

# Optimization of RNA extraction from laser captured microdissected glomeruli from formalin-fixed paraffin-embedded mouse kidney samples for Nanostring analysis

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**Summary.** Optimized protocols for the microdissection of specific areas from archival tissues and the subsequent RNA analysis are needed but challenging due to RNA degradation and chemical modifications. The aim of this study was to present the most appropriate protocol for utilizing mouse FFPE kidney for laser capture microdissection and Nanostring gene expression analysis.

We evaluated different section thicknesses (3, 5, 10  $\mu\text{m}$ ), 2 RNA extraction kits (Qiagen and Roche) and different H&E staining methods to optimize microdissection and RNA extraction from glomeruli and cortical tubules samples from FFPE mouse kidney. RNA quality and quantity were assessed via Nanodrop and Qubit.

The protocol providing the best results consisted of 5  $\mu\text{m}$  sections, a shorter protocol for H&E staining, and RNA extracted with the Roche kit. Higher Nanostring gene counts and lower qPCR cT significantly correlated with RNA concentrations measured with the Qubit, but not with measures obtained with the Nanodrop. The Nanostring data showed that none of the genes included in the panel was differentially expressed in the cortical tubule compartment compared to the whole kidney. However, 25 genes were differentially expressed in the glomerular compartment compared to the whole kidney. Our data showed that sufficient RNA can be extracted from small compartments like mouse renal glomeruli

from archival FFPE tissue, and that whole kidney analysis does not accurately represent the transcriptome state of the glomeruli, which comprise only a small proportion of the overall kidney volume.

**Key words:** Mouse glomeruli, Nanostring analysis, Laser capture microdissection

## Introduction

Examination of whole tissue sections for molecular pathology studies has been invaluable in increasing our knowledge of the molecular mechanisms of health and disease. However there is increasing emphasis in investigation of RNA or protein expression at the tissue sub-compartment level, as functional and pathologic changes often do not occur across the whole organ, but are localised to different regions of the organ. Laser capture microdissection (LCM) is a method which improves and simplifies the process of isolating cellular structures by using laser beams to dissect specific cells or structures from the tissue (Espina et al., 2006). LCM allows the user to select specific areas and delivers small pulses of UV laser to dissect and propel these from the slide into an adhesive tube cap, allowing their use for downstream applications such as gene expression. LCM studies using gene expression as a downstream application have mainly used frozen tissues sections due to their superior preservation of RNA (von Ahlfen et al., 2007; Erickson et al., 2009; Legres et al., 2014; Castro et al., 2016). However, LCM can also be used on formalin-fixed and paraffin-embedded tissues (FFPE).

FFPE is one of the most prevalent methods of preserving and storing both human and animal tissue samples. It is a method which preserves internal structures and proteins while allowing easier handling and sample storage, and has thus become a standard approach for histologic and immunohistochemical studies. However, the cross-linking properties of formaldehyde which so well preserves the proteins and internal architecture does come at a cost in the form of chemical modification to nucleic acids, such as RNA (Masuda et al., 1999). Thus, FFPE tissue has historically been considered a sub-optimal source of RNA for gene expression analysis due to low yield and high degradation. However, the large FFPE tissue archives available in many medical and research institutions represent an important resource that could potentially be tapped into in order to increase our understanding of molecular processes of disease. As a result, intensive research has yielded RNA extraction methods and gene expression techniques optimized for FFPE tissue, and the use of such material has become more commonplace in molecular pathology (Kokkat et al., 2013).

One such technique which has proved valuable for gene expression studies in FFPE tissue is the Nanostring nCounter, which relies upon hybridisation of a two-part probe system, each of which contains a fluorescent 'barcode' unique to a particular gene. Nanostring data has shown to match well between frozen and FFPE samples, with comparable data obtained from qRT-PCR (Reis et al., 2011), thus demonstrating its suitability for FFPE molecular analysis. Nanostring requires a minimum of 100 ng of RNA for its gene expression assays. However accurately quantifying RNA in FFPE samples has proved problematic. Nanostring recommends the use of the Nanodrop spectrophotometer. Another commonly used method for nucleic acids quantification is the Qubit fluorometer. Determination of quality has also proved problematic with this method. The traditionally used Agilent Bioanalyser 'lab on a chip' technology provides as an output a single number, the RNA Integrity Number (RIN). The RIN however does not consider the distribution of the differently sized fragments and as such, samples with similar RIN may in fact have different levels of degradation (Illumina, 2014). An alternative measure of quality, the DV200 or percentage of fragments <200 nt, can also be calculated from the trace generated by the RIN analysis and this has shown to correlate better with gene expression output in assays (Illumina, 2014).

Despite advances in gene expression technologies (Ludyga et al., 2012; Esteve-Codina et al., 2017) and the development of protocols to extract RNA from FFPE tissue (Gouveia et al., 2014; Tekin et al., 2016; FitzGerald et al., 2018) and microdissected FFPE tissue (Golubeva et al., 2013; Castro et al., 2016), the yield and quality of the RNA obtained from FFPE tissues remain critical factors for success of any downstream application (Aguilar-Bravo and Sancho-Bru, 2019). In addition, differences between different RNA quantity

and quality measurement methods on FFPE tissues could also have an impact on downstream applications, such as Nanostring analysis. Considering all these variables, a good initial pilot study to determine the steps required to achieve the target amount of RNA, in both qualitative and quantitative terms, for the selected downstream gene expression analysis, appears fundamental.

The present study describes a complete strategy for the extraction and quality control of RNA from stained, laser capture microdissected FFPE samples for Nanostring analysis. This strategy has been applied to the study of different compartments in mouse kidney using FFPE samples.

## Materials and methods

### Tissues

Formalin-fixed paraffin-embedded kidney samples from 7-week-old db/db mice (n=3) were used.

Experiments using 7-week-old db/db mice (Charles River) were conducted under a U.K. Home Office Project Licence in accordance with the U.K. Animal (Scientific Procedures) Act 1986 and in accordance with EU Directive EU 86/609. Kidneys were collected immediately after euthanasia and were immersion-fixed in 10% neutral-buffered formalin for 24 hours prior to processing and embedding in paraffin.

