Who pairs with whom?

High-throughput sequencing of the human paired heavy and light chain repertoire

Technical Journal Club September 15th

Christina Müller

nature biotechnology

High-throughput sequencing of the paired human immunoglobulin heavy and light chain repertoire

Brandon J DeKosky[1], Gregory C Ippolito[2], Ryan P Deschner[1], Jason J Lavinder[3], Yariv Wine[1], Brandon M Rawlings[1], Navin Varadarajan[4], Claudia Giesecke[5,6], Thomas Dörner[5,6], Sarah F Andrews[7], Patrick C Wilson[7], Scott P Hunicke-Smith[3], C Grant Willson[1,8], Andrew D Ellington[3,8] & George Georgiou[1–3,9]

TECHNICAL REPORTS

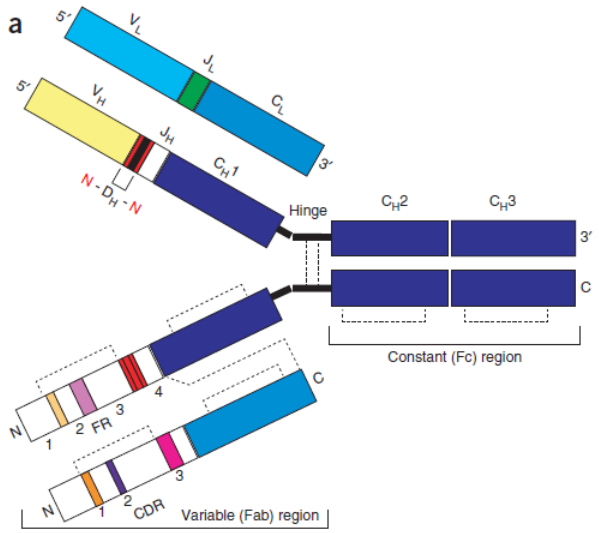
nature medicine

In-depth determination and analysis of the human paired heavy- and light-chain antibody repertoire

Brandon J DeKosky[1], Takaaki Kojima[1,2], Alexa Rodin[1], Wissam Charab[1], Gregory C Ippolito[3], Andrew D Ellington[4] & George Georgiou[1,3,5,6]

Background

- antibody repertoire is the sum of all circulating antibodies produced by the B cells
- total number of B lymphocytes ~ 1-2 x 10^{11}

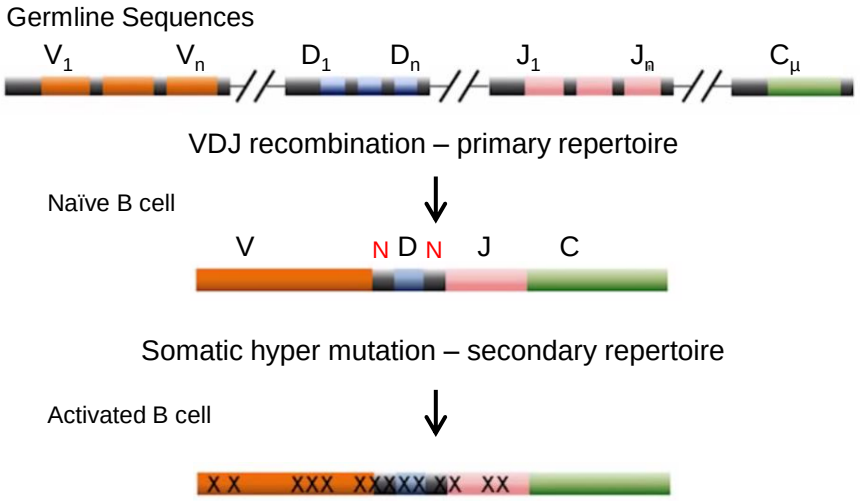


Georgiou et al. 2014

Generation of the antibody repertoire:

- V(D)J recombination
- addition or deletion of nucleotides in regions where junctions occur

Functional gene segments in human IgG loci			
Segment	Light chains		Heavy chain
	κ	λ	H
Variable (V)	34-38	29-33	38-46
Diversity (D)	-	-	27
Joining (J)	5	4-5	6
Constant (C)	1	4-5	9



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Why is it important to determine the antibody repertoire?

