RNA expression profiling in renal biopsies

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- What has gene expression profiling of mRNA in human renal biopsies (native/transplant) done for us? What more can it do?
    - 1. As a research tool:
        - Better understanding of pathophysiology
        - Drug target discovery
        - Biomarker discovery
    - 2. In the clinic
- I will not talk about
    - Animal models
    - Anything else than renal biopsies (blood, urine)
    - Anything other than mRNA analysis

Coding RNA

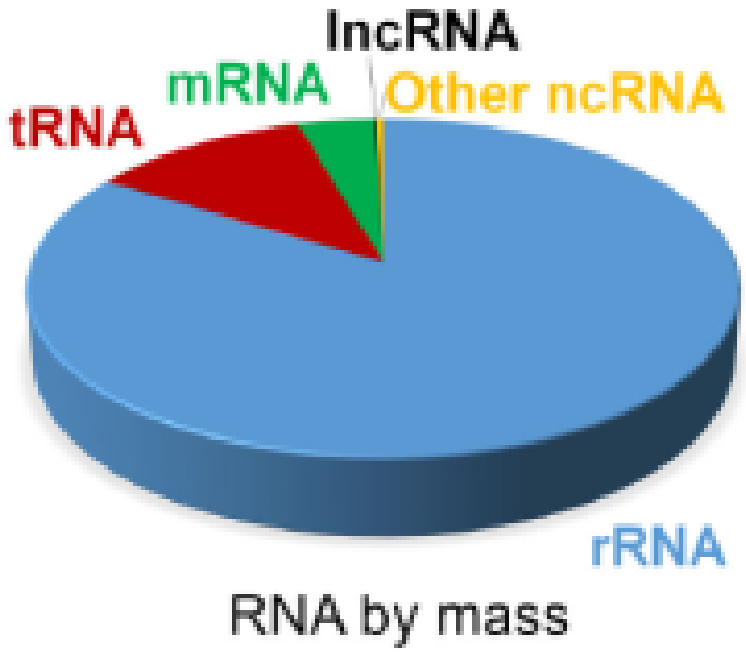
Non coding RNA

Table 6–1 Principal Types of RNAs Produced in Cells

TYPE OF RNA	FUNCTION
mRNAs	messenger RNAs, code for proteins
rRNAs	ribosomal RNAs, form the basic structure of the ribosome and catalyze protein synthesis
tRNAs	transfer RNAs, central to protein synthesis as adaptors between mRNA and amino acids
snRNAs	small nuclear RNAs, function in a variety of nuclear processes, including the splicing of pre-mRNA
snoRNAs	small nucleolar RNAs, used to process and chemically modify rRNAs
scaRNAs	small cajal RNAs, used to modify snoRNAs and snRNAs
miRNAs	microRNAs, regulate gene expression typically by blocking translation of selective mRNAs
siRNAs	small interfering RNAs, turn off gene expression by directing degradation of selective mRNAs and the establishment of compact chromatin structures
Other noncoding RNAs	function in diverse cell processes, including telomere synthesis, X-chromosome inactivation, and the transport of proteins into the ER

Table 6-1 Molecular Biology of the Cell 5/e (© Garland Science 2008)

A



RNA by mass

Palazzo 2015

- mRNA quantification is an imperfect reflection of what is actually happening
- Translational regulation:
  - Initiation
  - Elongation
  - Termination
- Importance of protein data (discovery phase)

Samples for gene expression analysis

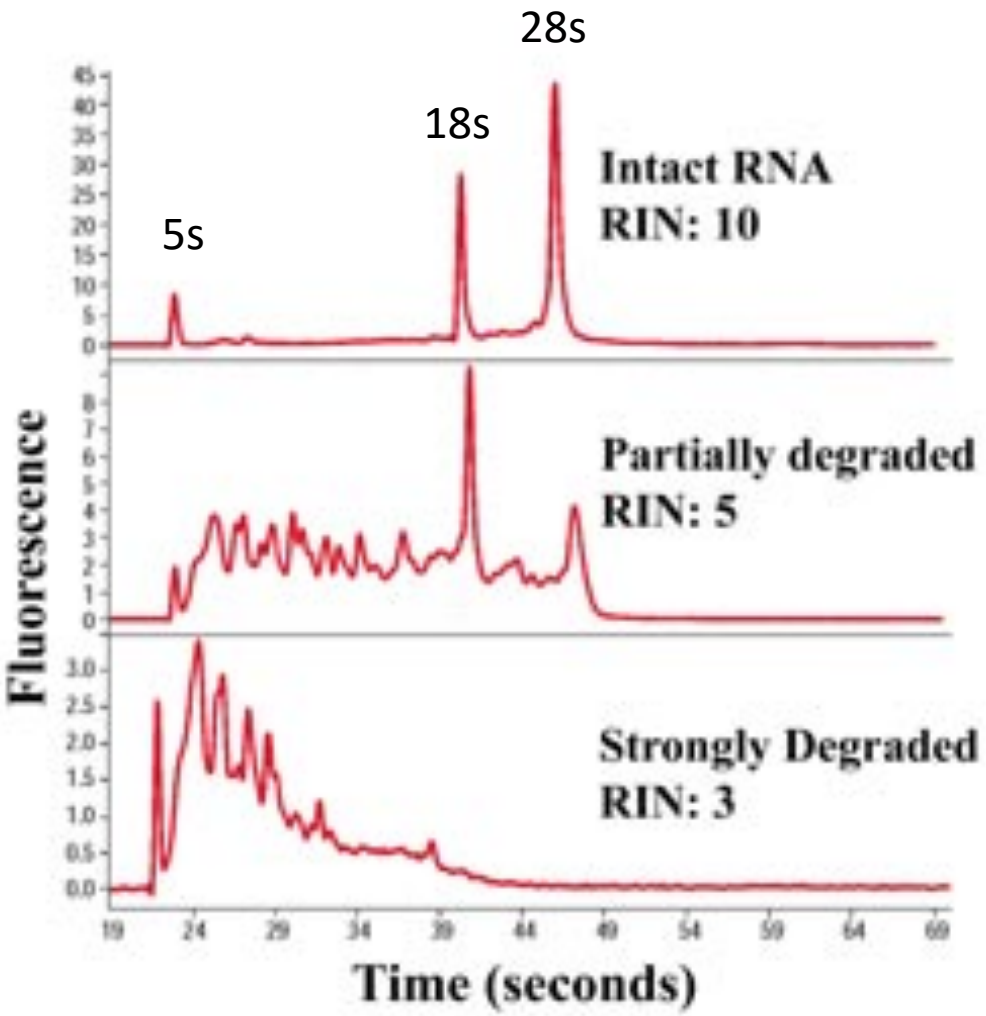
- Ideal experiment would use a high yield of pure, high quality (non degraded) RNA
  - E.g. 1 µg of RNA for microarray analysis
  - Spectrophotometric analysis: quantify (concentration) and purity (A260/280 for pure RNA = 1.9-2.1)
  - Capillary electrophoresis (e.g. Agilent 2100 Bioanalyser): quality of RNA (degradation): RIN value (RNA integrity number)

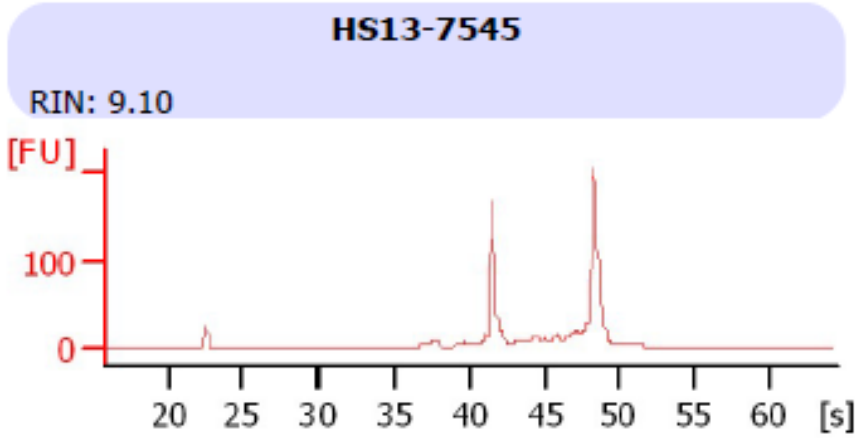
- RNAlater
- Frozen samples

<u>Loss/fragmentation of RNA</u>:

- time between acquisition and RNA purification (Rnase)
- during purification
- prolonged storage

Sample Quality





RNA later

- ~1500 samples in RNA later
- Up to 1 whole core obtained with 18G needle
- Biopsy size: 1-28mm
- Yield: 0-5800 ng; mean = 992ng

- Glomeruli can be teased out of RNA later samples [Hodgin et al. PLOS One 2014(9) (IgA NP)]

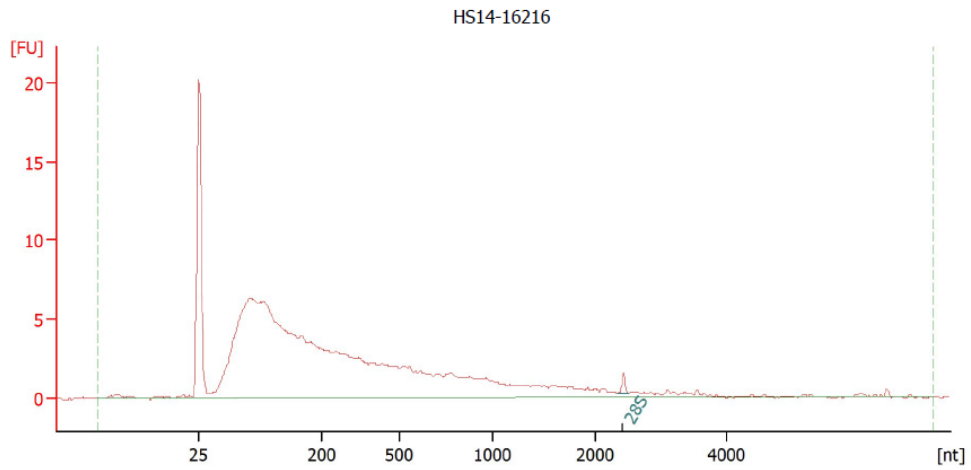
Formalin-fixation and RNA

- Embedding at high temperatures
- Formalin fixation cross-links RNA to DNA and proteins, by addition of methylol groups and methylene bridge formation
- Crosslink dissociation during RNA purification -> further fragmentation

- FFPE RNA fragments <300 bases (typically around 100-200)
- Disappearance of ribosomal RNA bands
- RIN ~ 2.0-3.0

HS14-16216
RIN: 2.4

FFPE



- ~ 159 transplant biopsies
- Yield: 0 to 2870; mean = 536 ng
- RIN: 1-6; mean = 2.13

- Microdissections of glomeruli from FFPE:
  - Pool cross sections of same glomerulus over several sections
  - And/or pool several different glomeruli on same section

  [Theophile et al. Diagn Mol Pathol (2008); 17: 101; Agustian et al. Transplantation 2013;95: 1242 (TMA); Bockmeyer et al. BMC Molecular Biol (2018) 19:2;]

Discovery
Compare to gold standard

Validation
Across platforms and across samples

RCT
Biomarker converted to assay suitable for clinical application

Multiple RCT

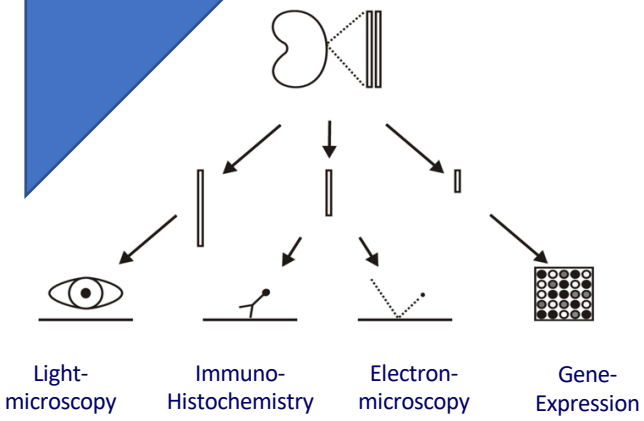
Health Technology Assessment

Evaluation in clinical setting

Implementation of biomarker

RNA sequencing
Microarrays

qPCR
Probe –based methods (Nanostring)