**Table 1.** Staining procedures used for visualizing kidney sections mounted on slides.

Step	Reagent	Time [m.s] – Protocol A	Time [m.s] – Protocol B
1	Xylene	5.00	5.00
2	Xylene	5.00	5.00
3	Xylene	5.00	N/A
4	IMS	3.00	0.30
5	IMS	3.00	0.30
6	IMS	3.00	/
7	95% IMS	3.00	0.30
8	Running Tap Water	3.00	1.00
9	Hematoxylin Gill II	3.00	3.00
10	Running Tap Water	4.00	1.00
11	1% Acid Alcohol	0.20	/
12	Running Tap Water	3.00	/
13	Eosin Y	0.30	/
14	Running Tap Water	3.00	/
15	95% IMS	1.00	0.30
16	IMS	0.45	0.45
17	IMS	0.45	0.45
18	IMS	0.45	/
19	Xylene	2.00	1.00
20	Xylene	2.00	1.00
21	Xylene	2.00	N/A
22	Air Dry (Room Temperature)	10.00	10.00

Protocol A is the standard procedure routinely used within the laboratory on the Leica ST5020 Autostainer (Leica Biosystems). Protocol B was modified from Espina et al. (2006). Incubation time for each step expressed as minutes (m). seconds (s).

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### Preparation of FFPE sections

The Leica RM2135 rotary microtome and water bath were cleaned using RNaseZap (Thermo Scientific, Leicestershire, UK) prior to sectioning and the water bath filled with Millipore water. Kidney sections were cut at 3, 5 and 10  $\mu\text{M}$  thickness and mounted on glass Superfrost plus slides (Thermo Scientific, Leicestershire, UK).

### Hematoxylin and Eosin (H&E) staining

Hematoxylin and Eosin staining was performed using the protocols illustrated in Table 1. Protocol B was also tested with the addition of 200  $\mu\text{L}$  of RNase inhibitor (Sigma-Aldrich, Gillingham, UK) in steps 4-16.

### RNA extraction

RNA extraction was performed using two different commercially-available kits: the column-based RNEasy FFPE Kit (Qiagen, Manchester, UK) and the High Pure FFPE Extraction Kit (Roche, Welwyn Garden City, UK), according to the manufacturer's instructions. In brief, tissue lysate was added to the column and RNA captured on the column membrane. The final RNA was eluted using RNase-free water.

### Laser capture microdissection

The glomeruli were microdissected using the 'AutoLPC' function under the PALM Microbeam ZEISS Microscope (Cambridge, UK) (Fig. 1). Laser parameters, namely energy and focus, were set to 69 and 81 respectively. Microdissected glomeruli were captured into the lid of AdhesiveCap 500 opaque (415190-9201-000, Zeiss, Cambridge, UK). Whole kidney sections were taken from the same tissue blocks, to use as comparator samples.

### Qualitative assessment of RNA: RIN and DV200

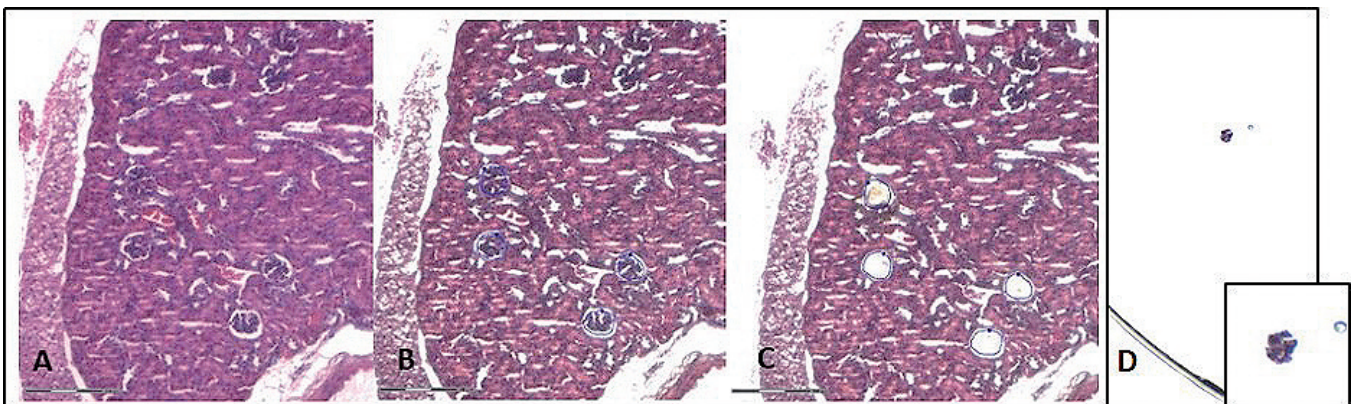
Total eukaryote RNA pico chips were run on the Agilent 2100 Bioanalyser according to the manufacturer's instructions. Additionally, the DV200 (percentage of fragments over 200 nt in length) was calculated.

### Quantitative assessment of RNA: Nanodrop and qubit

Quantity of RNA obtained was assessed using the Nanodrop 8000 spectrophotometer and the Qubit 2.0 according to the manufacturer's instructions. The ranges of RNA concentration measurement with NanoDrop and Qubit are between 2.5 and 3700  $\text{ng}/\mu\text{L}$  and between 0.25 and 1000  $\text{ng}/\mu\text{L}$  respectively.

### Nanostring

Nanostring gene expression analysis was performed using the nCounter XT Mouse Immunology code-set according to the manufacturer's instructions. As previously reported (Nanostring Website) (Butto et al., 2019) the nCounter data were normalized in two steps. In the first, we used the positive spiked-in controls provided by the nCounter instrument as per the manufacturer's instructions (NanoString Technologies). Second, we normalized the data of the Mouse Immunology code set to 10 (Alas1, Eefg1, G6pdx, Gusb, Hprt, Oaz1, Polr1b, Polr2a, Rpl19, Sdha) out of the 14 housekeeping genes present in the nCounter XT Mouse Immunology code set. These 10 housekeeping genes were selected based upon Pearson's correlation co-efficient and GeNorm calculations. Any housekeeping gene which exhibited a statistically significant t-test results ( $p < 0.05$ ), corresponding to an unstable expression pattern, was excluded. In this study the housekeeping gene excluded were Gapdh, Ppia, Tbp, Tubb5.



**Fig. 1.** LCM glomerular cross-sections using the PALM Laser-Microbeam System. After mounting, deparaffinization and staining of the sections with H&E, glomeruli were identified (A), then manually traced (B) and microdissected (C). Later, glomeruli are visualised in the lid (D).