- provides important information on protective and pathogenic immunity
- capturing the nature of a successful antibody response
- Ig-sequencing combined with other techniques such as the expression and isolation of antigen-specific antibodies, sequencing of multiple RNAs from single cells or proteomic analysis help to identify antibody properties mediating protection against infectious diseases or autoimmune response

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First steps – Low-throughput analysis of the antibody repertoire:

- determination of IgH and IgL V(D)J recombinants in a few hundred B cells per experiment based on Sanger sequencing (1990s)

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First steps – Low-throughput analysis of the antibody repertoire:

- determination of IgH and IgL V(D)J recombinants in a few hundred B cells per experiment based on Sanger sequencing (1990s)

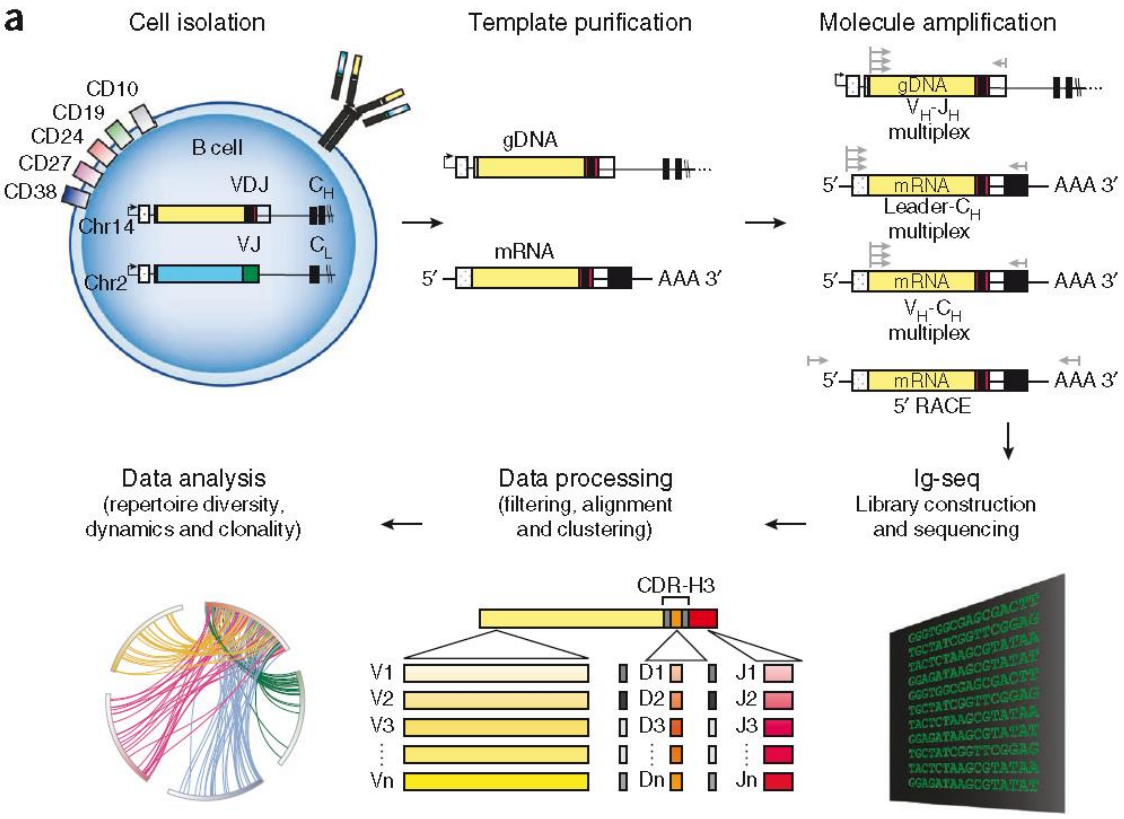
BUT

Low-throughput analysis provides only a small amount of information about the entire antibody repertoire & too labor intensive

