AvanSci Bio Technical Application:
Mesodissection of FFPE Tissue for Expression Analysis by Reverse Transcription and Quantitative PCR

This technical application presents the results of two experiments where we investigated relative RNA recovery efficiencies of different dissection parameters, as assayed by Reverse Transcription and Quantitative PCR (RT-qPCR). In the first experiment, we investigated floating tissue sections vs placing directly on slides, dissection of deparaffinized vs paraffinized tissue, different milling solutions, and crude vs purified template preps for the reverse transcription reaction. In the second experiment, we investigated manual dissection vs mesodissection of the same areas on serial sections.

Experiment 1:

- On the day of dissection, cut 5 micron serial sections from multiple year old human liver and kidney FFPE tissue blocks. Discard the first 10 sections, then place sections either onto 45^{o}C water (to spread) and recover onto surface of positively charged slides, or place sections directly onto slides (**"dry"**). Heat slides 65^{o}C 15 min to adhere tissue sections.
- Deparaffinize the indicated tissue sections (using AvanSci Bio's mineral oil-alcohol system) and stain with Analine Blue.
- Dissect the same 20 mm^{2} area from 8 liver serial sections. Do the same for 8 kidney serial sections.

Sample	tissue	prep
1 and 9	liver	paraffin
2 and 10	liver	deparaffin
3 and 11	liver-dry*	paraffin
4 and 12	liver-dry*	deparaffin
5 and 13	kidney	paraffin
6 and 14	kidney	deparaffin
7 and 15	kidney-dry*	paraffin
8 and 16	kidney-dry*	deparaffin

Milling solutions:
Samples 1-8 = Qiagen PKD buffer (Rinse xScisor with second volume of PKD to improve recovery.)
Samples 9, 11, 13, and 15 = mineral oil
Samples 10, 12, 14, and 16 = 2 mM Tris, pH 8.0, 0.2 mM EDTA, 0.1% tween-20

Notes:
The Qiagen RNeasy kit worked well for us. We presume similar kits from other manufactures would also work.

Tissue fragments were recovered from xScisor into 0.6 ml low binding surface tubes, the benefit of which is untested.

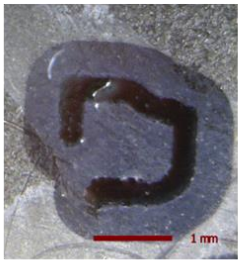
Milling solutions used in this study:

Qiagen PKD buffer – Can go directly into the Proteinase K step of the RNeasy FFPE spin column based clean up kit and relatively easy to visualize the dissection process. However, only weakly holds paraffinized tissue fragments in suspension, and RNA purification required before proceeding with downstream biochemistry.

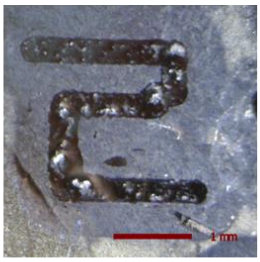
Mineral oil - Effective at aspirating and holding paraffinized tissue in suspension, easy to remove after Proteinase K step using AvanSci Bio's Wicking strips, non-inhibitory to most downstream biochemistry so crude template can be utilized (at least for DNA templates). However, can be messy to work with and difficult to visualize the dissection process. (Addition of hexanol to mineral oil can improve recovery efficiency, improves visualization, but is not compatible with Wicking Strips and has a strong smell.)

TE + 0.1% tween-20 - Non-inhibitory to most downstream biochemistry and easy to visualize, but not effective with paraffinized tissue.