### qPCR

qPCR gene expression analysis was performed in triplicate using the Taqman RNA-to-Ct 1-step kit (Applied Biosystems, Warrington, UK) on the Quantstudio 12K Flex Real-time PCR system (Thermo Scientific, Leicestershire, UK). Master mix was prepared using 2  $\mu$ L 4X master mix, 0.1  $\mu$ L reverse transcriptase and 0.4  $\mu$ L of the desired primers and 1.6  $\mu$ L of sample RNA (5ng/  $\mu$ L). Taqman gene expression assays used in this study were Podocin (Mm01292252\_m1), Cdh16 (Mm00483196\_m1) 18s (Mm03928990\_g1) and B2M (Mm00437762\_m1). Expression values for Podocin and Cdh16 were normalized using 18s.

### Statistics

Data for RNA quality and concentration were presented as mean values  $\pm$  standard deviations. Differences in the groups were tested using the paired t-tests or One way ANOVA with Tukey's multiple comparisons tests. Correlation of RNA concentrations measured by Nanodrop Spectrophotometry and Qubit fluorometry with Nanostring housekeeping gene counts or qPCR housekeeping gene cT values were analyzed via Pearson's correlation co-efficient and GeNorm calculations. Data analysis was done using GraphPad Prism (GraphPad Software Inc, California).

Differential expression analysis was performed using the Nanostring nSolver software. Benjamini-Yekutieli false discovery rate p-value adjustment was performed due to the high number of repeats for the same sample. A p value of less than 0.05 was considered statistically significant for all statistical analyses.

## Results

### Optimization of sectioning and staining method for laser capture microdissection and RNA extraction

Sections taken at 3, 5 and 10  $\mu$ M were assessed to determine which thickness was optimal for LCM based upon ease of sectioning, internal structure visibility and ease of microdissection. All sections thicknesses allowed effective and accurate microdissection of tissues, however the absence of a coverslip (necessary for microdissection) lead to decreased visibility of internal structures in the 10  $\mu$ M sections. There was no difference between the 3 and 5  $\mu$ M sections in terms of internal structure visibility, therefore 5  $\mu$ M was considered optimal as it would yield more tissue than 3  $\mu$ M for RNA extraction.

For the comparison of staining procedures and RNA extraction kits, 3 different FFPE blocks were sectioned and the areas microdissected were equal to 107  $\mu$ M<sup>2</sup> per slide. Three staining procedures were tested to ascertain which would be optimal for subsequent extraction of RNA using the Qiagen extraction kit (Table 1): all three protocols include initial steps in xylene for

deparaffinization, followed by dehydration in a series of alcohols, 100% and 95%. The 'standard' procedure (A) is the one used routinely within the laboratory on the Leica ST5020 Autostainer (Leica Biosystems). The 'fast' protocol (B) was modified from Espina et al., 2006 (B). Protocol B was also separately tested with addition of RNase inhibitor. RIN values were similar across all staining procedures (Table 2A). RNA concentration and sample purity (260/280 ratio) were better in protocol B compared to protocol A (RNA concentration 17.1 $\pm$ 6 ng/ $\mu$ L and 9.9 $\pm$ 5.8 ng/ $\mu$ L respectively; 260/280 ratio 1.81 $\pm$ 0.43 and 1.73 $\pm$ 0.14 respectively). RNA yields with Protocol B were significantly higher than with Protocol A (p=0.05). There was no statistically significant difference between protocol A and B for RNA quality (p= 0.2185) and purity (p=0.9366). The addition of RNase inhibitor to protocol B did not significantly affect RNA quality (p=0.8939), yield (p=0.8538) or purity (p=0.9105; Table 2A).

The Qiagen RNEasy FFPE kit and Roche High Pure FFPE extraction kit were compared to assess which would be optimal for RNA extraction of microdissected FFPE tissue. RIN values (p=0.9097), RNA yield (p=0.4362) and purity values (p=0.1127) were similar between the two kits (Table 2B).

### Quantitative and qualitative assays in the prediction of sample suitability for nanostring and qPCR gene expression analysis

RNA concentration was determined using the Qubit

**Table 2.** Comparison of RNA quantity, quality, and sample purity obtained with the 3 different H&E staining procedures and 2 different FFPE RNA extraction kits.

A) H&E Protocol	RIN	Concentration (ng/ $\mu$ L)	260/280
Protocol A	2.1 $\pm$ 0.1	9.9 $\pm$ 5.8	1.73 $\pm$ 0.14
Protocol B	1.9 $\pm$ 0.2	17.1 $\pm$ 6	1.81 $\pm$ 0.43
Protocol B + RNase Inhibitor	1.9 $\pm$ 0.1	22.9 $\pm$ 13.68	1.92 $\pm$ 0.32
B) RNA Extraction Kit	RIN	Concentration (ng/ $\mu$ L)	260/280
Qiagen RNEasy FFPE Extraction Kit	2.3 $\pm$ 0.1	10.8 $\pm$ 5.4	1.39 $\pm$ 0.06
Roche High Pure FFPE Extraction Kit	2.2 $\pm$ 0.3	15.0 $\pm$ 11.4	1.94 $\pm$ 0.30

For the comparison, 3 different FFPE blocks have been sectioned and the areas microdissected was equal to 107  $\mu$ M<sup>2</sup> per slide. Average RIN and concentration presented as values  $\pm$ standard deviation. RNA elution volume constant (25  $\mu$ L). A) RIN consistent across all staining procedures. RNA concentration and 260/280 ratio better with protocol B with addition of RNase inhibitor. One way ANOVA with Tukey's multiple comparisons tests indicated RNA yields with Protocol B were significantly higher than with Protocol A (p=0.05). B) RIN consistent between the two kits used however concentration and 260/280 values appeared to increase when RNA was extracted using the Roche kit, however paired t-tests indicated no significance in any of the comparisons.

