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HiFi-Slide spatial RNA-Sequencing V.1

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Tianyang Xu[1], Riccardo Calandrelli[1], Pei Lin[1], Diming Zhu[1], Sheng Zhong[1]

[1]Department of Bioengineering, University of California San Diego

Human BioMolecular Atlas Program (HuBMAP) Method Development Community
Tech. support email: **Jeff.spraggins@vanderbilt.edu**

Pei Lin

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We are still developing and optimizing this protocol

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Abstract

HiFi-Slide sequencing is a super-resolution spatial transcriptomics sequencing technology. This technique captures and spatially resolves genome-wide RNA expression in a submicron resolution for fresh-frozen tissue.

Troubleshooting

1 **Flow Cell Generation and Collection**

Recycled illumina flow cell was used to provide spatial coordinate for RNA molecules.
For MiniSeq flow cells, a pre-run modification is necessary to replace the NaOCl solution with the Tween-20 solution in the reagent cartridge. We replaced the NaOCl solution inside the MiniSeq reagent cartridge (MiniSeq Mid Output Kit (300-cycles) FC-420-1004 or MiniSeq High Output Reagent Kit (300-cycles) FC-420-1003) by washing well #31 on the cartridge with 0.1% Tween-20 Buffer twice, then adding 1.3 mL buffer to the well. In addition, to prevent carry-over contamination between experiments, a manual wash with NaOCl solution is performed each time before using the machine.
For NextSeq 2000, no modification is required.
Collected flow cells are stored in the 4℃ fridge prior to use.

2 **Surface Treatment**

Flow cells were placed on a craft cutting board with long edges aligned to be disassembled. Two transparent films were applied to each side of the flow cell to protect the cell surfaces. Laser absorbent materials surrounding the sequencing surface were cut off by a hand drill with a round diamond cutter. Since each flow cell has two surfaces, forces were applied to the shared plane to separate the two surfaces. Letters were then assigned to different surfaces to distinguish surfaces from each other, as well as the direction.

Slides were transferred into a 2 mL tube with 500 mL 2μM P7 matching primer and incubated in a preheated thermomixer to 95℃ at 400 RPM for 10 minutes. Surfaces were allowed to cool to room temperature once the reaction was complete and transferred to a plastic chamber to wash.

BbsI restriction enzyme digestion is then performed to cleave double-stranded P7 structures on the surfaces and expose a uniform sequence for later ligation with oligos that contains poly T tails. The digestion reaction was set up in a 2 mL tube: 50 μL NEB Buffer 2, 5 μL BbsI enzyme (NEB, R0539), and 445 μL nuclease-free water so that the total reaction volume is 500 μL. Slides were incubated overnight at 37℃ together in one tube for a higher throughput process and washed with water three times once the reaction was complete. A brief centrifuge was performed to remove the excess water remaining on the slides.

A 200 nM ligation reaction is then set up as follows in a new 2 mL tube: 200nM double-stranded poly T oligo with overhang, 10,000U/mL T4 ligase, and 1X ligation buffer (NEB, M0202). All the surfaces were transferred into the tube and incubated overnight at 16°C and 400 rpm. The wash process was repeated to clean the surface.

The exonuclease I procedure is incorporated into the protocol to minimize the presence of any single-stranded structure that can potentially decrease the capturing efficiency. Exonuclease I was diluted to 2 units/µL with 1X r2.1 buffer to a total volume of 800 µL in a new 2 mL tube. Surfaces were then transferred into the new tube for exonuclease I digestion at 37 ℃ for 30 minutes. Once the reaction is complete, surfaces are transferred into individual wells of the plastic chamber to be cooled and washed by 0.1N NaOH. To neutralize the acidity introduced by NaOH, surfaces were washed with 200 µL Tris-HCl, then with 200 µL water. The wash process is repeated three times. Modified surfaces were stored in a -20℃ fridge before applying tissue.

3 **Tissue Application**

In this procedure, the tissue of interest was sectioned into 20-30 um thin slices by a cryostat machine and applied to the surface. 20um to 5um OCT embedded tissue sections were applied to the slides. Once all surfaces were applied with the sample, they were immersed separately in a 2 mL tube with methanol pre-chilled in a -20 ℃ fridge for 20 minutes.