Adapted from Menon et al. Transplantation 2016

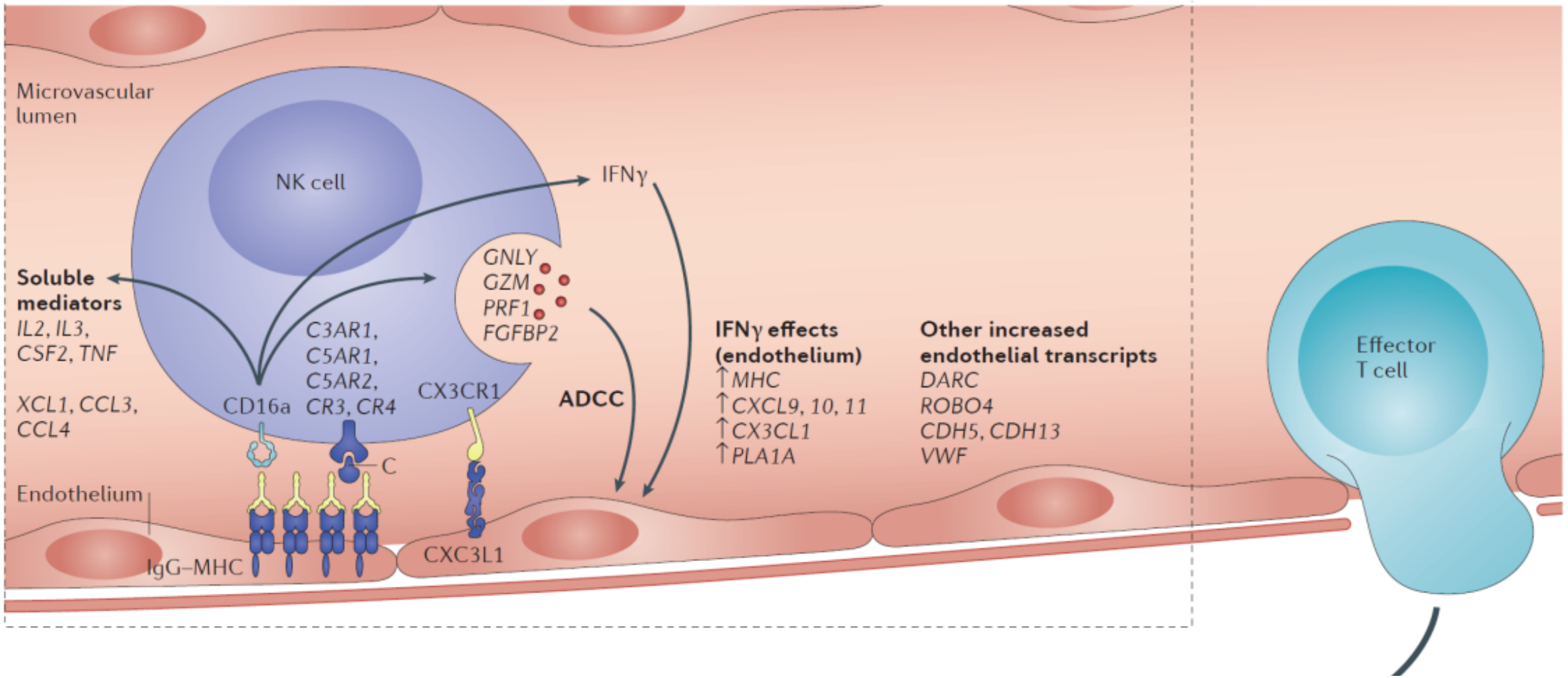
1. Discovery

- Transcriptome analysis: genes currently being expressed (mRNA content) and their expression level in given condition
- 20-25K genes (human)
- High throughput technology:

- Microarray: 1 µg high quality RNA
  - For lower amounts (e.g. microdissected glomeruli): modified amplification protocol [Schmid H et al. NDT 2004; Hodgin et al. PLOS One 2014 (RNA later/IgA); Peterson et al. JCI 2004(113) (frozen/SLE)]
  - For low quality (FFPE) – adapted technique and arrays (Stewart et al. BBA - Reviews on Cancer 1868:258)
- RNA seq: 10ng mRNA (single cell applications) high quality RNA
  - For low quality (FFPE) (e.g. ribosomal depletion, increased number of reads, decreased lengths of reads etc.)[Francina Webster A et al. Tox Sciences 2015, Vukmirovic et al. BMC Pulmonary Medicine (2017)(lung)]

Group		Reference	Findings
Sarwal (UCSF)	Microarrays	Sarwal NEJM 2003	B cell signature in biopsy associated with severe rejection
ATACG – Alberta Transplant Applied Genomics Centre (Genome Canada) + INTERCOM(EX); The Molecular Microscope ™	Microarrays	Reviewed in Halloran P Rev Nat Med 2016 Sis AJT 2007; Sellares AJT 2013 Loupy 2014 Einecke AJT 2018	Pathogenesis of T-cell and antibody-mediated rejection Central role of ABMR in poor allograft outcomes Molecular signature for arteriolar hyalinosis (Mah) in biopsies with rejection associated with non-adherence
GoCar – Genomics of chronic allograft rejection	Microarrays	O'Connell Lancet 2016	13-gene set predicts development of fibrosis
GTCRG - Genomics of Transplantation Co-operative Research Group (7 centres, USA, NIH funded)	Microarrays and RNAseq	Modena AJT 2016 Kurian AJT 2017	Most samples with IFTA have on-going immune injury; in bland IFTA without inflammation this is associated with poor outcomes Blood/biopsy transcript comparison
Cornell, New York	RNAseq	Thareja AJT 2018	Quantifying donor cell infiltration in graft
Montefiore Einstein, New York	Microarrays	Hayde KI 2014; Gupta KI 2016 Kamal Transplantation 2015	DSA-associated gene signature in biopsy Genes predict allograft loss in TG
Vienna, Austria	Microarrays	Wilflingseder PLOS One 2014	AKI molecular signature
Mayo Clinic	Microarrays	Dean AJT 2014	Rejection signature in +XM biopsies

Molecular landscape of AMR



Halloran P Nat Rev Nephrol 2016

Single-Cell Transcriptomics of a Human Kidney Allograft Biopsy Specimen Defines a Diverse Inflammatory Response

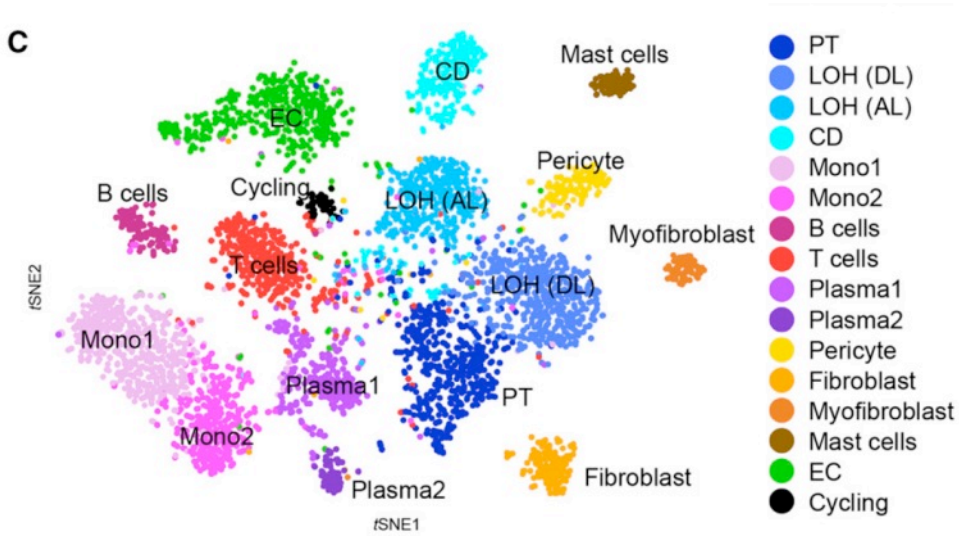
Haojia Wu,[1] Andrew F. Malone,[1] Erinn L. Donnelly,[1] Yuhei Kirita,[1] Kohei Uchimura,[1] Sai M. Ramakrishnan,[1] Joseph P. Gaut,[2] and Benjamin D. Humphreys[1,3]

1 adult healthy kidney and 1 transplant biopsy with mixed rejection (fresh sample)

Dissociation using 0.25% trypsin, 35min
75,000 cells, 91% viable

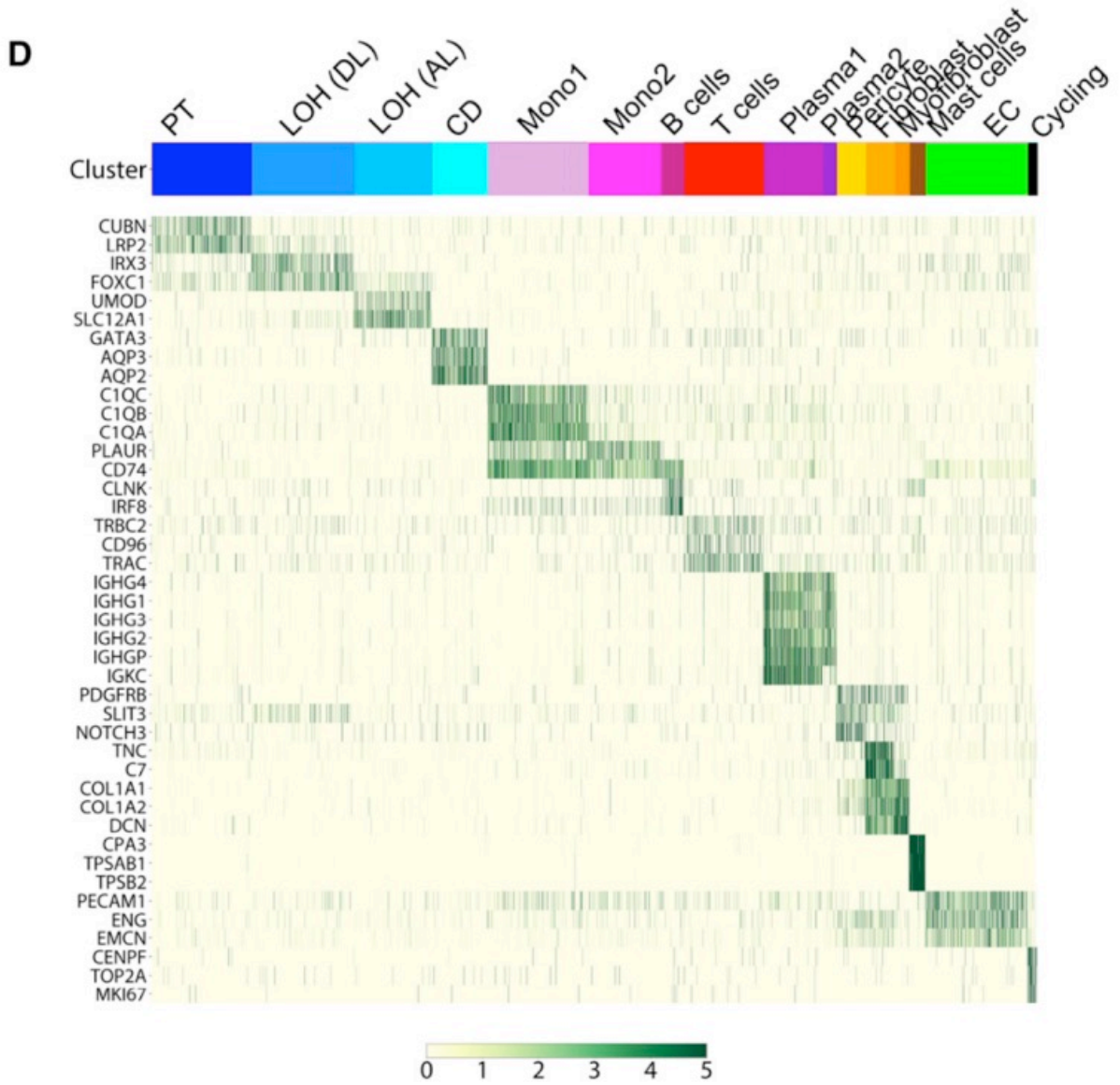
Droplet based microfluidic cell separation (inDrops technology), library construction and sequencing to generate
4,487 cells passed quality control
16 cell type clusters

T-SNE plot (t distributed stochastic neighbour embedding)
16 distinct cell types



Wu et al. JASN 2018

Recursive hierarchical clustering



4 tubular epithelial cell types
7 inflammatory cell types
3 stromal cell types
Endothelial cells

<u>Key findings:</u>

2 sub-clusters in monocyte cell populations non-classical CD16+ and classical CD16-