Background

High-throughput sequencing of the antibody repertoire

- NGS allows in-depth antibody repertoire studies



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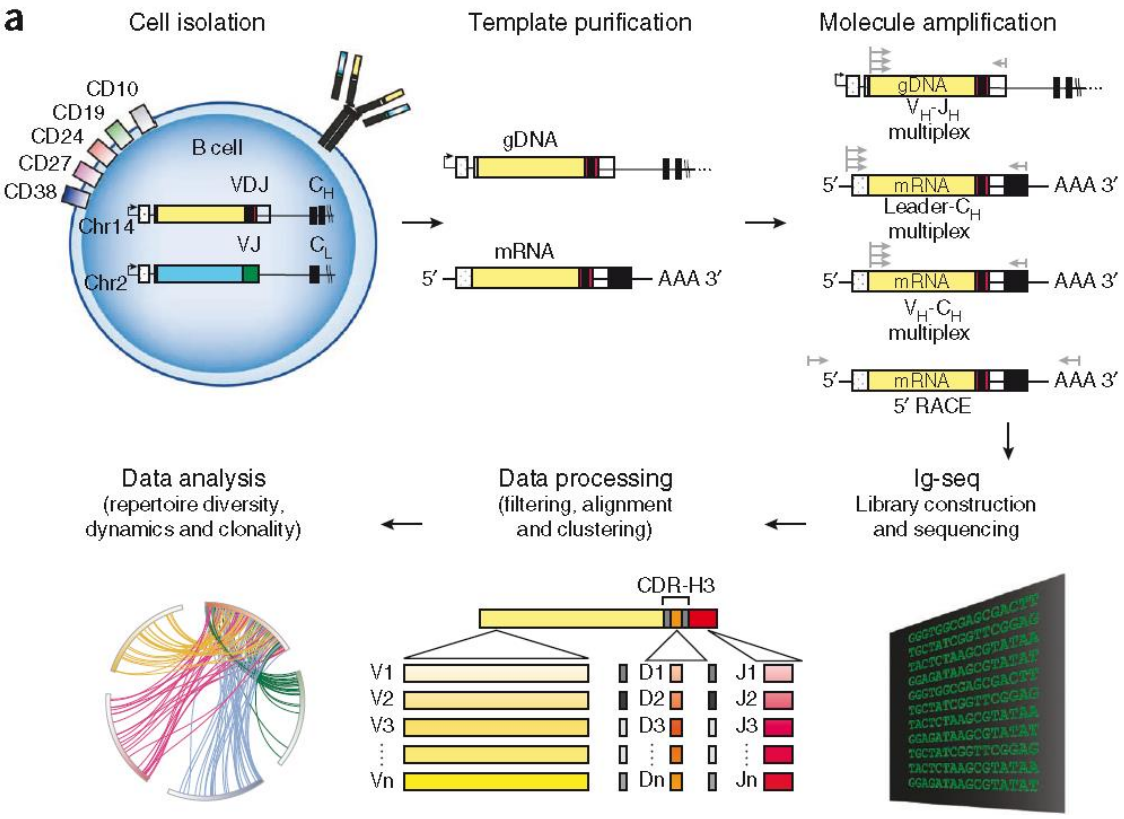

Georgiou et al. 2014

Information about the endogenous pairing of the V heavy (V_H) and V light (V_L) chain is lacking

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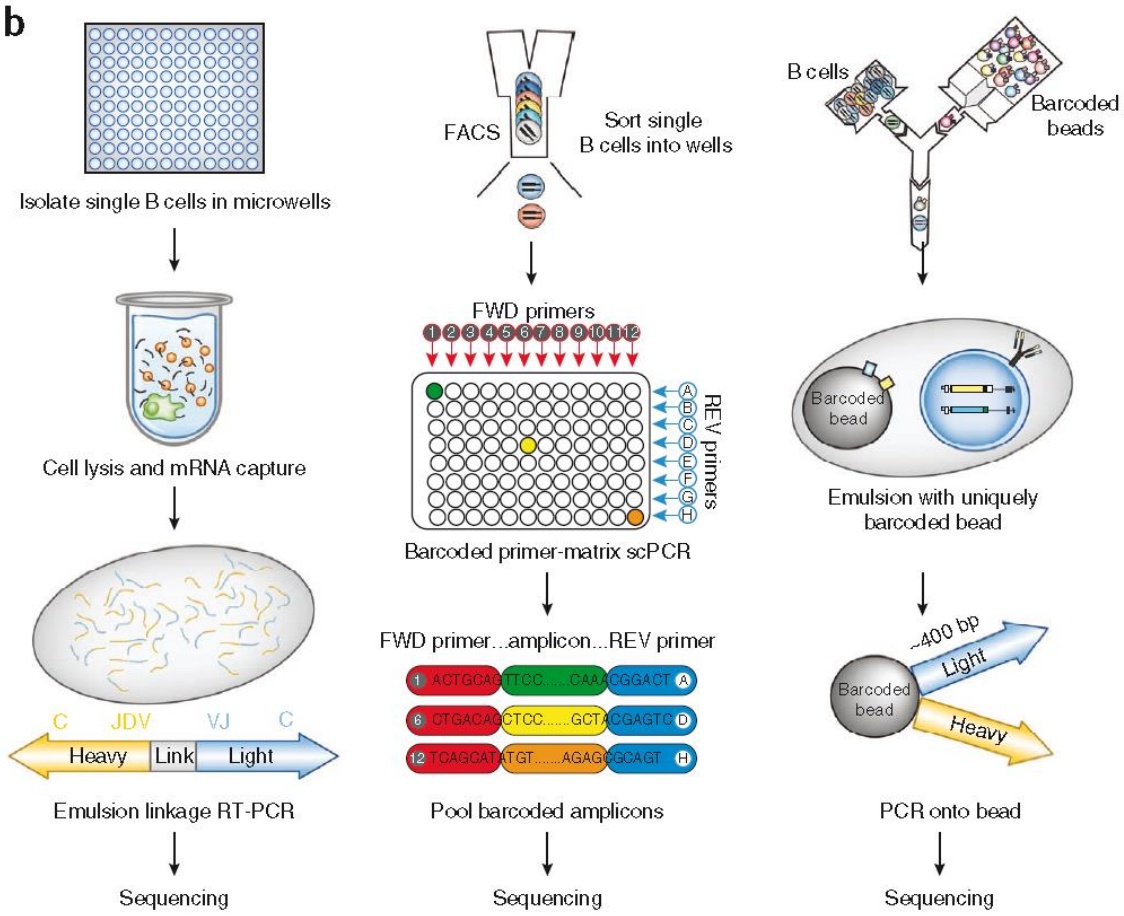