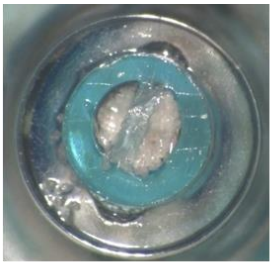
Mesodissection of paraffinized tissue



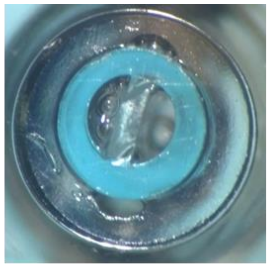
Using mineral oil



Using PKD buffer



Suspension of paraffinized tissue and Qiagen PKD buffer in end of xScisor



Single rinse in PKD buffer improves recovery

RNA Preparation Methods

Samples 1-8: purify using Qiagen RNeasy FFPE kit

- Add 10 ul Qiagen Prot K, mix gently
- Incubate on Thor 60oC 15 min 800 rpm, then 80oC 15 min 1200 rpm (the small amount of paraffin melts and disappears, presumably onto the sides of the container)
- Place on ice 3 min, then centrifuge top speed 5 min (only went 8k rpm because 0.6 ml tubes)
- Transfer supernatant to new tube (look for pellet before and after transfer)
- Add 16 ul DNase booster buffer, mix, then 10 ul DNase I, invert tube and quick spin to mix
- Incubate 37 for 15 min (400 rpm)
- Add 320 ul RBC, mix, then 720 ul 100% ETOh, mix again
- Add 700ul to spin column, spin 15 sec 8000g, discard flow through, repeat until all bound
- Add 500 ul RPE, spin 15 sec 8000g, discard flow through
- Add 500 ul RPE, spin 2 min 8000g, discard flow through
- Transfer column to new tube, open lid, centrifuge top speed 5 min
    - Transfer column to new tube, add 40 ul RNase free water, spin top speed 1 min to collect

Samples 9, 11, 13,and 15: make crude prep from paraffinized tissue
- To recovered tissue in mineral oil, add 40 ul TET with 8 ug fresh Prot K
- Incubate on Thor 56C 15 min 1200 rpm, then 80oC 15 min 1200 rpm
- Quick spin, then remove mineral oil with wicking strip
- Perform Qiagen recommended DNase protocol

Samples 10, 12, 14, and 16: make crude prep from deparaffinized tissue
- Centrifuge tube, remove supernatant to leave 36 ul
- Add 4 ul TET containing 5 ug fresh Prot K, then 40 ul mineral oil
- Incubate on Thor 56C 15 min 1200 rpm, then 80oC 15 min 1200 rpm
- Quick spin, then remove mineral oil with wicking strip
- Perform Qiagen recommended DNase protocol

Experiment 1: RT-qPCR results

- Make cDNA using Applied Biosystems High Capacity Reverse Transcription kit follow the recommended protocols. Used either 10 ul Qiagen purified RNA or 10 ul crude lysate.
- Perform qPCR using Applied Biosystems Power SYBR reagent and the StepOne system using the recommended protocols.

PCR amplicons were housekeeping gene primer pairs from Galiveti et al., (RNA 16:450, 2010)

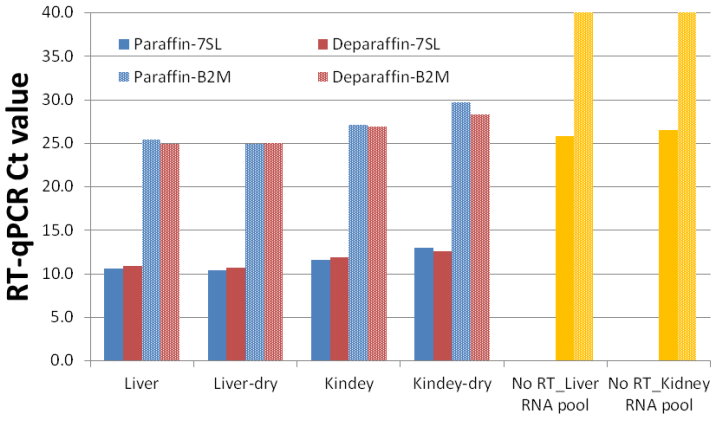
7SL = snRNA (Signal recognition particle RNA, universally conserved)

B2M = Beta 2-microglobulin (part of MHC class 1 complex, found on all nucleated cells)

cDNA from Qiagen purified RNA

Sample	tissue	prep
1	liver	paraffin
2	liver	deparaffin
3	liver-dry	paraffin
4	liver-dry	deparaffin
5	kidney	paraffin
6	kidney	deparaffin
7	kidney -dry	paraffin
8	kidney -dry	deparaffin