Novel tubular segment-specific pro-inflammatory responses in rejection

3 sub-clusters of endothelial cells: resting, activated with FcR activation + Ig internalisation genes, second active type

Genes previously associated with rejection mapped to single cell types

Wu et al. JASN 2018

RNAseq : genes differentially expressed between ABOi C4d+ vs ABOc C4d-

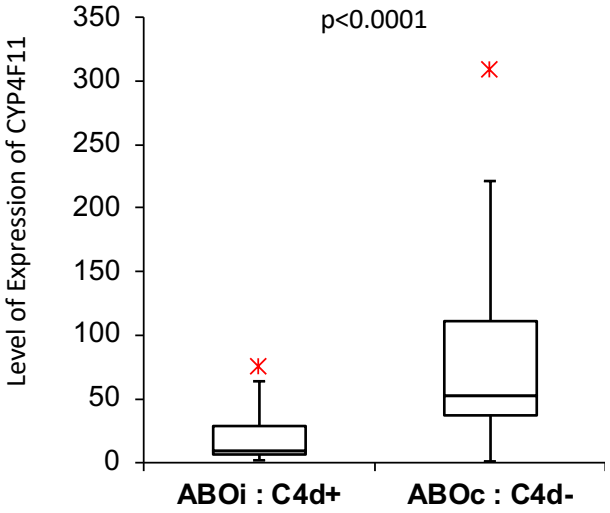
gene name	fold change	adjusted p-value
CYP4F11	0.073555905	0.001163486
CD1E	0.200836589	0.066779101
XCR1	0.204472163	0.014424859
ANKRD1	0.230692697	0.092492137
MMRN1	0.261905211	0.015196747
TRPA1	0.264436733	0.08924559
TNFSF9	0.271182226	0.08924559
CTD-2162K18.4	0.307053594	0.017600998
B3GAT1	0.321210909	0.087601691
NCMAP	0.322171065	0.053848132
ARNTL2	0.330775153	0.053848132
THEMIS	0.337059086	0.074524114
SEMA3A	0.342610983	0.094026951
ARHGAP40	0.345298678	0.085215778
GPR141	0.350711828	0.087601691
KLHL1	0.354763545	0.000748187
CYSLTR2	0.356635001	0.090746061
ZNF831	0.373115368	0.008291799
PIK3CG	0.406901174	0.002377361
ARHGAP20	0.407917762	0.013261326
CDH22	0.418092301	0.056968223
IQCJ-SCHIP1	0.429726322	0.001058308
KRT80	0.430207443	0.04483793
SLAMF1	0.430989291	0.08924559
ZC3HAV1L	0.433731297	0.013284185
MYO1G	0.443374449	0.001446233
UBASH3A	0.447961353	0.014424859
CSF2RB	0.454975256	1.33775E-06
TLR6	0.459386264	0.014608791
CREB5	0.47223602	0.030585426
C11orf21	0.473417479	0.001216717
ZNF724P	0.486757245	0.008708638
PTPRC	0.496874448	0.012273611
ITGA4	0.53465468	0.002898773
FSBP	0.536526104	0.087601691
EOMES	0.548288056	0.063127026
TNFRSF11B	0.554388526	0.011080186
GCNT1	0.566734944	0.020968162
CEP192	0.589420501	0.034013847
SPN	0.612008747	0.005440584
RASSF9	0.615455597	3.39588E-07
FLRT2	0.617681585	0.098302857
LILRB1	0.618606357	0.040652965
STAC	0.629351703	0.017546834
PLEKHG1	0.641362034	0.074524114
ARHGAP30	0.654172705	0.053848132
ANKRD45	0.665120361	1.19658E-06
DOCK2	0.666554956	0.01543222
PRELID2	0.667288468	0.04575183
TNFAIP3	0.68412522	0.050097268
ARHGAP29	0.685865868	0.014496002
MEX3A	0.698750199	0.014424859
PCDHGA7	0.703277171	0.081892822
FIBIN	0.718258586	0.014496002
KALRN	0.723566905	0.020968162
GUCY1A3	0.728652064	0.009606719
LRRC34	0.748581434	0.093861429
ZNF510	0.750521372	0.014496002
ERI2	0.761115889	0.014496002
GTF2H2C	0.765549867	0.032103096
ZNF175	0.783143669	0.097847645
TMEM246	0.785926003	0.014424859
MBP	0.819034387	0.001216717
C1orf198	0.832630538	0.049081141
NAP1L3	0.83693157	0.014424859
ORAI2	0.842391601	0.072552495
DENND5A	0.843847798	0.097847645
DDHD2	0.848205497	0.089876875
ZNF234	0.86609031	0.014496002
TMEM248	1.067436119	0.041128259
ING5	1.161698185	0.009237958
MRPS25	1.166671019	0.002377361
MOGS	1.174358223	0.087601691
GPN2	1.184743454	4.87881E-08
OTUD5	1.193069678	0.008291799
ORAOV1	1.207159284	1.33775E-06
CDAN1	1.208685192	0.086490649
BLOC1S3	1.213520185	0.066706967
MOB3A	1.213618573	0.067883868
USP20	1.223697347	0.036777562
ZNF839	1.238193914	0.040265975
MAP2K5	1.251530127	0.018590537
RAD23A	1.256169969	0.05920969
CTDNEP1	1.264204002	0.001258308
ELMOD3	1.265671572	0.043292194
ALS2CL	1.265681713	0.037917182
FBXO10	1.30644792	0.087601691
SGK2	1.31070262	6.22837E-05
TBC1D10A	1.314493787	0.007842126
VPS4A	1.314946286	0.053293433
TNIP1	1.318283702	0.022617646
DEDD	1.32144742	0.063127026
FAM134C	1.339233088	0.008291799
PCBP2	1.345656288	0.013284181
SMTNL2	1.346936959	0.087601691
AP006621.5	1.347322116	0.045826765
PDLM	1.352756623	0.008921829
FAM65A	1.395662892	0.089876875
PLD6	1.404931635	0.014608791
MRPL53	1.439333878	0.02481416
THAP8	1.441137591	0.02169579
CPNE1	1.447853759	0.044914733
SUV39H1	1.491659104	0.014798833
AVIL	1.513123474	0.066779101
PPP5D1	1.548198896	0.05920969
MBLAC1	1.570716502	0.044914733
SLC25A18	1.6072554	6.22837E-05
YBEY	1.698209437	0.098301139
SLC13A4	1.734063179	0.013284181
C2orf73	1.868612311	0.002908773
KLHL41	1.985958436	0.014424859
C2orf66	2.154714446	0.096977996
DDTL	2.563393127	0.056968223
FPR2	3.254955186	0.016147884
C4orf22	4.020603722	0.045656894
GSTT2B	5.457272299	0.038575902
CRYBA4	6.396707772	0.036230379

- RNA later samples (4 ABOi vs 4 ABOc)
- 117 genes differentially expresses between the 2 conditions

- 16 genes selected by biological plausibility, p value and fold change (0.5-2), and included in nanoString Panel

Gene	(A) RNA-seq		(B) Nanostring
	Fold Change	P	P ABOi C4d+ vs ABOcC4d-
ANKRD1	**0.26**	**1.21E-07**	0.065
ANKRD45	0.82	**5.72E-03**	0.726
C11orf21	**0.41**	**2.71E-04**	0.184
C4orf22	**3.83**	3.89E-02	0.239
CD1e	**0.32**	**2.16E-06**	
CRYBA4	**22.06**	**8.19E-04**	0.669
CSF2RB	0.55	**3.37E-05**	0.599
CyP4F11	**0.15**	**5.72E-03**	**0.0001**
FPR2	**4.07**	**2.67E-06**	0.841
GSTT2B	**6.06**	**7.74E-03**	0.652
KLHL1	**0.4**	**8.55E-04**	0.495
MMRN1	**0.35**	**6.41E-03**	0.930
MYO1G	**0.33**	7.80E-02	0.206
RASSF9	0.82	**3.39E-07**	0.940
SLC25A18	1.56	**4.57E-04**	0.679
XCR1	**0.17**	3.41E-02	0.239

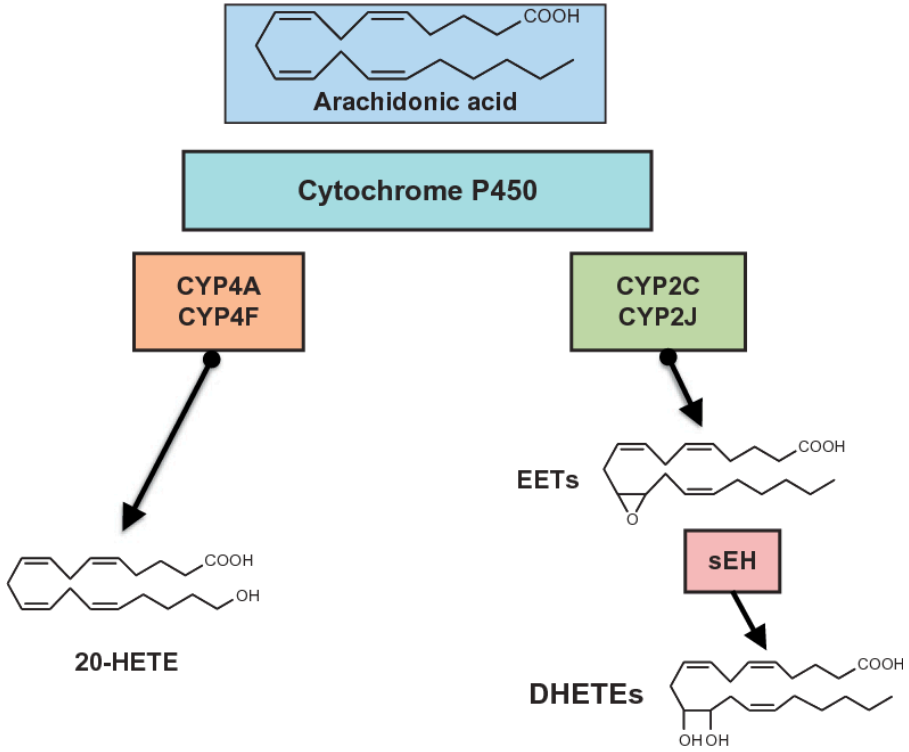
CyP4F11

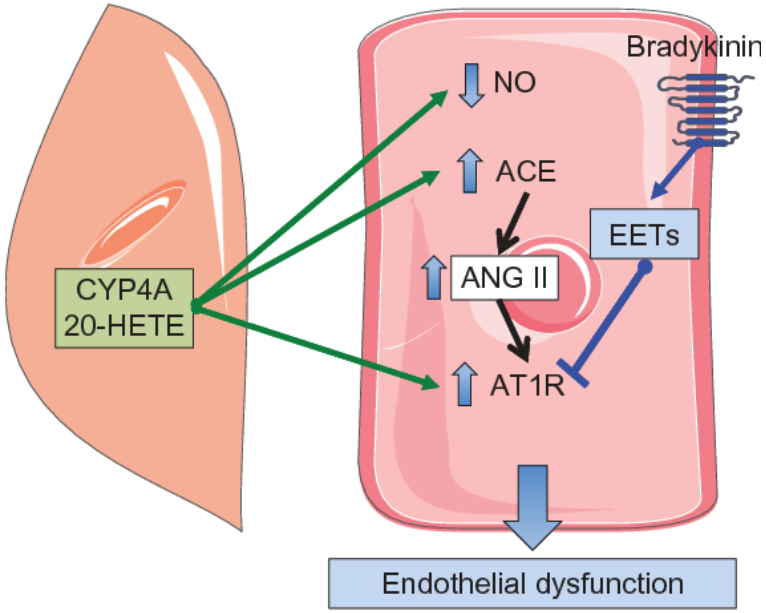


ASN Kidney Week 2018 poster PO1802-3

Cyp4F11

Member of the cytochrome P450 superfamily of enzymes
Metabolize arachidonic acid to 20- hydroxyeicosatetraenoic acid (20-HETE).





Roman RJ J Am Soc Nephrol 2017
Imig JD Advances in Pharmacology, 2016

ASN Kidney Week 2018 poster PO1802-3

2. Molecular clinical test

- Which approach?
  - Performed in parallel to routine work-up of biopsies giving independent diagnostic information
    - High throughput (microarray)
  - Add-on procedure after completion of the routine diagnostics for defined differential diagnostic problems
    - Limited number of cDNAs
- Is it worth it?
  - What information does it add?
    - Diagnostic
    - Prognostic
  - How much does it cost?