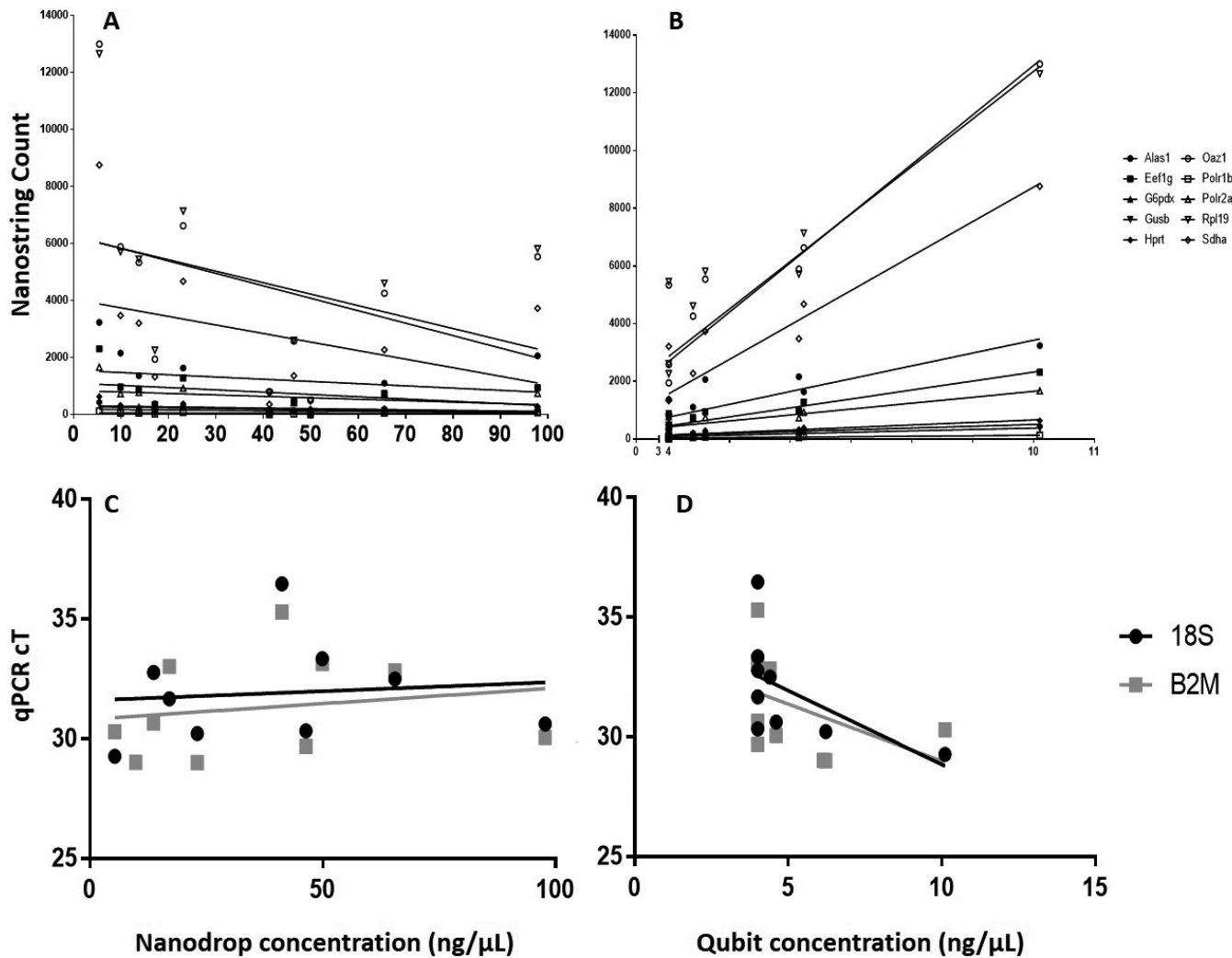
## Nanostring analysis on mouse glomeruli

fluorometer and Nanodrop spectrophotometer and plotted against the Nanostring housekeeping gene counts and qPCR housekeeping gene cT values. Nanodrop concentrations did not correlate with the Nanostring counts or qPCR cT values (Fig. 2A,C). Qubit RNA concentrations significantly correlated with all individual Nanostring counts ( $p \leq 0.05$ ) and with qPCR output data (Fig. 2B,D). Similarly, quality of RNA was determined using RIN or the percentage of fragments over 200bp (DV200) and plotted against the Nanostring housekeeping gene counts and qPCR housekeeping gene cT values. No correlation was observed for the RIN (Fig. 3A,C) and the DV200 values (Fig. 3B,D) with Nanostring counts and cT values.

## Validation of the purity of kidney compartment samples extracted by LCM via qPCR

We microdissected and extracted the RNA from whole sections and from different areas (glomeruli and cortical tubules) of mouse kidney.

qPCR using glomerulus and tubule specific genes was performed to determine whether LCM was successfully extracting specific samples of the two compartments. As represented in Fig. 4A, podocin expression was significantly higher in the glomeruli samples than in both the whole kidney and cortical tubule samples ( $p=0.0007$  for both). CDH16 expression was lower in the glomeruli compared to the whole



**Fig. 2.** RNA quantification in the prediction of sample suitability for Nanostring and qPCR gene expression analysis. Correlation of RNA concentrations measured by Nanodrop Spectrophotometry and Qubit fluorometry with Nanostring housekeeping gene counts or qPCR housekeeping gene cT values. Nanodrop concentrations did not correlate with Nanostring counts ( $p > 0.05$ ) (A) or qPCR cT values ( $p > 0.05$ ) (C). All genes reported significant Pearson's correlation coefficients for Qubit vs Nanostring count ( $p < 0.05$ ) (B) and with qPCR output data (18S  $p = 0.059$ , B2M  $p = 0.028$ ) (D).

