quality. Therefore, understanding the RNA quality of tissue samples before imaging with MERSCOPE is vital to successful MERFISH measurements.

RNA Electrophoresis

Scientists originally performed RNA electrophoresis on samples and analyzed the bands of the highly abundant, mature 18S (1.9 kb) and 28S (5 kb) ribosomal RNA species to determine the quality of RNA.[8] Samples showing weaker 18S and 28S bands with more smaller fragments were considered to have lower RNA quality than samples with strong 18S and 28S bands.[8] A 18S:28S ratio of 1.8 to 2 or greater generally indicated that a sample had good quality RNA.[8] However, scientists eventually realized that this manual procedure introduced a large degree of error and variability between experiments.[8,9]

Over time, the methods for determining RNA quality in samples have evolved with the development of new technologies. Vizgen's scientists found that it is informative to determine the RNA quality of a tissue block using RNA Integrity Number (RIN) or DV200 values via commercially available instruments such as the BioAnalyzer or TapeStation platforms.

RNA Integrity Number

The RIN algorithm was developed by scientists from Agilent Technologies in 2006 to create a standardized method for determining RNA quality.[9,10] Different features that contribute to RNA integrity, including the 18S:28S ratio, were used as a foundation for the algorithm. Additionally, inherent smaller RNA such as the 5S and 5.8S rRNAs, as well as transfer RNAs (tRNAs), were taken into consideration. The team applied Bayesian probability machine learning to select the most probable model for accurately measuring RNA quality. The metric they developed, RIN, is expressed in values that range from 1-10, where 1 indicates a sample has shorter and more degraded RNA whereas 10 reflects longer and less degraded RNA. Higher RIN scores will have more intact 18S and 28S RNAs. In the model, the 5S, 5.8S, and tRNAs were determined to not reflect RNA degradation.

Figure 2 shows an example of the RIN readout portion of the data generated by an Agilent TapeStation system for 16 different tissue samples. The samples have RIN scores ranging from 2.5 to 8.2. The tissue samples with

lower RIN scores have more bands on the electrophoresis gel that are less than 1000 nucleotides in length, which reflect more degraded RNA fragments. The higher RIN scores have stronger signals for nucleotide length greater than 1000, as well as fewer and lower signals for smaller RNA fragments. Strong and clear signals for the 28S and 18S bands are also notable for the lanes that achieved the higher RIN scores, such as samples C2, D2, and B1.

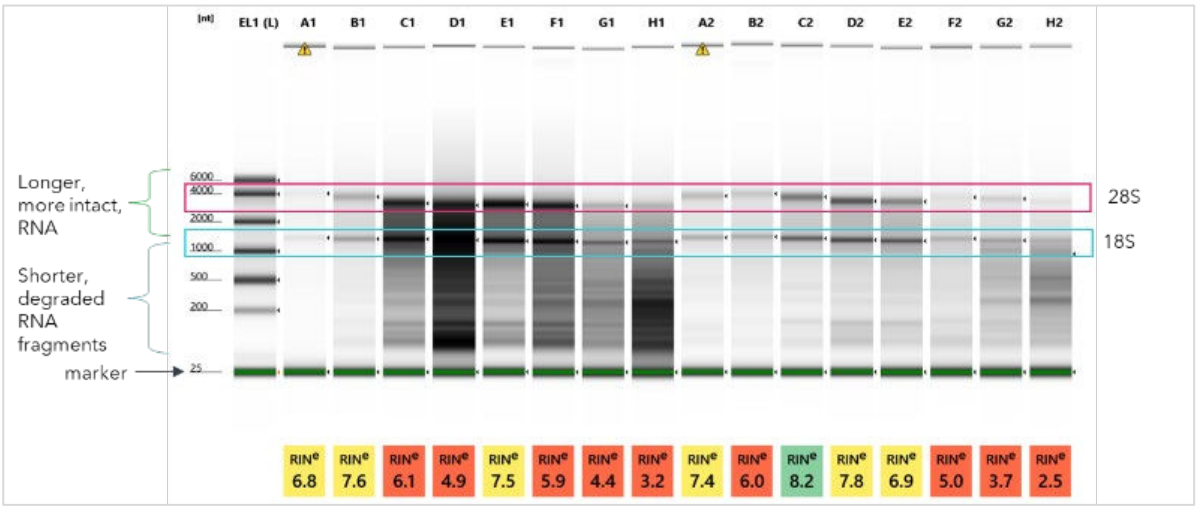

Figure 2. Example of RIN Readout Summary from the Agilent Tape Station- The more degraded the RNA in a sample, the more bands of smaller fragments appear on the electrophoresis gel readout. The samples with higher quality RNA have stronger and clearer bands for the 28S and 18S rRNA molecules, indicating more intact RNA.