Performed in parallel to routine work-up of biopsies giving independent diagnostic information

Real time assessment of kidney transplant indication biopsies by microarrays: first results of the INTERCOMEX study (Halloran et al. AJT 2017)

- Multicentric observational prospective study
- Feasibility of real time central molecular assessment as adjunct to diagnosis
- Large gene sets and machine learning to define scores

Alberta Transplant Applied Genomics Centre
250 Heritage Medical Research Centre, University of Alberta
Edmonton, AB T6G 2S2, ph. 780-407-8880, fax 780-407-3417

INTERCOM Study: Molecular Diagnostic Report

Patient ID:	BCN3322
Biopsy Site ID:	BCN22
INTERCOM Study ID:	BCN22
Attending Physician:	
Date Reported:	
Date Received:	3-Dec-11
Date of Transplant:	20-Mar-91
Date of Biopsy:	14-Jul-11

ATTACH PATIENT LABEL HERE

Clinical Information

Time of Biopsy post-Transplant:	20.3 years	Biopsy Indication:	investigate proteinuria
Primary Disease:	IgA nephropathy	Transplant Type:	Deceased-heart beating
GFR (CG) at Biopsy:	29	Treatment before Biopsy:	No
Proteinuria:	Pos	DSA Status at Biopsy	Not done
Haematuria:	neg		

Local Histopathology Phen

Inflammatory/TCMR Lesion
i: 2
t: 0
v: NA

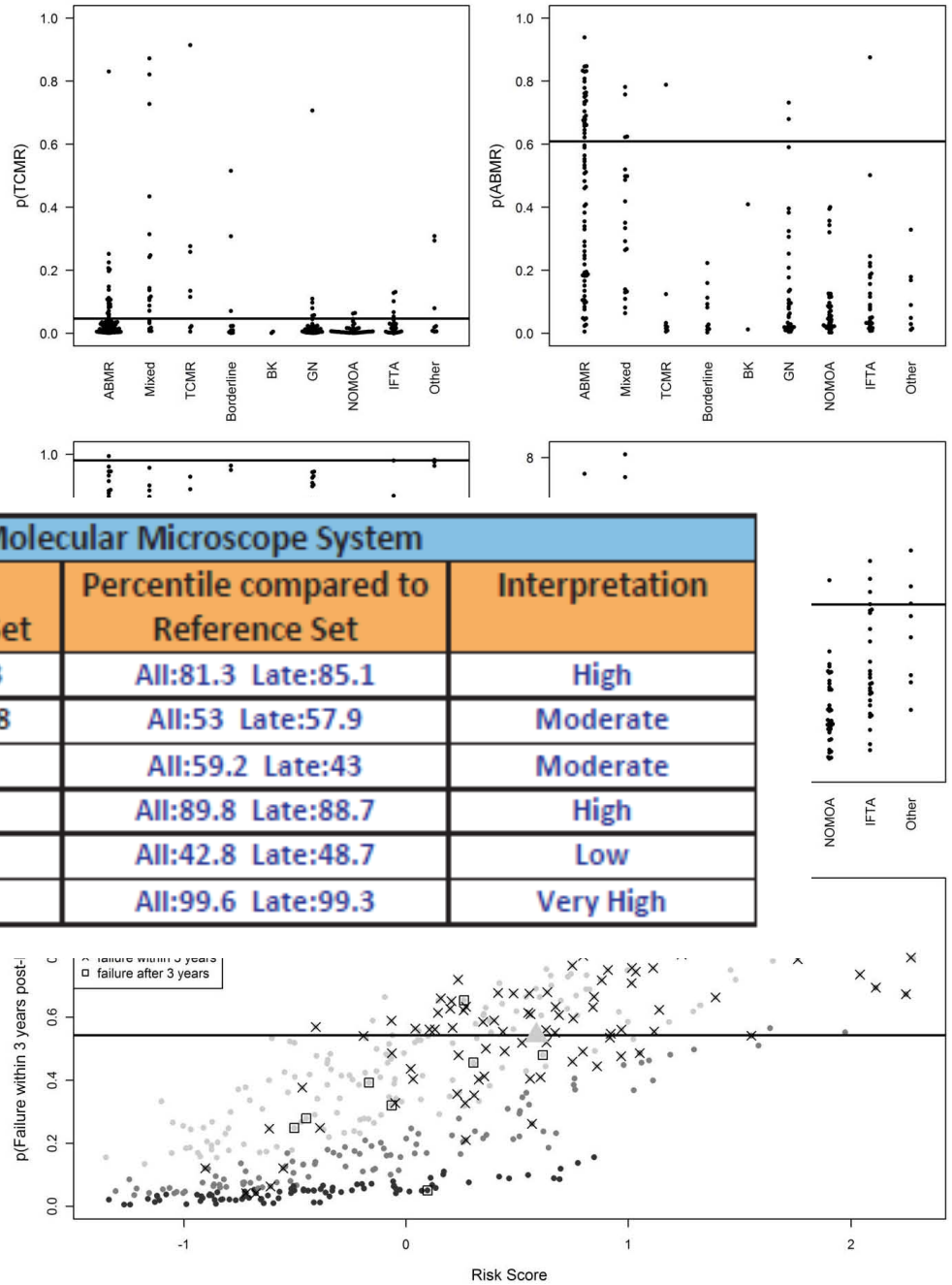
Banff Diagnosis:
2) TG

Molecular phenotype: the

Classifier		
Global Disturbance		
Injury Repair Response		
Atrophy-Fibrosis		
Rejection		
TCMR		
ABMR		
Failure in <3 years	0.54	0.19

Molecular Phenotype: the Edmonton Molecular Microscope System

Classifier/PBT	Biopsy Score	Range in Reference Set	Percentile compared to Reference Set	Interpretation
Global Disturbance Score	2.16	-5.6 -> 9.33	All:81.3 Late:85.1	High
Acute kidney injury (AKI) Score	0.46	-0.92 -> 1.98	All:53 Late:57.9	Moderate
Atrophy-Fibrosis Score	0.35	0.0 -> 1.0	All:59.2 Late:43	Moderate
Rejection Score	0.77	0.0 -> 1.0	All:89.8 Late:88.7	High
TCMR Score	0.00	0.0 -> 1.0	All:42.8 Late:48.7	Low
ABMR Score	0.98	0.0 -> 1.0	All:99.6 Late:99.3	Very High



Comments

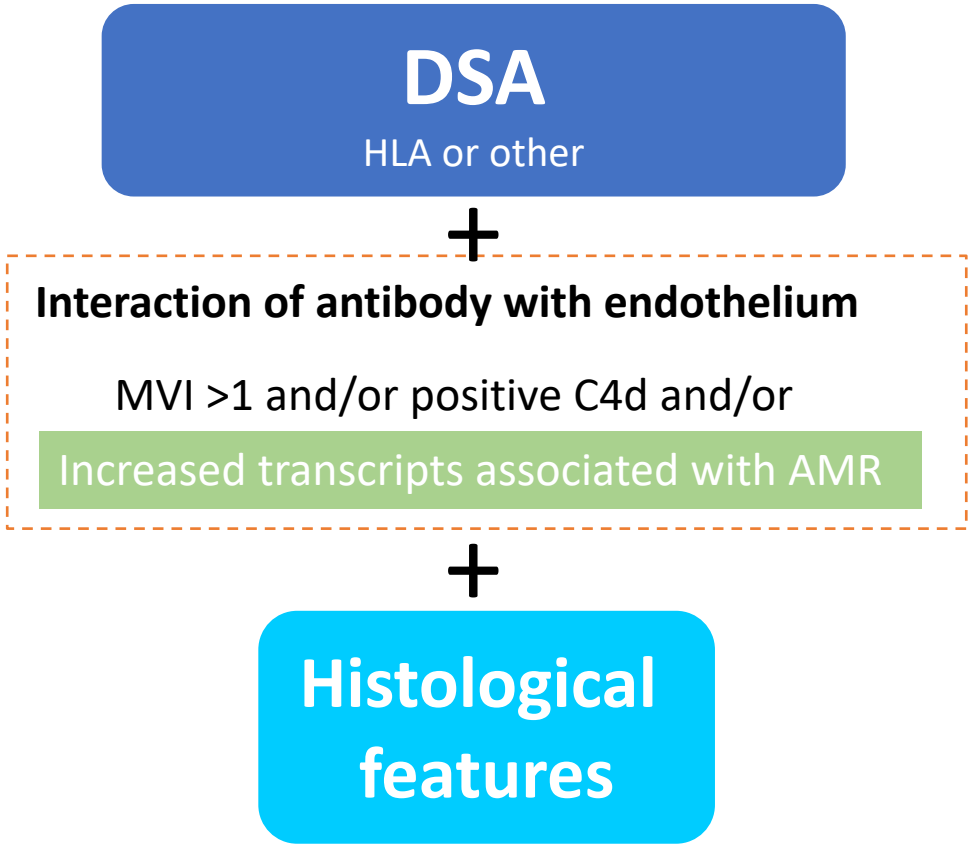
Grade 2/3 inflammation score, Grade 2/3 injury score, Grade 3/3 atrophy-fibrosis score, Grade 3/3 rejection score, Grade 0/3 TCMR score, Grade 3/3 ABMR score, Diagnosis: active ABMR with significant probability of failure

Signed out by Dr. P. F. Halloran

Legend: NA = not available, ti = total i-score, ABMR = antibody mediated rejection, TCMR = T cell mediated rejection

DRAFT: FOR RESEARCH PURPOSES ONLY

Add-on procedure after completion of the routine diagnostics for defined differential diagnostic problems



- Diagnosis of ABMR in Banff Classification of Allograft Pathology
- Other potential areas:
  - TCMR vs BL [deFreitas D. et al AJT 2012]
  - BK +/- TCMR [Sigdel T et al. Transplantation 2016]
  - Bland IFTA: look for molecular rejection signal [Modena et al 2016]

- Which genes?
  - Banff Working group for Adjunct Technologies: Generating Consensus in Molecular Transplant Diagnostics
    - Gene sets: few dozen? Few hundred?
    - How do we combine them?

- Which technique?
  - Applicable to paraffin?
  - Routinely take sample for RNA later?

qPCR

- Established “gold standard” for quantitative gene expression analysis
- High quality RNA preferred, frozen/RNAlater
- ~ 5 ng mRNA (cDNA) for 1 gene
  - For lower amounts e.g. microdissected glomeruli [Peterson JCI 2004 (frozen/SLE); Schmid NDT 2004]
  - For FFPE [Reich J Mol Diag 2011 (SLE); Sigdel KI Reports 2018 (Tx); Theophile et al. Diagn Mol Pathol (2008)]

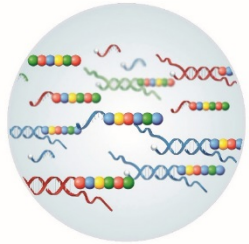
nanoString TECHNOLOGIES®

Workflow

CodeSets are color-coded "barcodes" with two 50bp probes that hybridize to the mRNA target in solution. The Reporter Probe carries the fluorescent barcode signal and the Capture Probe immobilizes the hybridized complex for data collection. Detection is direct, digital, and the assay does not require cDNA synthesis or amplification.

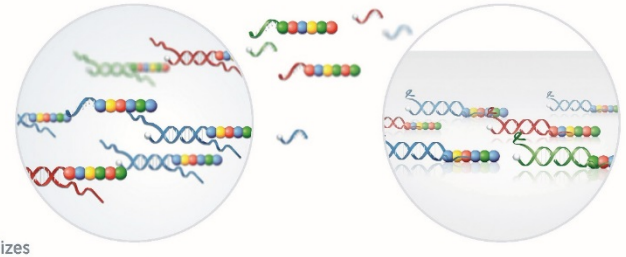
1 Hybridize

1. Buffer
2. CodeSet
3. Sample



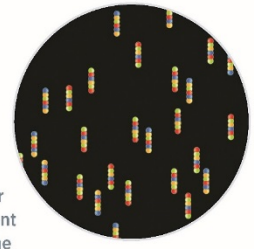
Sample material is mixed with excess CodeSet and hybridized overnight.

2 Purify



The Prep Station removes excess CodeSet and immobilizes CodeSet/RNA complexes in the nCounter Cartridge for data collection.