### Nanostring analysis on mouse glomeruli

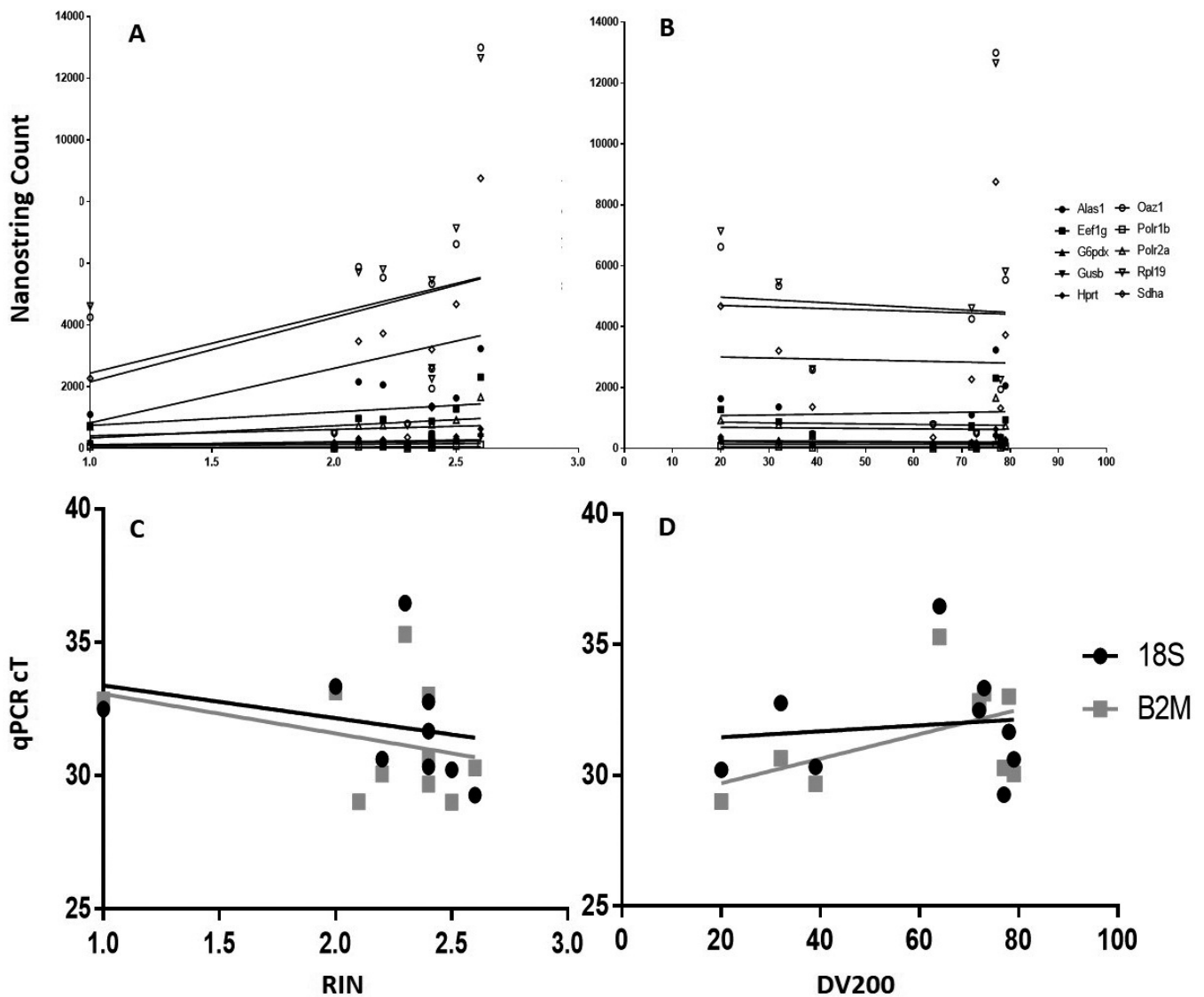
kidney or cortical tubule samples ( $p=0.564$  and  $p=0.284$  respectively; Fig. 4B), but this difference was not statistically significant.

#### Nanostring gene expression analysis

Using the protocol that gave us the best results (5  $\mu\text{m}$  sections, a shorter protocol for H&E staining, and RNA extracted with Roche kit), RNA from glomeruli and cortical tubule was extracted from healthy FFPE mouse kidney and subjected to Nanostring gene expression analysis of 547 immunology related genes. In

order for the desired downstream gene analysis techniques to be performed, 100 ng of RNA was required. The minimal glomeruli threshold to reach the adequate RNA amount was calculated (data not shown) and 200 glomeruli/sample were microdissected.

Expression in each of the compartments was compared to that of the whole kidney. None of the gene included in the panel was differentially expressed in the cortical tubule compartment compared to the whole kidney. However for the glomerular compartment, 25 genes were differentially expressed compared to the whole kidney (C1ra (NM\_023143.3), Casp1



**Fig. 3.** RNA assessment in the prediction of sample suitability for Nanostring and qPCR gene expression analysis. Correlation of RIN or percentage of fragments over 200bp (DV200) with Nanostring housekeeping gene counts and qPCR housekeeping gene cT values. RIN values seemed slightly to correlate with Nanostring counts (A) and qPCR cT values (C), but this difference was not statistically significant ( $p>0.05$ ). The DV200 did not correlate with Nanostring counts ( $p>0.05$ ) (B) and qPCR cT values ( $p>0.05$ ) (D).

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(NM\_009807.2), Cd34 (NM\_001111059.1), Cd55 (NM\_010016.2), Cd97 (NM\_011925.1), Cd99 (NM\_025584.2), Cdh5 (NM\_009868.3), Dpp4 (NM\_001159543.1), Ets1 (NM\_001038642.1), Fyn (NM\_008054.2), Icam2 (NM\_010494.1), Ifngr1 (NM\_010511.2), Il2rg (NM\_013563.3), Il4ra (NM\_001008700.3), Il6ra (NM\_010559.2), Itgb1 (NM\_010578.1), Map4k4 (NM\_008696.2), Notch1 (NM\_008714.2), Pdgfb (NM\_011057.3), Pdgfrb (NM\_008809.1), Ptger4 (NM\_008965.1), Tgfb1 (NM\_011577.1), Tlr4 (NM\_021297.2), Tnfsf12 (NM\_011614.3), Zeb1 (NM\_011546.2); Fig. 4).

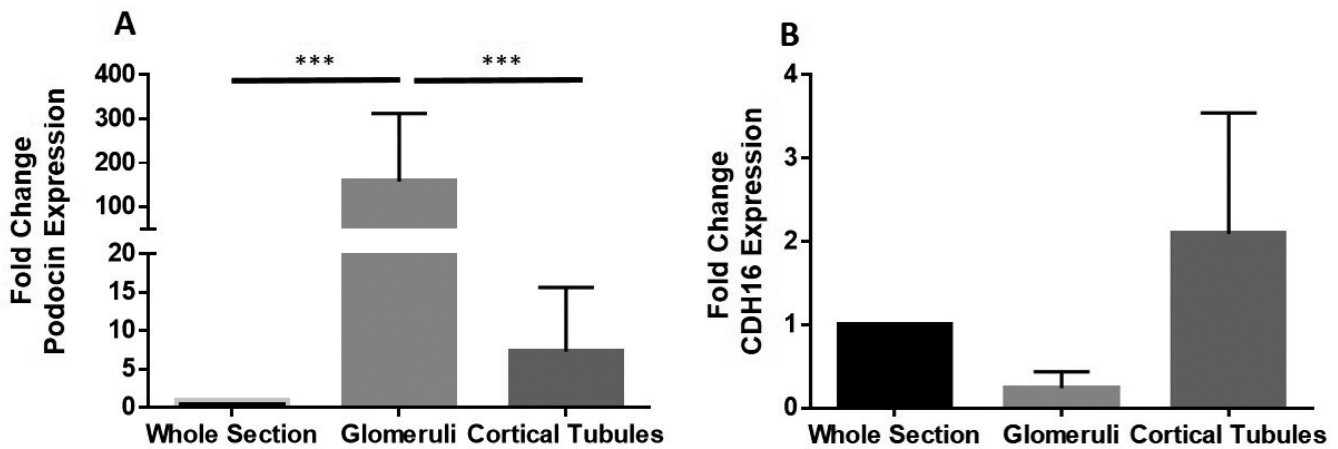
### Discussion

Large FFPE tissue archives available in many medical and research institutions represent a fundamental resource in biomedical research. Intensive research has yielded RNA extraction methods and gene expression techniques optimized for FFPE tissue, and the use of such material has become more commonplace in molecular pathology (Golubeva et al., 2013; Kokkat et al., 2013; Castro et al., 2016; Amini et al., 2017; Aguilar-Bravo and Sancho-Bru, 2019).