DV200 Metric

Illumina developed the DV200 metric in 2015 to determine the percentage of RNA fragments greater than 200 nucleotides in length in tissue samples.[11] Tissues with lower DV200 percentages have shorter and more degraded RNA; conversely, a higher percentage indicates longer, and less degraded RNA molecules present in the tissue. This method aimed to address the challenges of checking the RNA quality of formalin-fixed paraffin-embedded (FFPE) tissue and was reported by Illumina to successfully predict RNA quality more accurately in those samples. Beyond this scope, DV200 is still a reliable metric by which to measure the quality of RNA in both fresh frozen and fixed frozen tissue samples.

Figures 3A and 3B show the data used in the determination of DV200 values for sample wells C2 and H2 from Figure 1. Well C2 sample has a RIN score of 8.2 and a DV200 value of 80.17. On the other hand, the well H2 sample has a RIN score of 2.5 and a DV200 value of 74.64. When the graphs for both samples are compared, there is a shift towards shorter nucleotide length for the sample in H2 (Figure 3A), which is reflected in the lower DV200 value and RIN score for sample well H2. As can be seen from these two samples, the RIN and DV200 scores may not have the same levels of sensitivity to specific signs or modes of degradation, but they often point towards the same conclusion. For FFPE samples, Vizgen recommends using DV200 values as the major metric to evaluate sample RNA quality, as RIN scores for most FFPE samples are low.

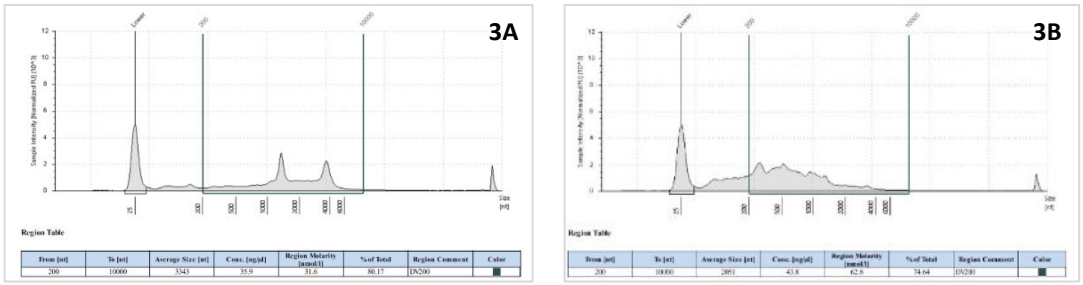

Figure 3. Example of DV200 Readout from Agilent TapeStation. 3A) Example of the DV200 Readout of well C2 from the Agilent TapeStation. The sample's RIN is 8.2 and DV200 values is 80.17. 3B): Example of the DV200 Readout of well H2 from the Agilent TapeStation. The sample's RIN score is 2.5 and DV200 values is 74.64.

How We Analyze Initial Sample RNA Quality at Vizgen

Before performing a MERFISH experiment with MERSCOPE, Vizgen recommends to first evaluate the RNA quality by measuring RIN and/or DV200 values from a few sections to decide if the tissue block is suitable for MERSCOPE imaging or not.

We use the *Agilent TapeStation* in our lab to calculate the RIN and DV200 values in tissues before they undergo sample preparation and imaging with MERSCOPE. The TapeStation is a self-contained system that facilitates a quick and reproducible evaluation of these metrics. Both the RIN and DV200 values are automatically calculated by the system and our scientists use these analyses to ensure only high-quality tissue samples are selected to continue to sample preparation (Figure 4).

RNA Integrity Number Recommendation	
RIN >7	Optimal sample quality, the higher the better
RIN 5-7	May be used, but detection efficiency may be compromised; DV200 important
RIN <5	Unacceptable sample quality
DV200 Recommendation	
DV200> 70%	Optimal sample quality, the higher the better, fresh, and fixed frozen tissue

Figure 4: Table of Vizgen Recommendations for RIN and DV200 Values

Performing Post Sample Preparation Tissue Verification

Since different sample types may require optimization of certain steps or conditions during the sample preparation process, it is important to check the tissue quality before imaging. Furthermore, RIN or DV200 measurements require the extraction of RNA from many cells. Some clinical samples may be precious and not readily allow such measurements. Therefore, Vizgen developed the MERSCOPE™ Sample Verification Kits to directly evaluate the RNA quality in prepared tissue sections. These reagent kits verify that the sample will result in adequate MERFISH imaging quality with the MERSCOPE Instrument.

How MERSCOPE Sample Verification Kits Work

Sample verification is based on a set of single molecule fluorescence in situ hybridization (smFISH) probes that target essential housekeeping genes expressed in all cell types at low levels. Research over time has determined that housekeeping genes have different expression benchmarks in different tissue types. The MERSCOPE™ Sample Verification Kit (Human) 10400007 includes encoding probes targeting the human housekeeping gene Elongation factor 2 (EEF2). For mouse samples, The MERSCOPE™ Sample Verification Kit (Mouse) 10400008 includes encoding probes targeting the mouse homolog of this same gene, Eef2.