3 Count



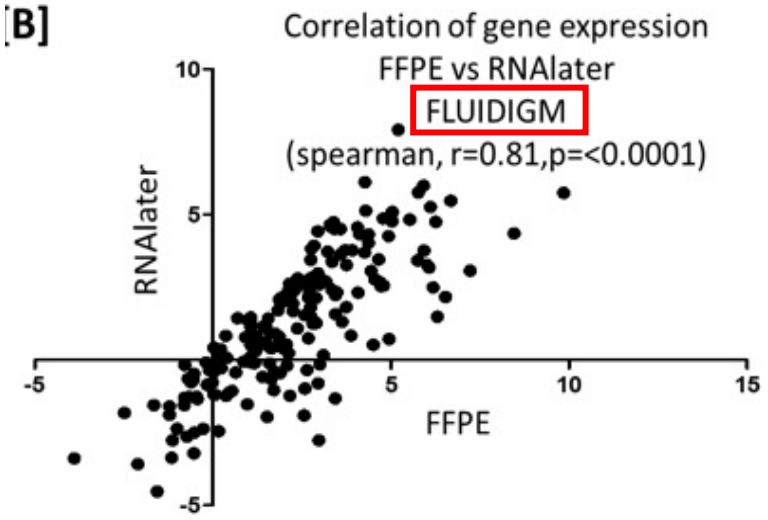
Barcode	Counts	Identity
	3	XLSA
	2	FOX5
	1	INSULIN

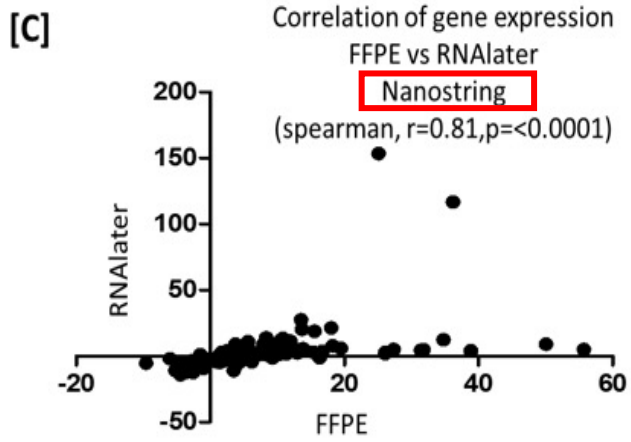
Sample cartridges are placed in the Digital Analyzer for data collection. Fluorescent barcodes on the surface of the cartridge are counted and a running total of each target is tabulated.

Minnie Sarwal Laboratory – University of California, San Francisco

- Common rejection module (CRM) = 12 genes elevated in all rejected transplants (kidney, heart, lung, liver) [Khatri et al. 2013][Sigdel et al. PLOS One 2015]

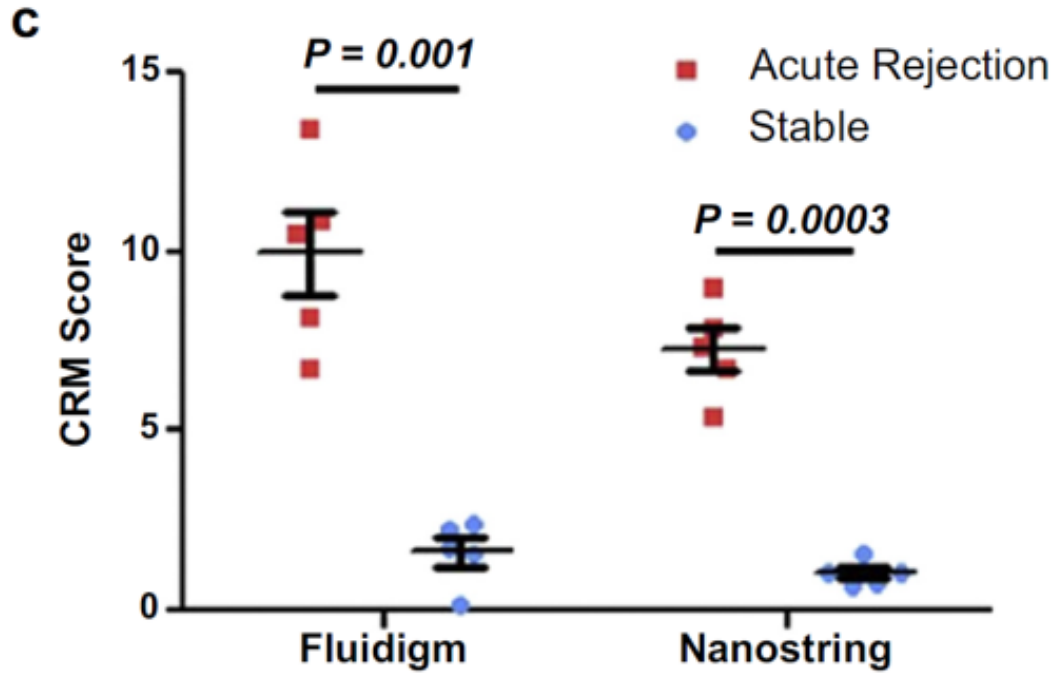
	Tissue size	Yield	260/280	RIN
RNA later	¼ 16G needle core (n=10)	2.8 ± 1.9 µg	1.98 ± 0.12	9.24 ± 0.51
FFPE	4 x 10µ sections	1.7 ± 1.0 µg	1.95 ± 0.06	2.53 ± 0.94





Mean expression all genes

Sigdel et al. KI Reports 2018



Common rejection module score
FFPE samples
5 = acute rejection
5 = non AR

Sigdel et al. KI Reports 2018

Nanostring nCounter technology

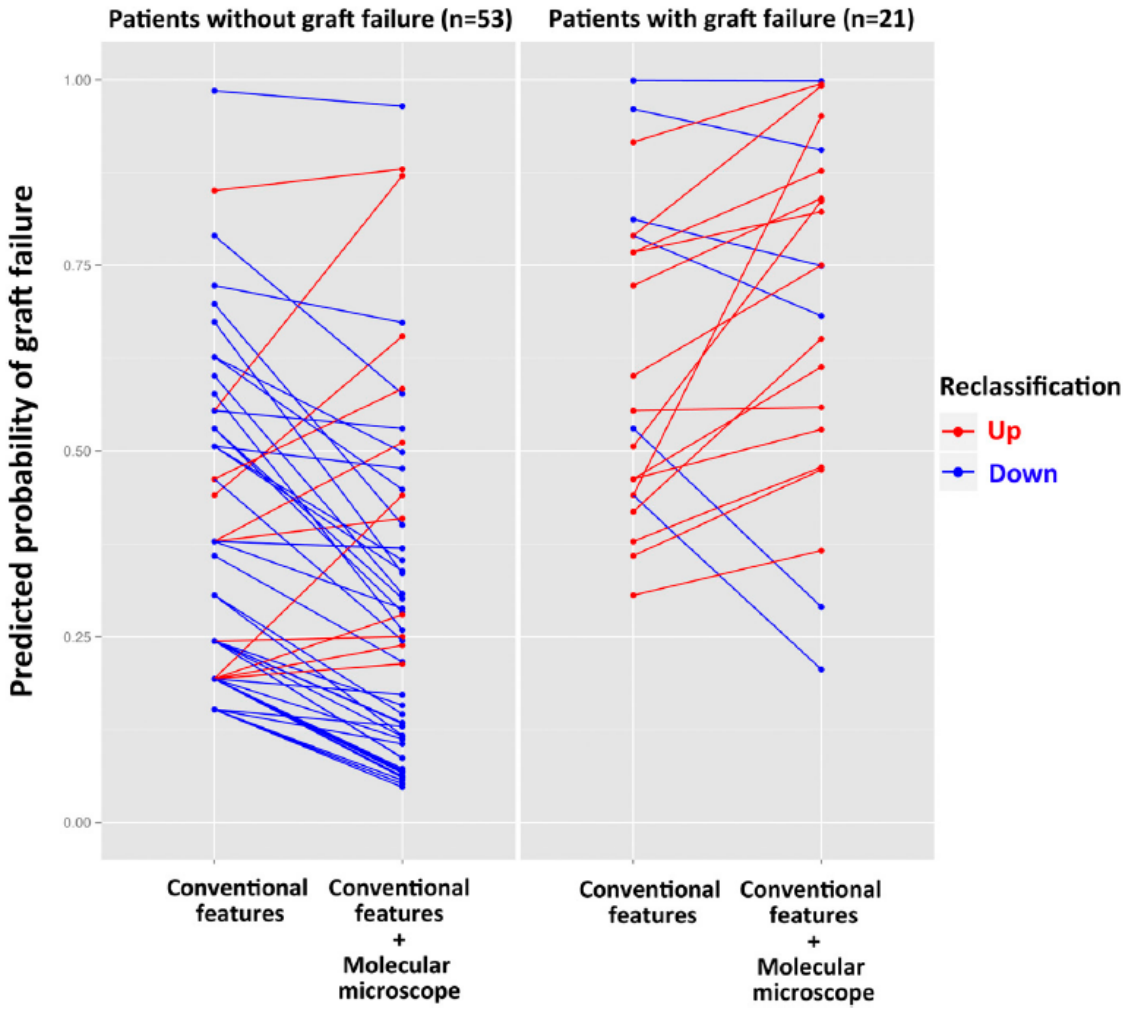
- Multiplexed Color-Coded Probe-Based Gene Expression Assessment for Clinical Molecular Diagnostics in Formalin-Fixed Paraffin-Embedded Human Renal Allograft Tissue. B Adam et al. Clin Transplant 2016

- Characterising the Immune Profile of the Kidney Biopsy at Lupus Nephritis Flare Differentiates Early Treatment Responders from Non Responders. Parikh et al. Lupus Science Medicine 2015

2. Molecular clinical test

- Which approach?
  - Performed in parallel to routine work-up of biopsies giving independent diagnostic information
    - High throughput (microarray)
  - Add-on procedure after completion of the routine diagnostics for defined differential diagnostic problems
    - Limited number of cDNAs
- Is it worth it?
  - What information does it add?
    - Diagnostic
    - Prognostic
  - How much does it cost?

Molecular Microscope Strategy to Improve Risk Stratification in Early Antibody-Mediated Kidney Allograft Rejection



Early ABMR; high risk patients

Independent predictors of graft loss: donor age, humoral histological score (g+ptc+v+cg+C4d), ENDAT or ABMR molecular score

Adding gene expression score to traditional risk model improved stratification of patients at risk

Loupy A et al AJT 2014

Conclusions

- Gene expression analysis has taught us a lot about pathophysiology of renal disease (esp. in transplantation); there is still a lot to do
- Usefulness as a clinical test remains to be proven

- Progress is hampered by technical difficulties
  - Nature of samples: FFPE
  - Nature of disease: affecting only certain compartments (e.g. glomeruli)
- Choice of technique will depend on nature of samples and question being asked: discovery versus clinical test development

Imperial College Biomedical research Centre

Immunology Theme

Transplantation Programme

Dr Kathy Dominy
Dr Frederic Toulza
Dr Michelle Willicombe
Dr Adam McLean
Prof Terry Cook

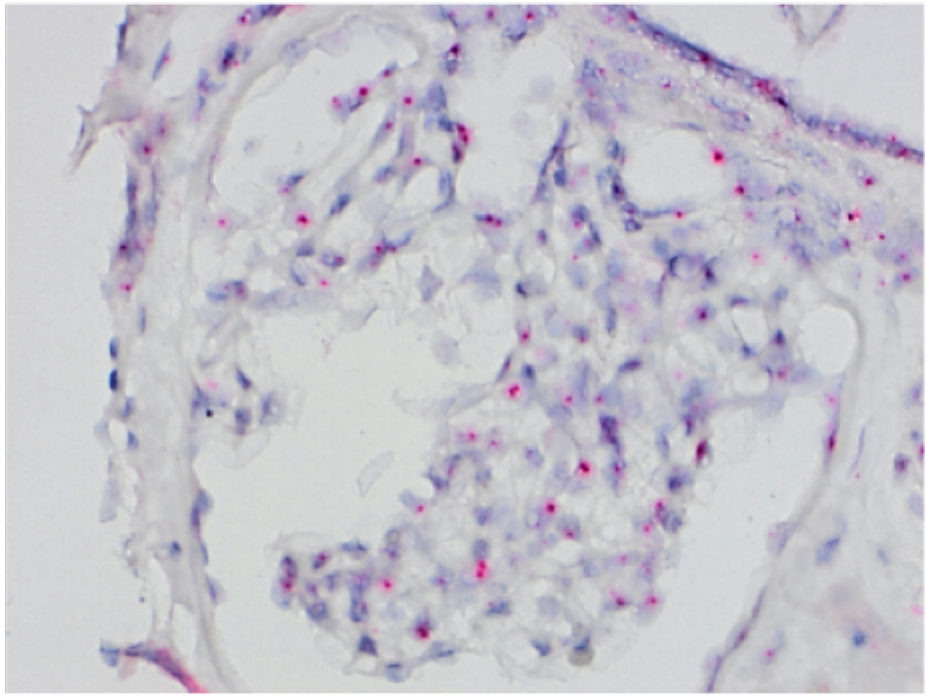
Staff and patients at the Imperial College Healthcare NHS Trust

Lack of spatial resolution

- Transplantation – may be less relevant (Madill-Thomsen KS et al. AJT 2017 Aug;17(8):2117-2128)
- Native biopsies – glomerular signals

  - Microdissection
  - Validation using techniques with spatial visualisation e.g.
    - RNA scope
    - Nanostring Digital Spatial Profiling
    - Etc..