Optimization of protocols is a vital step in the set-up of any new technique, whether that is in-house validation of an assay from the literature or establishment of a brand-new assay. In this study, we present a complete strategy for utilizing mouse FFPE kidney for laser capture microdissection and Nanostring gene expression analysis. In addition, we evaluated different factors that could influence the results such as section thickness, RNA extraction kits, H&E staining method and RNA quality and quantity assessment method.

The selection of the tissue-staining protocol should

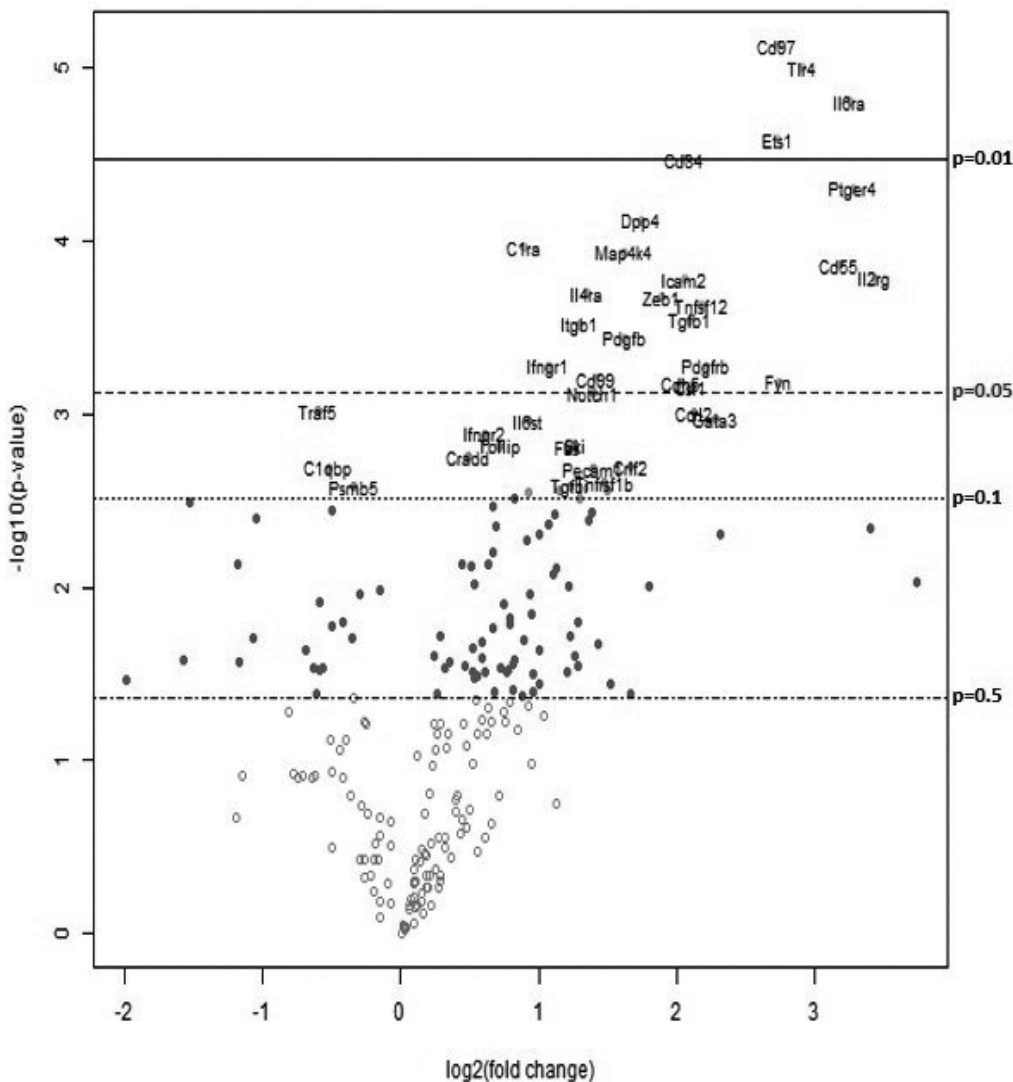
be based on compatibility with the downstream analysis to be performed with the microdissected tissue. Hematoxylin and eosin is the most popular staining method for LCM of FFPE tissue specimens (Liu et al., 2014), but other staining methods, such as methylene blue, Wright-Giemsa or toluidine blue are considered compatible with LCM experiments (Golubeva et al., 2013). In this study, to evaluate the effect of H&E staining methods on RNA quality and quantity, we tested a standard H&E procedure used for routine histological staining (Feldman and Wolfe, 2014), a shortened version described by Espina et al. (2006), and this shortened method with the addition of RNase inhibitor. Compared with the traditional H&E staining for pathology, the second protocol, thanks to shorter incubation times and elimination of unnecessary steps (eosin and acid alcohol), offered the possibility to reduce exposure to water and the risk of molecular degradation by endogenous RNases. This protocol selected for the downstream analysis was similar to others reported in the literature for FFPE tissue (Espina et al., 2006; Golubeva et al., 2013; Liu et al., 2014), it allowed a complete deparaffinization of the sections and good visualization of tissue morphology, maintaining only the essential steps and limiting the potential nucleic acid alterations as result of contact with the staining reagents. In general, the quality of RNA derived from a paraffin embedded tissue is influenced by several parameters. These include the pre-fixation time where ischemia and autolysis occur (Miyatake et al., 2004), the duration and conditions of the actual fixation process, the paraffin embedding procedure, sample storage and age (Liu et al., 2014), and the RNA extraction protocol (Srinivasan et al., 2002; Chung et al., 2006). These successive pre-analytical procedures influence the quality of the biomolecules derived from tissue samples and cannot be