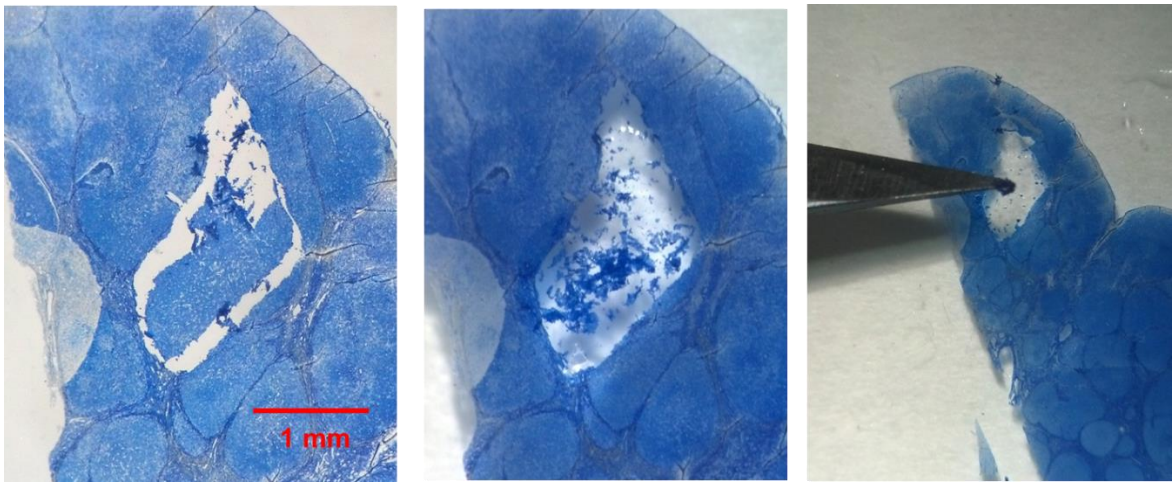
(Lower Ct =~ more RNA isolated)



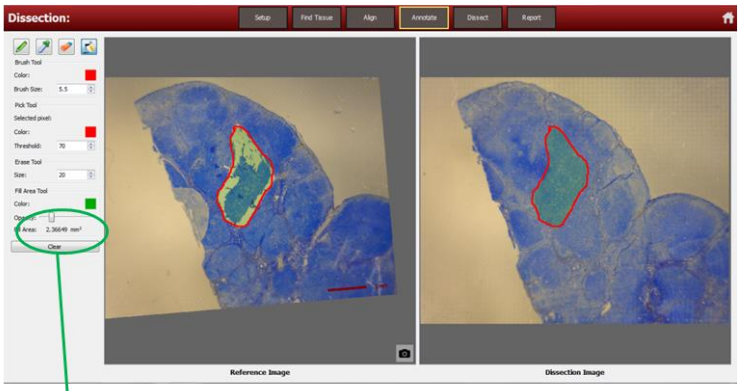
Samples 9-16 did not amplify earlier than No Reverse Transcription controls suggesting that cDNA was not made in Reverse Transcription reaction from crude lysates.

Experiment 2: Hand Dissection vs. Mesodissection

Hand dissection of deparaffinized and Analine Blue stained human liver tissue using dissection microscope, scalpel, and 3% glycerol solution