CyP4F11



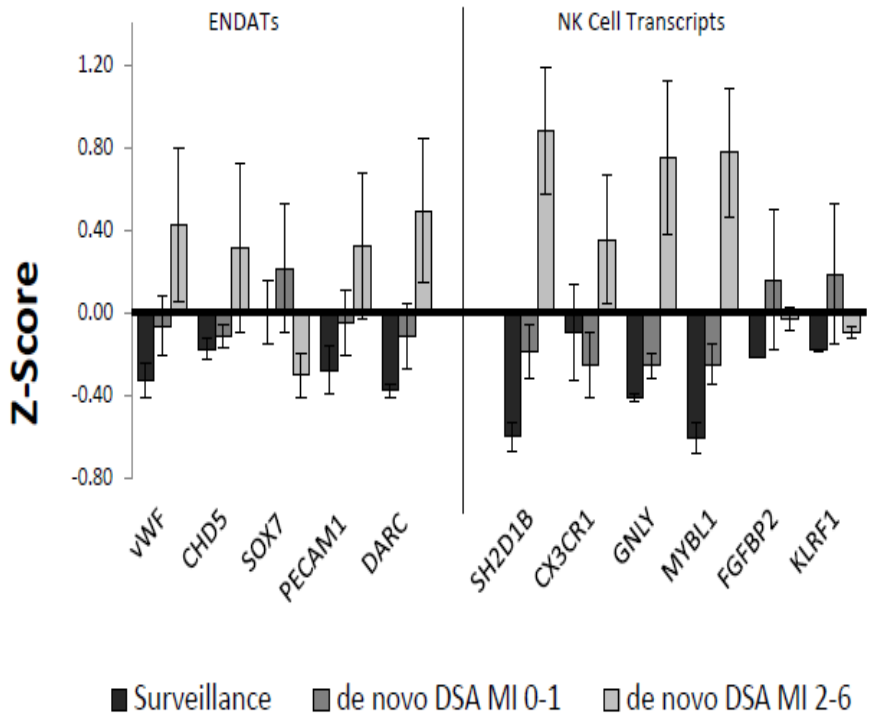
Transcript analysis of renal transplant biopsies at Imperial College

- Focus on validation of antibody-mediated rejection associated gene signature

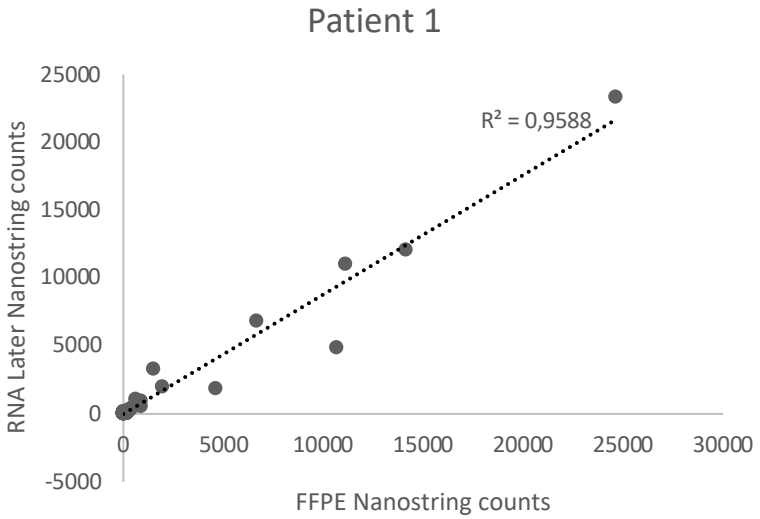
Use of quantitative real time PCR to assess gene transcripts associated with antibody-mediated rejection of kidney transplants.

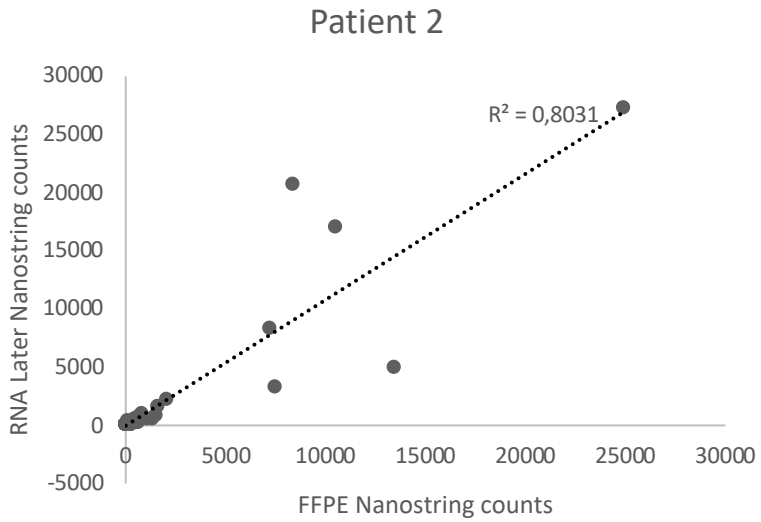
K Dominy et al. Transplantation. 99:1981-8; 2015

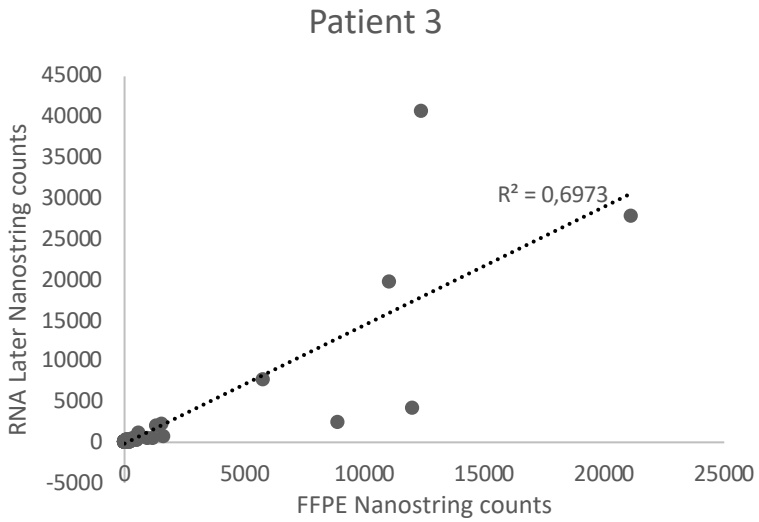
10-12 transcript panel
Endothelial and NK-related transcripts

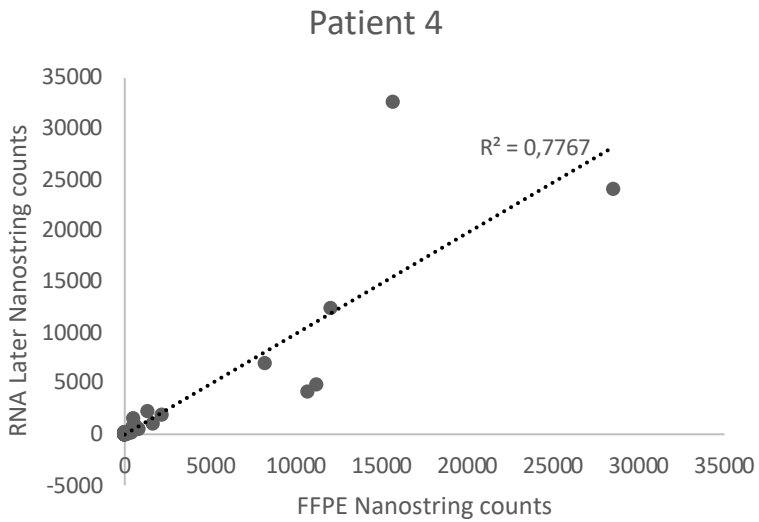


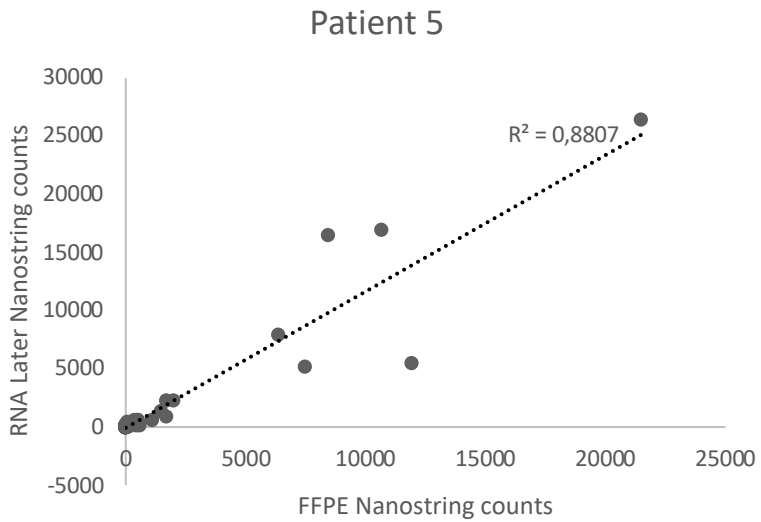
N = 39 biopsies from patients with de novo DSA
MI score 0/1 = 21
MI score 2-6 = 18
N = 18 near-normal 1-year surveillance biopsies (MI = 0)











Dominy et al KI reports in press

Nanostring nCounter technology

Characterising the immune profile of the kidney biopsy at lupus nephritis flare differentiates early treatment responders from non-responders

Samir V Parikh,[1] Ana Malvar,[2] Huijuan Song,[1] Valeria Alberton,[3] Bruno Lococo,[2] Jay Vance,[1] Jianying Zhang,[4] Lianbo Yu,[2] Brad H Rovin[1]

Lupus Science Medicine 2015

250 ng from FFPE full sections

Immunology panel (511 immune response genes) + RT PCR

19 proliferative LN (flares) -> 5 CR, 10 PR, 4 NR

4 normal controls

CR clusters with interferon pathway genes (STAT1, IRF1, IRF7, MX1, STAT2, JAK2)

NR clusters with complement genes (C1R, C1QB, C6, C9, C5, MASP2)