**Fig. 4.** Expression of podocin and CDH16 in tubules and glomeruli compartments compared to the whole kidney. Fold change in expression of podocin (A) and CDH16 (B) in the tubules and glomeruli of the kidney compared to the whole kidney sample. Average + standard deviation shown. Podocin expression significantly higher in the glomeruli ( $p=0.0007$ ) compared to the whole kidney or cortical tubule samples. CDH16 expression lower in the glomeruli compared to the whole kidney or cortical tubule samples ( $p=0.564$  and  $p=0.284$  respectively).

easily standardized between laboratories. Consequently, RNA extracted from FFPE tissue often shows unpredictable variations in quantity and quality which may lead to varying and poorly reproducible gene expression data. In our experiment, we used precise conditions to minimize these effects. The pre-fixation time was minimal, as the kidney samples were immediately immersed in 10% formalin buffer after resection. We used a standard fixation procedure recommended in the literature (Hewitt et al., 2008; Liu et al., 2014): 24 hours incubation at room temperature in a 10% buffered formalin solution in a flask with a diameter larger than the tissue specimen and containing at least at 10:1 volume ratio of fixative to tissue. Once embedded in paraffin, the blocks were stored at controlled (21-23°C) room temperature in a closed block archive and sectioned just prior to microdissection, within 6 months from collection. It has been reported

that paraffin-embedded tissue stored in appropriate conditions suffers little decay in molecular integrity. Storing FFPE tissue at room temperature in a dry environment, protected from direct sunlight, allows to maintain them for decades since the proteins are locked due to the crosslinking induced by the formalin (Xie et al., 2011). The effects of storage condition have been evaluated (von Ahlfen et al., 2007), where freshly prepared blocks were stored at different temperatures and for different amounts of time before sectioning. Samples stored for up to 12 months at room temperature or 4°C did not show a significant decline in RNA extraction and yield performance. Only after storage for more than 12 months or at elevated temperatures, RNA integrity becomes the limiting factor for RT-PCR performance (von Ahlfen et al., 2007). Similar results were found by Kokkat et al. (2013), showing no significant difference between macromolecules extracted



**Fig. 5.** Nanostring gene data. Volcano plot indicating the 25 genes differentially expressed in the glomeruli compared to the whole kidney at  $\text{adj-}p < 0.05$ . Log representations of the fold change in expression compared to the whole kidney and the p-value were plotted on the x and y-axes respectively. Due to the high number of repeat testing of the same sample Bejamini-Yeukateili false discover rate p-value adjustment was performed and these thresholds are indicated by the horizontal lines on the graph. From top to bottom  $\text{adj-}p = 0.01, 0.05, 0.10, 0.50$ . Those genes above the  $\text{adj-}p = 0.05$  line were considered statistically significant. No genes were differentially expressed at the same significance threshold in the tubular compartment compared to the whole kidney.



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from blocks stored over 11-12 years, 5-7 years, or 1-2 years in proper conditions.

Precut tissue sections already mounted on slides seem to be more affected by exposure to the environment, particularly humidity and oxygen, which induce molecular oxidation reactions leading to decay, a phenomenon that may affect certain epitopes for immunohistochemistry and DNA extraction more so than others (Xie et al., 2011). Therefore, the recommendation is to keep the tissue protected in the paraffin block and avoid cutting the sections until they are ready to be used.

Despite the chemical alterations induced by formalin, with the above precautions biomolecules can still be recovered and analyzed using specific molecular extraction protocols and downstream analyses optimized for FFPE tissues (Liu et al., 2014).

Regarding the RNA extraction method, previous literature (Landolt et al., 2016) has indicated that Qiagen RNEasy FFPE and Roche High Pure FFPE extraction kits were optimal for extracting RNA from FFPE microdissected tissues. We tested these two kits and our results were similar, in that the Roche kit appeared to provide somewhat better RNA yield and purity compared to the Qiagen kit, although these differences did not reach statistical significance, perhaps because of the small number (n=3) of samples used in the current study. A promising addition to the commercially available kits for RNA extraction could be the use of focused ultrasonication for efficient tissue disruption prior to the proteinase K treatment step. As recently showed by Amini et al. (2017), commonly used RNA isolation protocols are mostly inefficient due to insufficient initial steps of tissue disruption using proteinase K, which most of the time leaves behind macroscopically visible pieces of tissue. Using this approach, the total RNA yields could be increased by 8- to 12-fold compared to a commonly used protease-based extraction technique, enabling successful analysis of more difficult FFPE clinically relevant specimens (Amini et al., 2017).

Other aspects that could affect RNA extraction performance and that should be maintained constant for all the samples are the area of tissue microdissected and the laser beam intensity. The quantity and the quality of the RNA extracted from a tissue for LCM directly correlates with the amount of tissue used, the cellularity of the tissues and the presence of necrosis, inflammation, autolysis (Espina et al., 2006; Liu et al., 2014). All these factors should be minimized by including an appropriate histopathological evaluation (Liu et al., 2014).

The effect of the laser beam on the RNA yield is more relevant for IR capture laser based LCM method than for UV cutting laser systems. The near-IR capture laser is the original LCM system invented at the National Institutes of Health. During this technique, an IR laser (810 nm) activates a thermo-sensitive polymer (Vandewoestyne et al., 2013). The activated polymer attaches to the cells on the slide underneath, embedding

and capturing them in the polymer. Although the heat produced by the laser in the polymer is transient in the order of milliseconds, the heat (90°C) generated by the IR system potentially might be harmful for nucleic acids. The UV-cutting laser system uses a high-energy laser in the UV spectrum (355 nm) that is capable of cutting the tissues. The UV laser cuts around the target cells, leaving them intact, compared with an IR capture laser that focuses directly on the cells (Vandewoestyne et al., 2013).

It has been suggested that methods used for quantification of RNA yield and quality may not be as reliable for FFPE tissue-derived RNA as for frozen or fresh tissue RNA (Kashofer et al., 2013). Spectrophotometry techniques such as those used by the Nanodrop are commonly used to determine the concentration of RNA samples, though this may not be the most accurate method in FFPE-derived samples. Fluorometry techniques such as those used by the Qubit, which relies upon selective fluorescence when the fluorophore binds RNA, have been shown to be a more accurate measure of DNA concentration (Simbolo et al., 2013) and this could also be true for RNA. In the present study, we compared RNA concentration measurements for all samples with both the Nanodrop and the Qubit. As reported by Deben et al. (2013), RNA measurements with the Qubit were significantly lower than those reported by the Nanodrop. These values were then plotted against the Nanostring housekeeping gene counts or qPCR housekeeping gene cT to verify which quantification method better correlated with expression results. As expected, higher RNA concentrations measured with the Qubit correlated with higher Nanostring gene counts and lower qPCR cT. The same correlation was not reported for the RNA concentration measures obtained with the Nanodrop. Our data confirmed previously described results (Deben et al., 2013), showing that the Qubit fluorometer is a more accurate method for determining concentration for such downstream applications. NanoDrop is likely a less reliable quantitation method due to contamination or imprecise measuring of very low RNA concentrations delivering false high concentrations (Landolt et al., 2016).