The verification encoding probes are assigned with 2 unique readout bits and are imaged in a single imaging round using 2 fluorescent channels. The resulting foci are analyzed for colocalization between the fluorescent

channels. A higher colocalization ratio is indicative of higher RNA quality in tissue samples, as shown in Figure 5. Furthermore, zones that appear during the sample verification process can be used to guide areas of interest for MERFISH analysis. This is especially useful for precious or high-value samples that may have regions of high and low quality. By using the MERSCOPE Sample Verification Kits, researchers can make RNA-informed decisions about how and where to cut tissue blocks to generate the highest quality samples possible for real MERSCOPE runs. The MERSCOPE™ User Guide Sample Verification Kit (91600004) explains in full detail how to use these reagent kits.

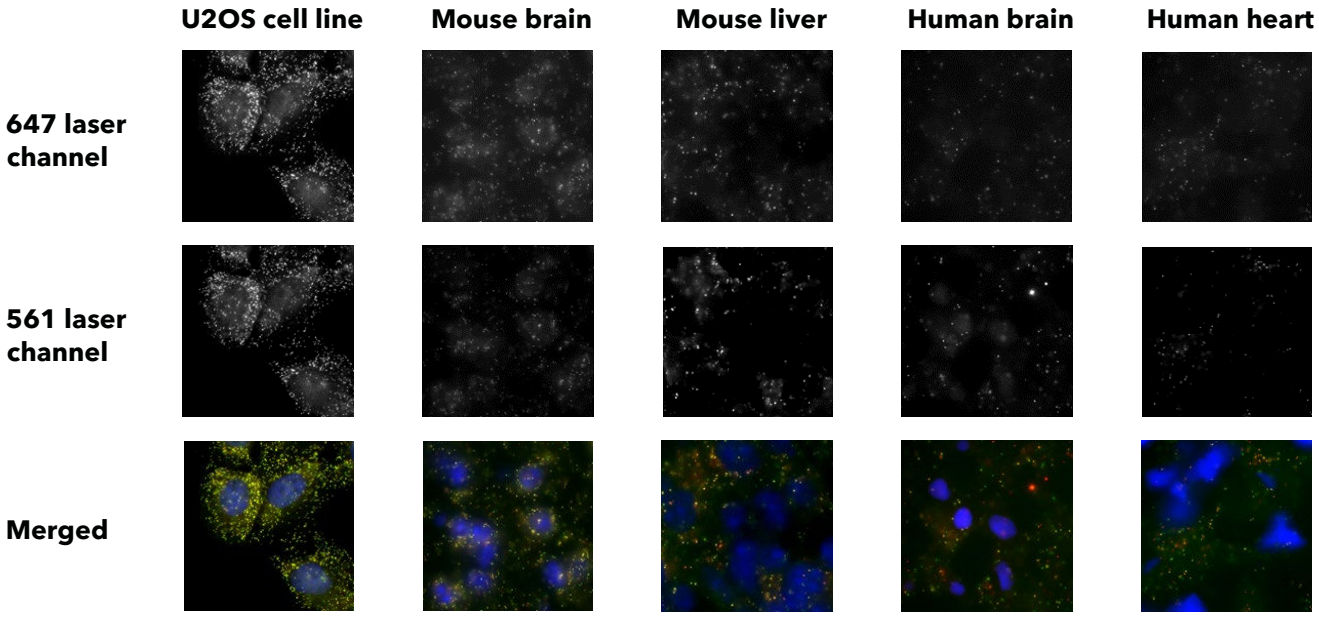

Figure 5. MERSCOPE Sample Verification Example. U2OS cell line was stained with the Sample Verification Kit (Human). Mouse brain and liver tissue sections were stained with the Sample Verification Kit (Mouse). Human brain and heart tissue sections were stained with the Sample Verification Kit (Human). smFISH signal of the verification probe EEF2 is shown in the individual channels and when merged.

Conclusion

To generate the best samples possible and subsequently the best data using our MERSCOPE Platform, researchers must assess the RNA quality of their samples at multiple points in the MERSCOPE workflow. Unfortunately, RNA is easily degraded by RNAses when samples are mishandled or treated in ways that do not specifically protect against RNA degradation. Maintaining an RNAse-free environment, properly preserving tissue samples, rigorously evaluating the sample RNA quality, and carefully cutting tissue blocks all help protect samples from RNA degradation. By initially measuring the RIN and DV200 values of tissue samples and subsequently using MERSCOPE Sample Verification Kits, researchers can ensure that only high-quality samples are imaged by the MERSCOPE instrument.

Once sample verification is completed, users can continue the MERSCOPE workflow and prepare samples for MERFISH imaging. The MERSCOPE™ Instrument User Guide (91600001) describes how to load and run the MERSCOPE Instrument. Should you need any assistance, our team is ready to support you. Please contact support@vizgen.com with your questions.

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