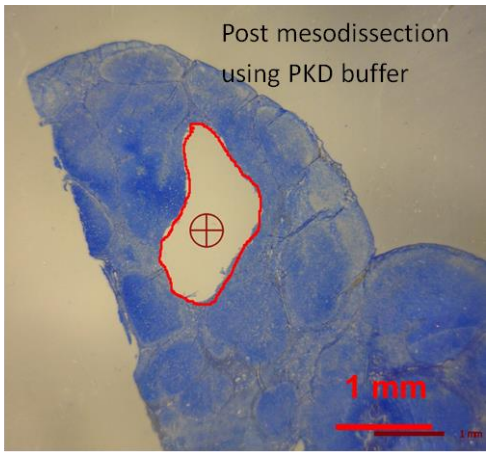


Mesodissection of Same Area from a Serial Section



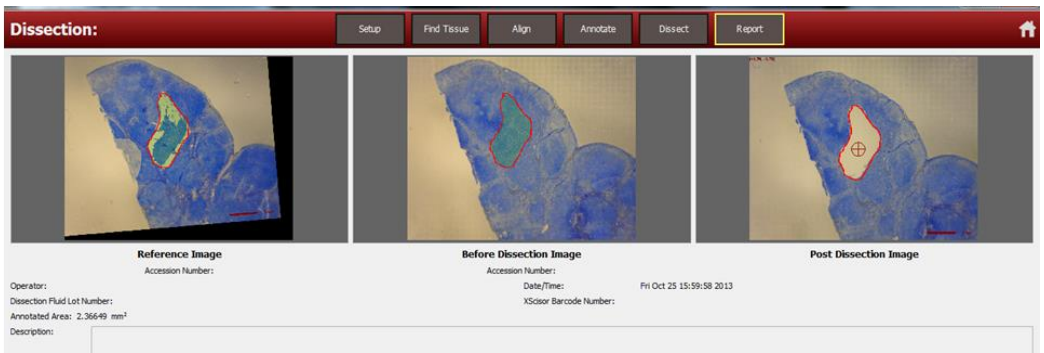
Saved image from hand dissection

Live image from serial section



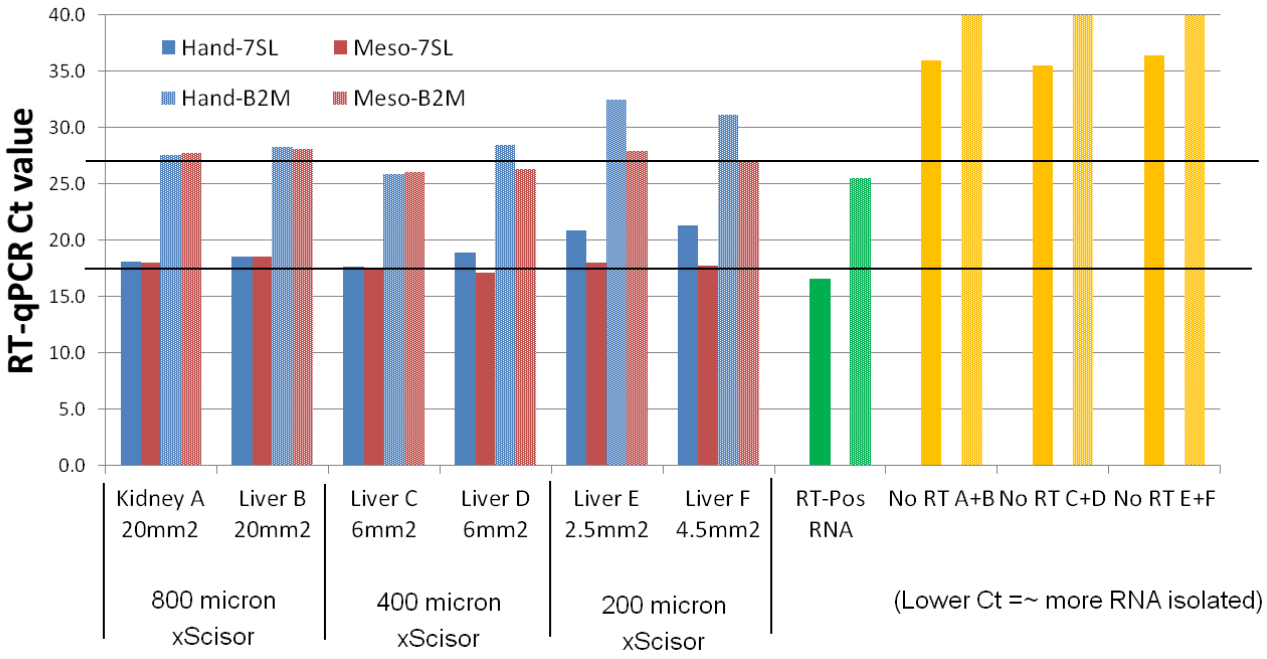
2.36 mm^2

Digital dissection report



Hand dissection vs. Mesodissection by RT-qPCR

- Prepare RNA using Qiagen FFPE RNeasy protocol
- Make cDNA using Applied Biosystems High Capacity Reverse Transcription kit
- Perform qPCR using Applied Biosystems Power SYBR reagent and the StepOne system (qPCR amplicons were 7SL and B2M amplicons described previously)



This data indicates RNA yields obtained using the mesodissection system are at least equivalent and in some cases (small areas) slightly better than using hand dissection.

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