In addition, two methods for measuring RNA quality were similarly assessed, as it would be expected that higher quality might increase the number of transcripts detectable by Nanostring and qPCR. The RIN values appeared to correlate better with both gene expression outputs from Nanostring and qPCR, however none of the correlations were significant. The range of RIN obtained was in line with previous literature, and reflected the fixation time prior to processing (24 hours). While the ratio of the rRNA bands (28S:18S) or the shape of the electropherogram in the RIN value are well established assays to assess RNA quality from fresh or cryopreserved tissues, it is also known that the two distinct rRNA peaks are compromised in RNA extracted from formalin-fixed tissues (Kashofer et al., 2013). Furthermore it is unclear

how well the fragment size distribution of ribosomal RNA predicts the behavior of the messenger RNA (mRNA) subsequently used as source material for gene expression studies. Because rRNA is a structural component of the ribosome, it could be extensively cross-linked to the amino acids of ribosomal proteins, leading to more extensive degradation of rRNA (Kashofer et al., 2013). In addition, the structural integrity does not necessarily correlate with chemical modifications and performance of RNA in enzymatic reactions. Several groups have already reported that RIN values did not provide reliable information on RNA quality of FFPE samples and subsequent performance in qRT-PCR assays (Kashofer et al., 2013; Sonntag et al., 2016; Patel et al., 2017; Carlsson et al., 2018). It is also important to note that DV200 values represent relative, rather than absolute, amounts of fragments > 200 bp and thus do not necessarily reflect the performance of nucleic acids for use in downstream reactions such as PCR (Patel et al., 2017).

In this study, all the samples tested gave evaluable results on both the Nanostring and the qPCR experiments. With particular reference to the Nanostring platform, which has been optimized for use on FFPE tissues, it is plausible that quality of RNA is not the largest determining factor in performance and that concentration is more important. In conclusion for the first experimental part of our study, the protocol that yielded the best results consisted in 5  $\mu$ m sections, shorter protocol for H&E staining, and RNA extracted with Roche kit. The most reliable method to quantify RNA for subsequent analysis was the Qubit fluorometer. This protocol was then applied for the microdissection of the different kidney compartments (glomeruli and cortical tubules) from FFPE mouse tissue samples.

After microdissection and RNA extraction, qPCR was performed to determine whether there was any cross contamination of the compartment samples. Podocin (NPHS2), a gene solely expressed by podocytes, cells that reside within the glomeruli (Relle et al., 2011) was selected as marker for the glomeruli compartment. Similarly, Cadherin-16 (CDH16), expressed solely in the membrane of proximal tubule epithelial cells (Igarashi et al., 1999), was selected as marker of the tubular compartment. Podocin expression was significantly higher in the glomerular samples than in the cortical tubule samples. The slightly higher expression of podocin in the cortical tubule samples compared to whole kidney was unexpected, as cortical tubules are not expected to have significant podocin expression. All efforts were made during dissection to isolate only cortical tubules and not glomeruli, however it is possible that portions of glomeruli may have been captured inadvertently, which could account for the minimal expression observed. Expression of CDH16 was lower in the glomerular samples compared to the whole kidney or the cortical tubule compartment, indicating low proportion of tubular tissue in the dissected glomerular samples, however the difference in expression levels was

not statistically significant. CDH16 is expressed solely in the membrane of the proximal tubules, thus this high variability is likely a result of varying amounts of proximal tubules being captured in the random sampling of the cortex (Igarashi et al., 1999). In combination, these results showed an overall low level of cross contamination between tissue compartment samples, suggesting that laser capture microdissection is an accurate isolation method providing gene expression data that are representative of specific renal compartments.

The microdissected glomeruli and cortical tubule RNA from healthy mouse kidney were subjected to Nanostring gene expression analysis of 547 immunology related genes. The immunological panel is one of the few genetic panel available for mouse tissues on the Nanostring platform and we selected it for the high number of gene included. The results indicated a differential expression of 25 genes in the glomeruli compared to the whole kidney, illustrating that whole kidney analysis does not accurately represent the native transcriptome state of its sub-compartments, particularly glomeruli, which comprise only a small proportion of the overall kidney volume.

Some of the differently expressed genes, such as FYN, PDGFB, PDGFRB, ICAM2 have been previously reported as associated with glomerular function and development (He et al., 2008; Lindenmeyer et al., 2010). Some other, such as Il6ra, Cd55, Dpp4, Ptger4, Ifngr1, Tnfrsf12a, Cd99, C1ra, Ets1 have been identified as upregulated in mouse glomeruli in large scale cDNA libraries analysis (Takemoto et al., 2006; He et al., 2007). Studying the reason why these genes are differently expressed in the glomeruli was not the purpose of this study, but considering that this is in the healthy kidney, the results and the implications in a disease-altered environment could potentially be even more interesting. Moreover, the fact that some of these genes have been already identified as glomerular-specific, reinforces the efficacy of the method described in this paper as valid for the dissection and analysis of glomerular population from mouse FFPE samples.

In conclusion, this study represents a comprehensively optimized protocol for the use of LCM with FFPE kidney tissue including downstream gene expression applications. It has also been shown that the Qubit method of quantifying RNA is a more accurate predictor of gene expression output than the traditionally used Nanodrop when working with FFPE derived RNA samples.

As proof of concept this protocol was successfully applied to gene expression investigations which indicated that LCM could provide samples of kidney tissue compartments of adequate purity, and that gene expression in the glomeruli is different to that of the whole kidney with reference to immunoinflammatory genes. This study paves the way for future investigations into transcriptomics of renal disease states using isolation of kidney compartments through LCM.

## Nanostring analysis on mouse glomeruli

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**Author Contributions.** EM and CMQ conceived and designed the research project. AH performed the experiments. AH, JML and EM analyzed the data. EM and AH wrote the manuscript. All authors edited and reviewed the manuscript.

**Conflict of interest.** The authors declare no competing financial interests.

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