Surfaces were then transferred out of the tube and washed with water, allowed to air dry in a petri dish. The staining procedure was accomplished by pipetting just enough volume of staining reagent to cover the sample and washing with water after the incubation. Depending on the sample size, the staining volume ranges from 50 to 100 µL. Hematoxylin was first added onto the slide for 7 minutes, followed by a treatment of bluing buffer for 2 minutes and a brief treatment with Eosin. Stained surfaces were then imaged under a microscope. All the structural details were captured and archived.

Surface space without any tissue applied is covered by a plastic film that was cut into an appropriate shape to block the large empty area without tissue application. This further simplifies the convolution of sequences and the tissue image.

Proteinase K was used as a final step to digest the sample and release its RNA. 200 µL 8U proteinase K solution was prepared and used to cover the surface residing in a chamber. The chamber was sealed with aluminum foil and placed in a 37℃ incubator for overnight incubation.

A reverse transcription procedure was carried out to convert RNA into cDNA with all RNA released from the sample and captured by poly T tails. The reverse transcription reaction was set up as follows: 80 µL 1mM dNTP, 200 µL RT buffer, 80 µL 3.75 µM template switching oligo, 80 µL RT enzyme mix, and nuclease-free water until volume reached 800 µL. The reaction mixture was then divided equally and added to the surface. Each surface received an equal amount of reaction mix and was incubated overnight at 42℃. Around 100 µL of exonuclease I reaction mix described previously was added to each

surface individually and incubated at 37 ℃ 550 RPM for 30 minutes to remove any single-stranded structure.

4 **Visualization of RNA Capturing**

In this step of our protocol, we relied on the interaction between biotin and R-phycoerythrin labeled streptavidin (SAPE) to visualize the capture efficiency of our surfaces. Enough volume of 5 ug/mL SAPE solution was added to the surfaces and incubated in a dark environment for 30 minutes. SAPE solution was pipetted off the surfaces, and 1X PBS buffer was used to wash all the surfaces three times. All the surfaces were then transferred into a 1.5 mL tube and centrifuged briefly to allow them to dry. Surfaces were then imaged under a fluorescence microscope in a dark room. Images were taken and archived. SAPE was then removed by a wash step described previously with NaOH for 5 minutes, followed by 200 mMTris-HCl and water wash.

5 **Library Construction**

With the SAPE tag confirmed capture efficiency, this part of the protocol aims to construct a library that various platforms can later be sequenced. The second strand DNA synthesis mix was produced using DNA Polymerase I mix and incubated at 37℃ for 2 hours, with a random primer-adapter extension conjugate of custom read 2 sequence and P7 sequence.

A previously described NaOH wash step is performed to elute the DNA library to 100 uL. The eluted library was then purified with a column (Qiagen, 28004), where it is neutralized with sodium acetate. A PCR reaction containing adapter extension primers was then performed. PCR product was then rewashed with column (Qiagen, 28004), followed by gel electrophoresis where the library between 300bp to 1500bp was selected and purified. Bioanalyzer with high DNA sensitivity detection was used to ensure the quality of the library with quantification (Agilent, 5067-4626). The library was then sequenced with custom reading primers on the MiniSeq platform.

6 **Data processing and analysis**

HiFi Slide sequencing basecell files were converted to raw reads by illumina software bcl2fastq (version 2.20.0.422).
HiFi Slide R1 reads were indexed by BWA (version 0.7.17-r1188).
Then de-duplicated spatial barcodes from the recycled flow cell were mapped to R1 reads using BWA-MEM. It is necessary to invoke the BWA-MEM parameter "-a".
HiFi Slide R2 reads were mapped to human genome by STAR (version 2.7.5c).
Reads uniquely aligned to human genome were extracted from the resultant sam file.

We used bedtools (version 2.30.0) to identify genes mapped by HiFi Slide R2 reads. Summary statistics such as number of spatially resolved HiFi Slide read pairs were calculated by in-house script. Visualization of results were performed by R (version 4.1.2).