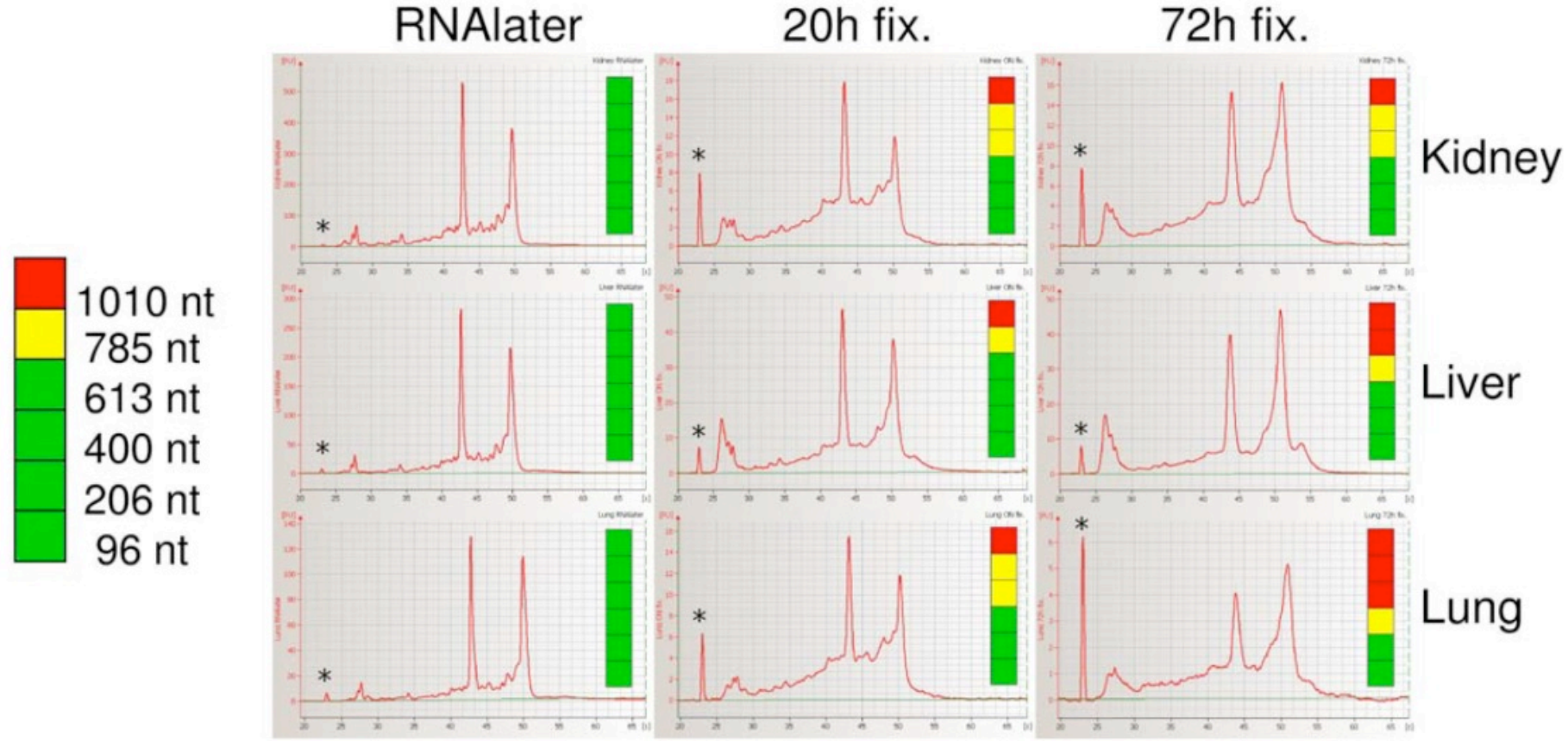
- NR: suppression of BAFF, April, NFKB, Il-6

- For applications that rely of cDNA synthesis (microarrays, qPCR)
  - Fragmented RNA not compatible with oligo-dT priming: leads to a 3’ bias in cDNA generated
  - Cross-linking of RNA hampers primer annealing and elongation during reverse transcription
  - Leads to reduced sensitivity and reproducibility

- Strategies:
  - Demodification of RNA (Wehmas et al. Tox Sci 2018:162)
  - Random Hexamer priming
  - Gene-specific reverse transcription
  - Gene-specific pre-amplification/TaqMan (Zeka F et al. Sci Rep. 2016 Feb 22;6)(Sigdel KI Report 2018)
  - For qPCR: aim to produce shorter amplicons (ideal amplicon = 50-200 bases)

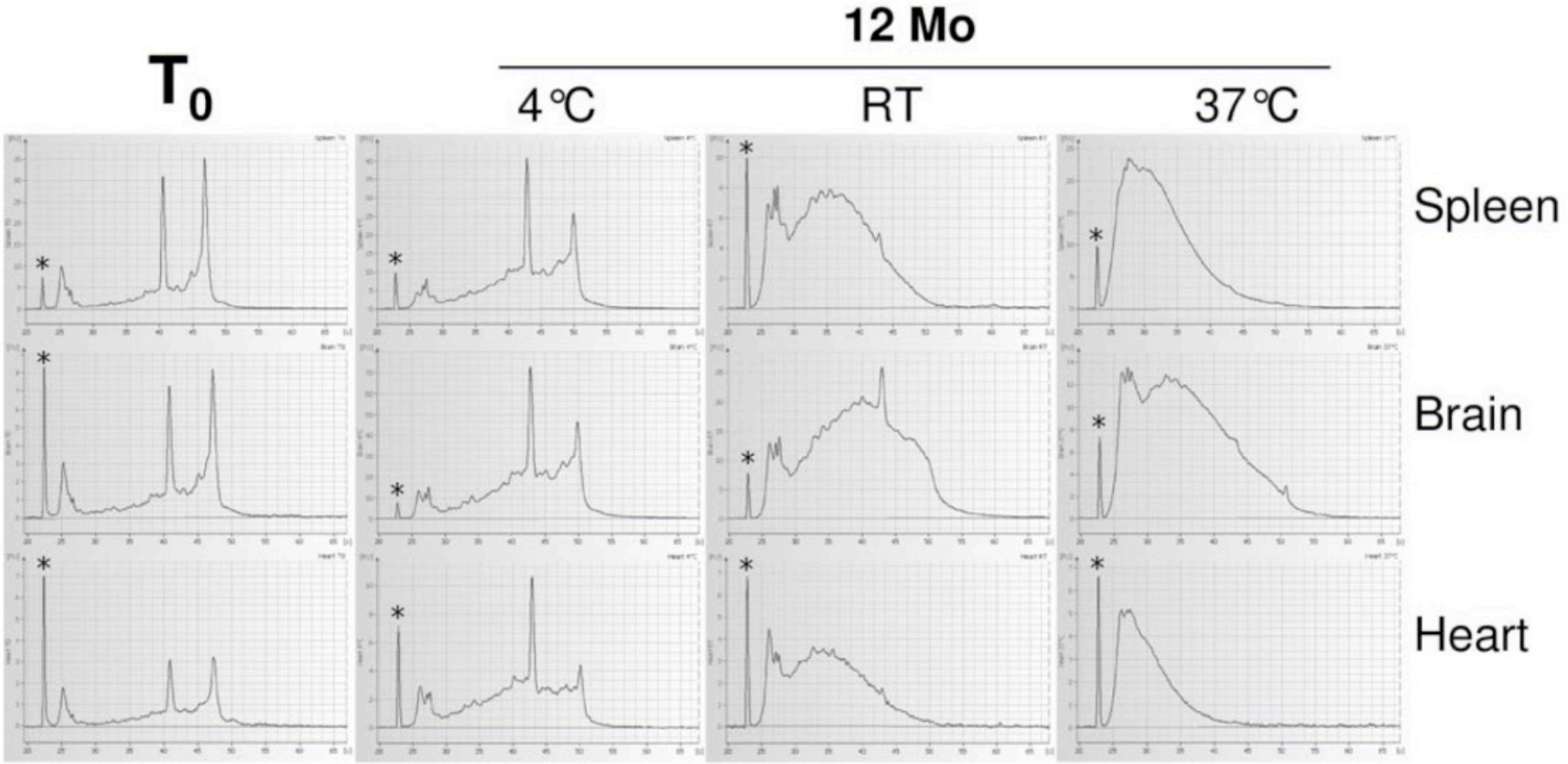
Prolonged fixation results in fragmentation and compromises qPCR according to product length (worse results for long amplicons)

Red= no amplification; Yellow = low amplification



Von Alfen PLoS One 2007

Prolonged storage at RT results in RNA degradation
Samples taken few days after FFPE embedding have RIN ~7
If stored at 4C – RIN ~5 -6 at 1 year



T0 = 3 days post embedding

Von Alfen PLoS One 2007

Table 2. Considerations for sample handling to improve RNA yields and quality from FFPE samples.

Problem	Recommendation
Tissue autolysis	Start fixation as soon as possible
Tissue autolysis	Cut samples into thin pieces (preferrably 5 mm or thinner)
Efficiency of cDNA synthesis	Avoid overfixation
Efficiency of cDNA synthesis	RNA isolation should include crosslink reversal step
RNA integrity	Isolate RNA preferrably within 1 year after fixation and embedding
RT-PCR performance	Use of random priming or primer mix instead of oligo-dT

<u>Potential positives for renal biopsies</u>

Fixation is complete rapidly - tissue very quickly in formalin and thin

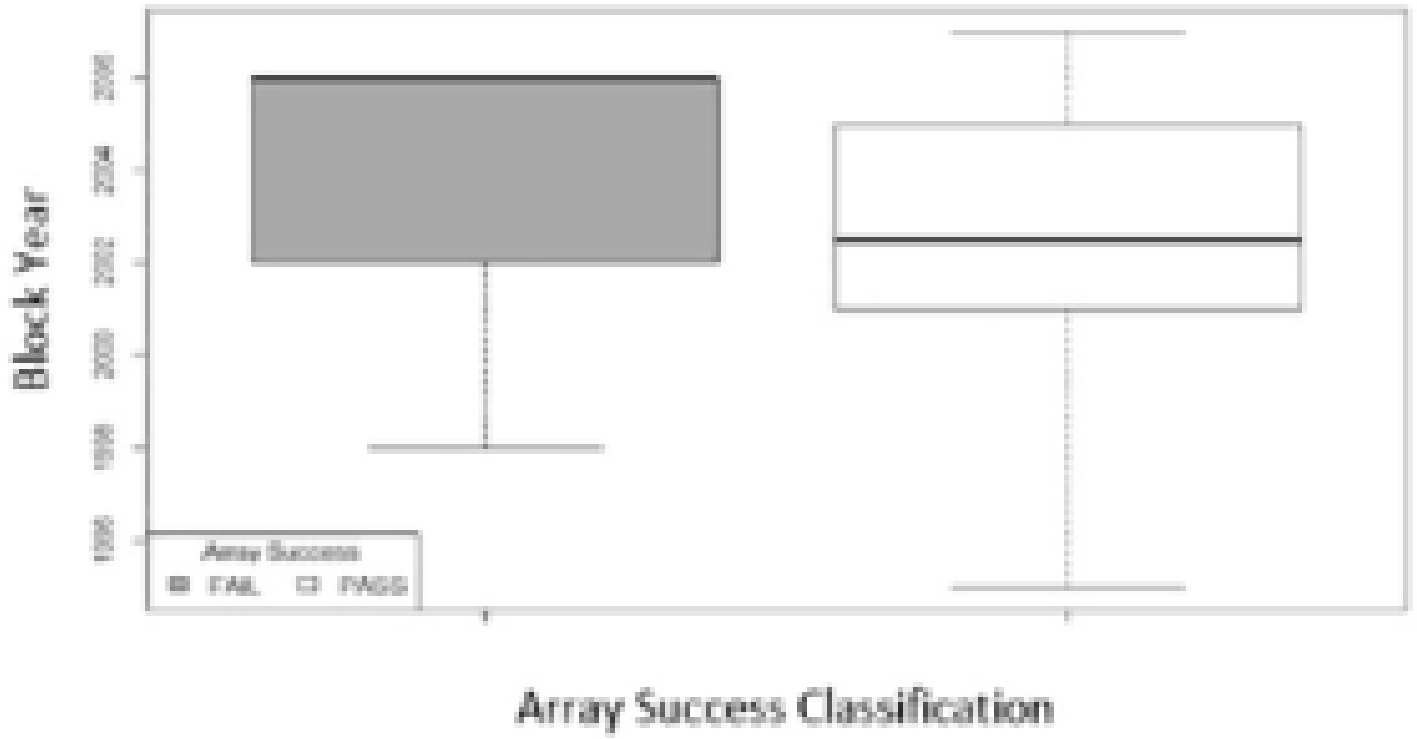
Fixation is not prolonged: most centres process rapidly or overnight

<u>Down sides</u>

Small core – low yields

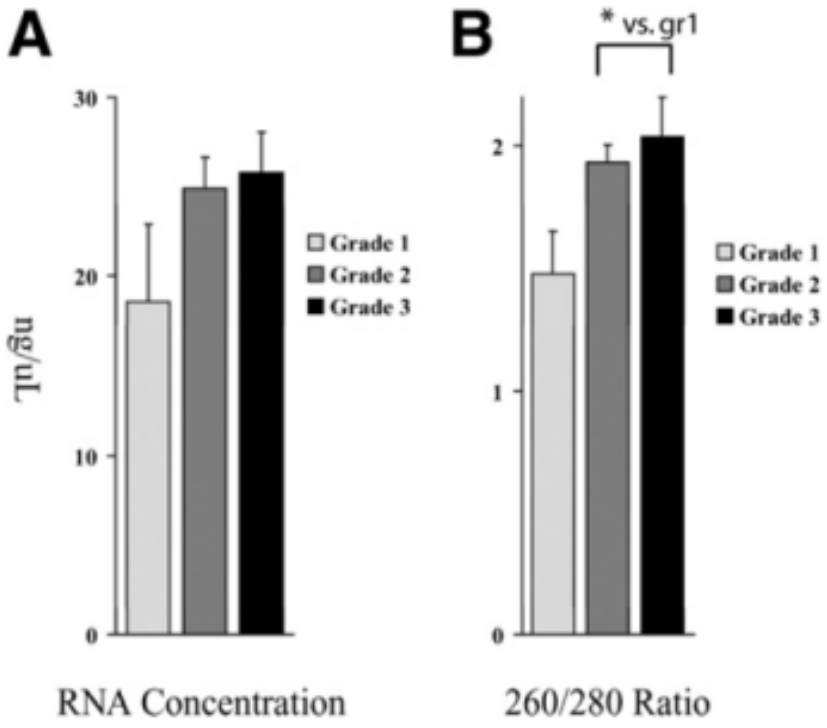
Von Alfen PLoS One 2007

F



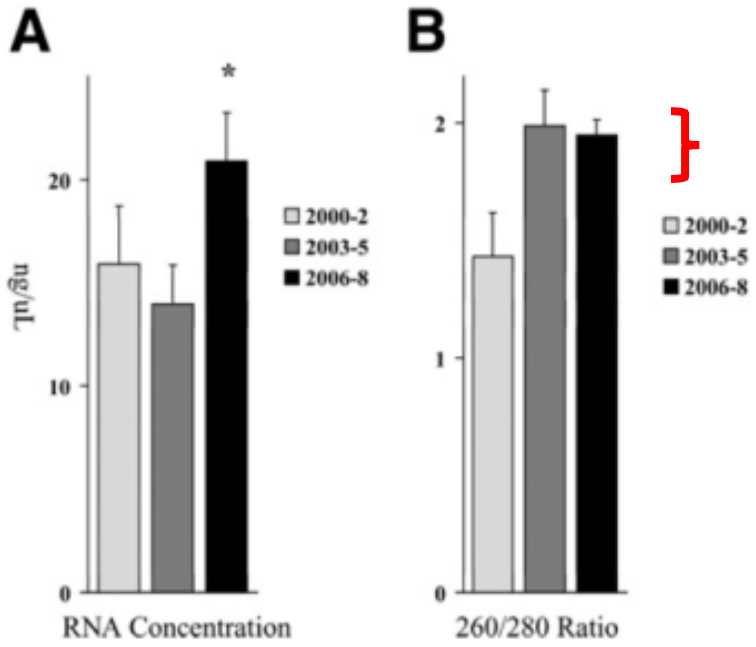
RNA extraction FFPE; microarray analysis (Affymetrix)

RNA yield and purity depends on what is left in FFPE block



Grade 1	<5mm
Grade 2	5-10 mm
Grade 3	>10mm

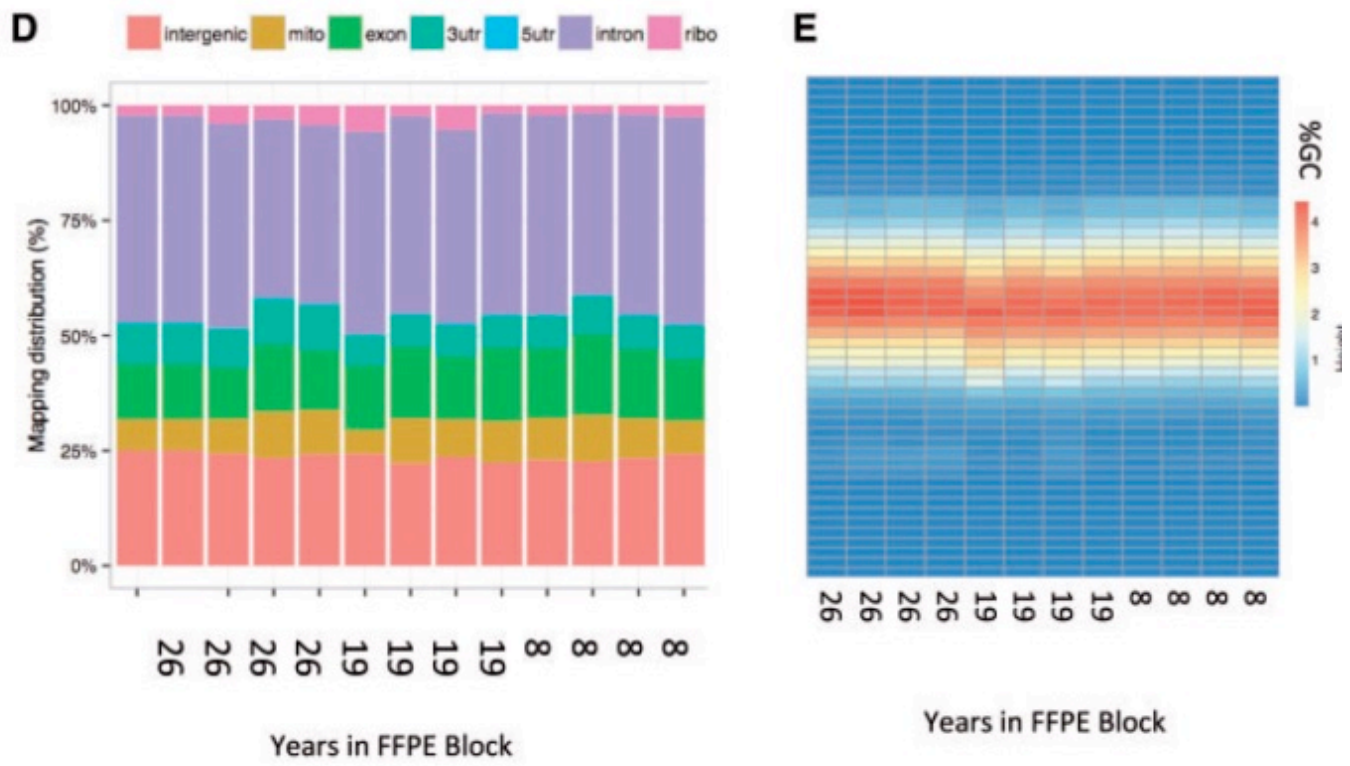
RNA yield and purity depends on storage time



Ideal 260/280

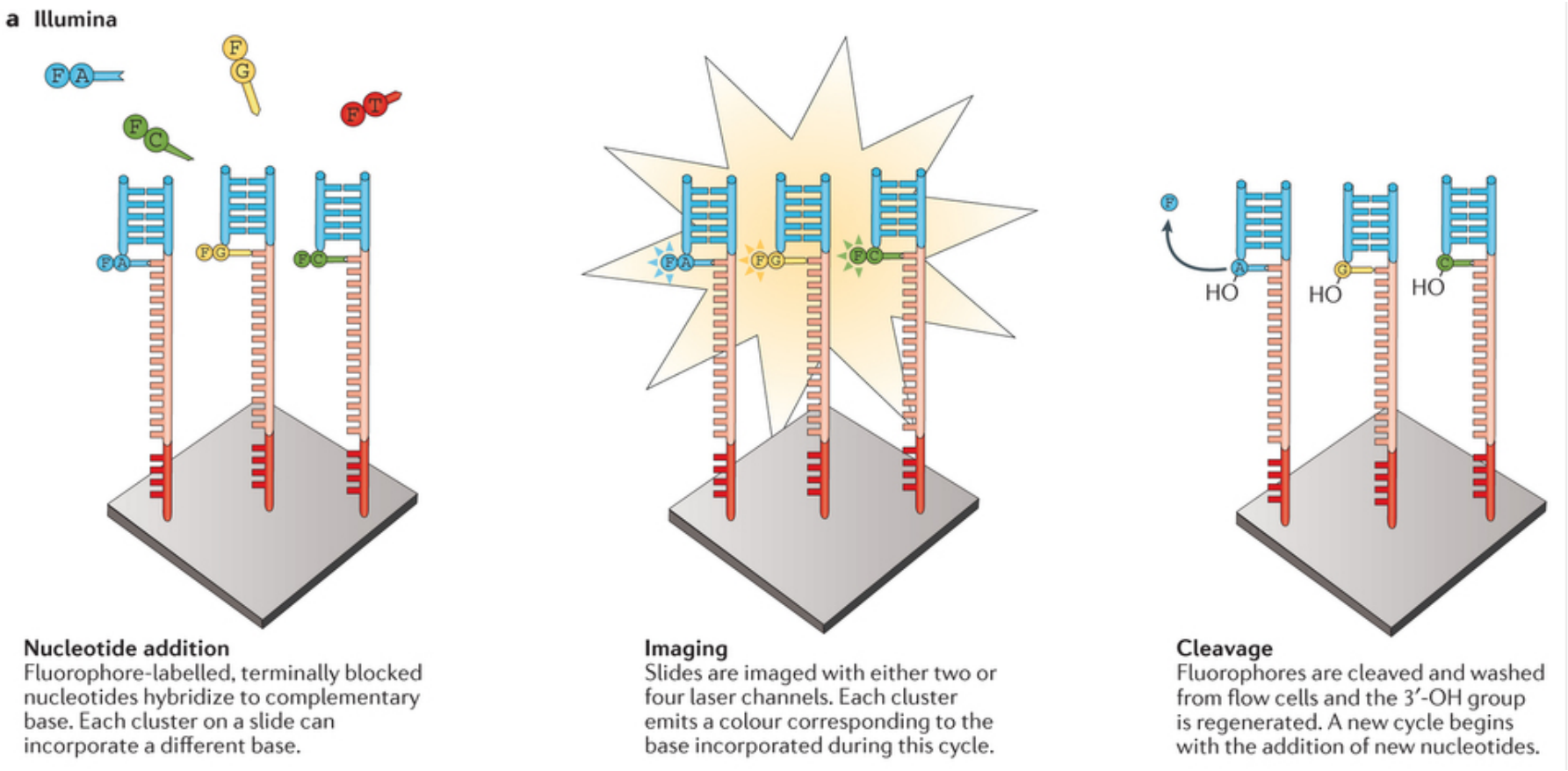
Reich JMD 2011: 13(2): 143-

Ribo-depletion RNA-seq analysis of 8-, 19-, and 26-year-old control blocks resulted in comparable quality metrics, including expected distributions of mapped reads to exonic, untranslated region, intronic, and ribosomal fractions



Francina Webster

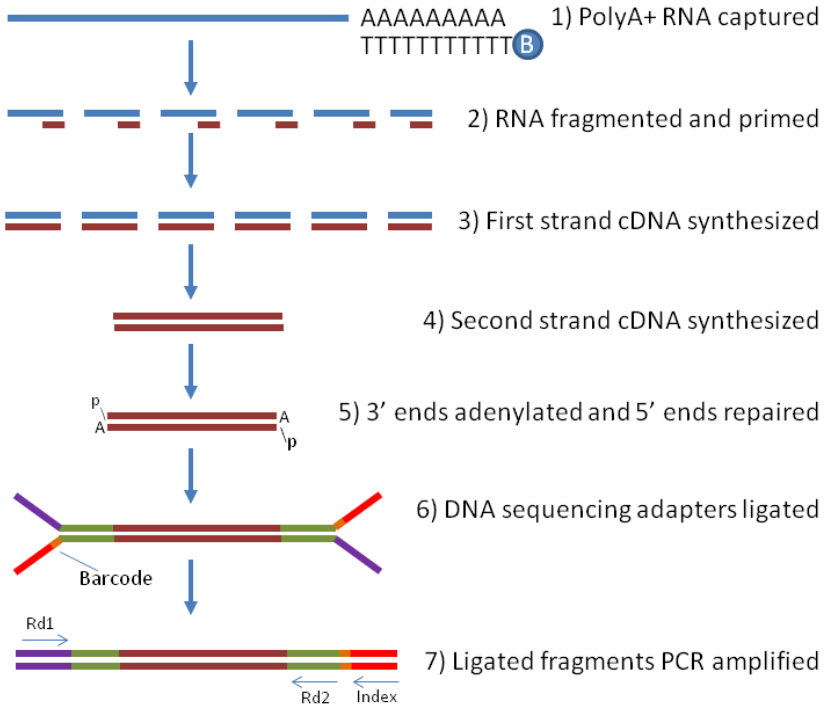
MiSeq - Illumina



RNA sequencing

Library preparation

Illustrated for polyA used for RNA enrichment



Poly A RNA is fragmented then converted to cDNA fragments(NA: sometimes cDNA is fragmented instead) with adaptors at both ends

Amplification step if needed

Sequencing

Sequence cDNA library (reads 30-400bp)

Align reads to reference genome or reference transcripts

Produces a transcription map with structure and level of expression