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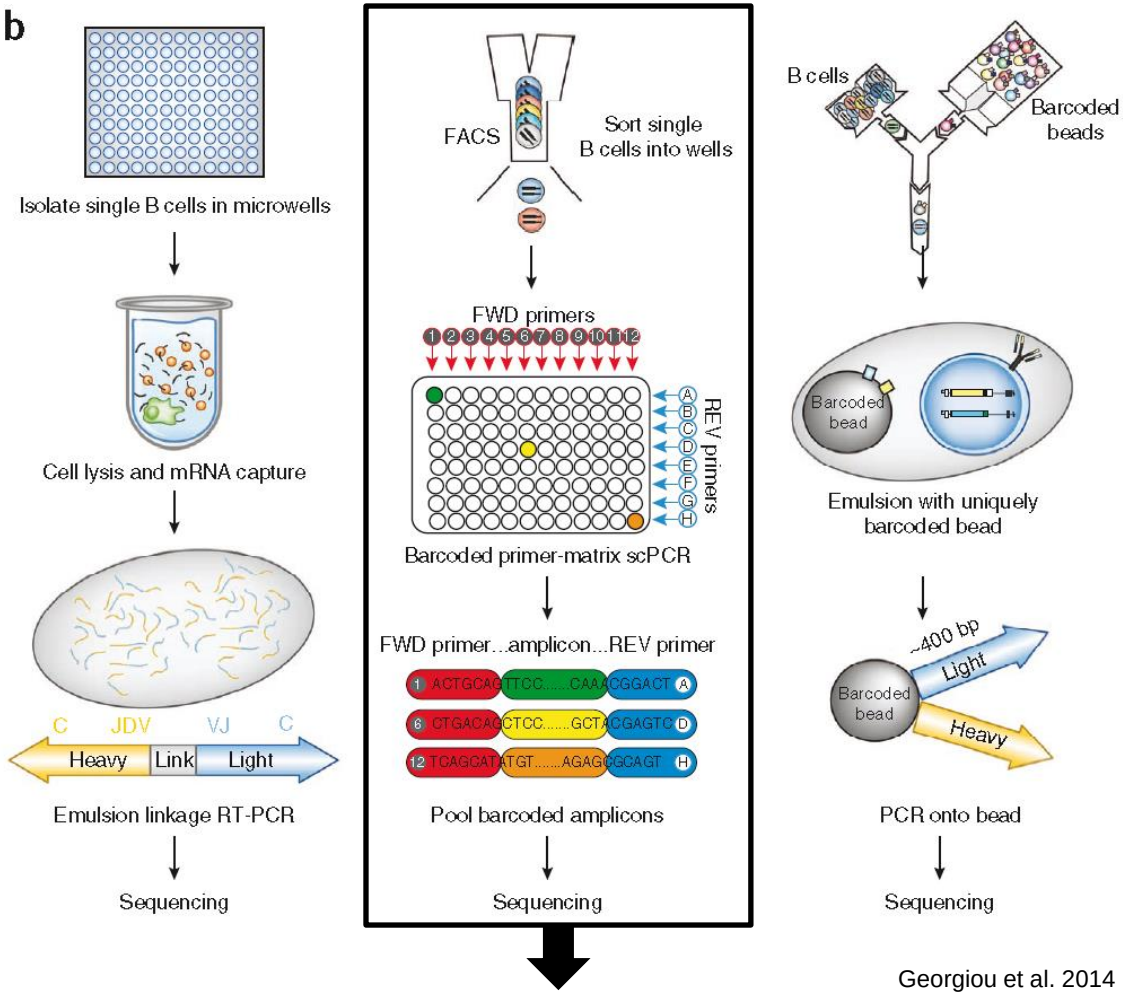
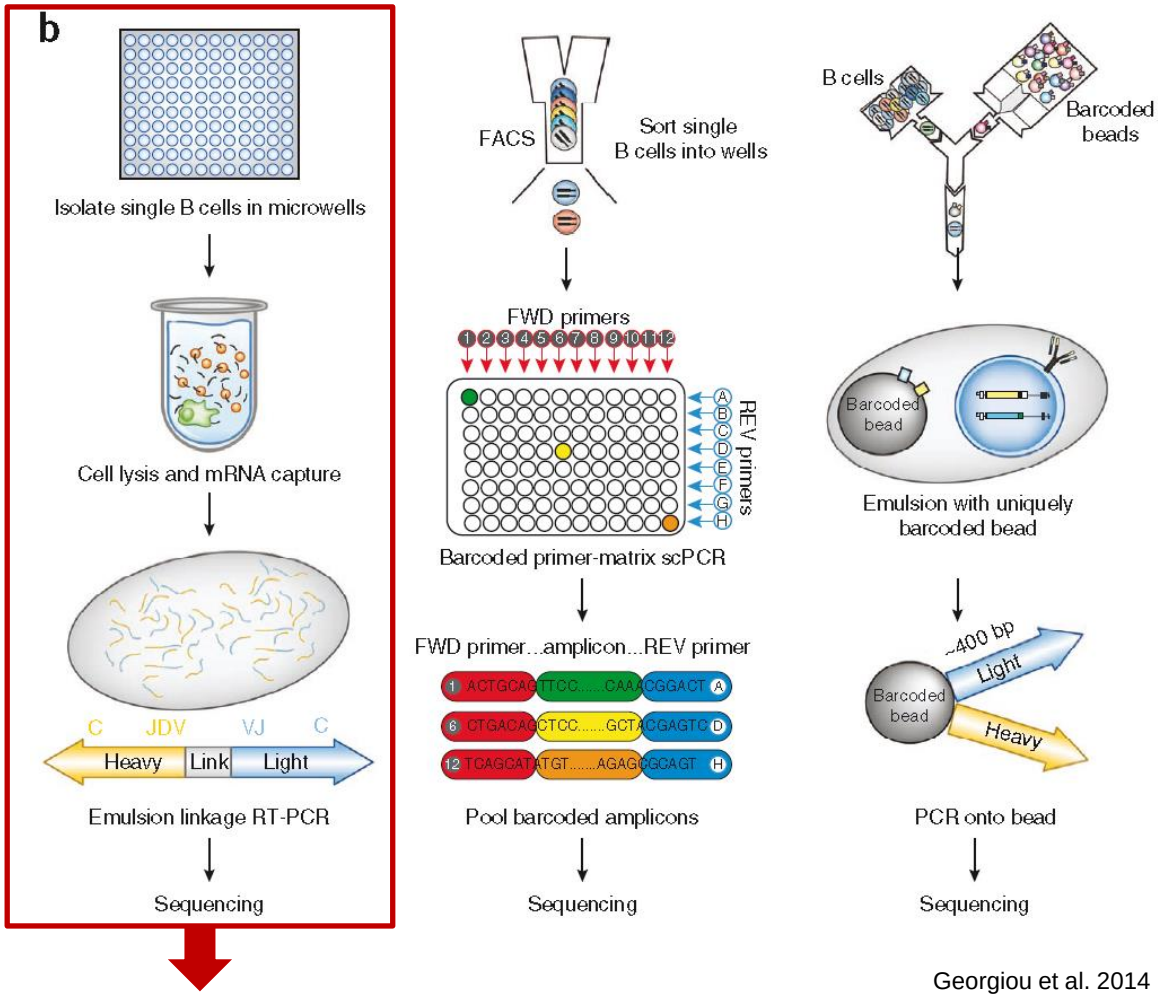


Georgiou et al. 2014

~46 000 individual B cells
(Busse et al. 2013)

Background

High-throughput sequencing of the antibody repertoire



~50 000 individual B cells
(DeKosky et al. 2013)

Georgiou et al. 2014

LETTERS

nature biotechnology

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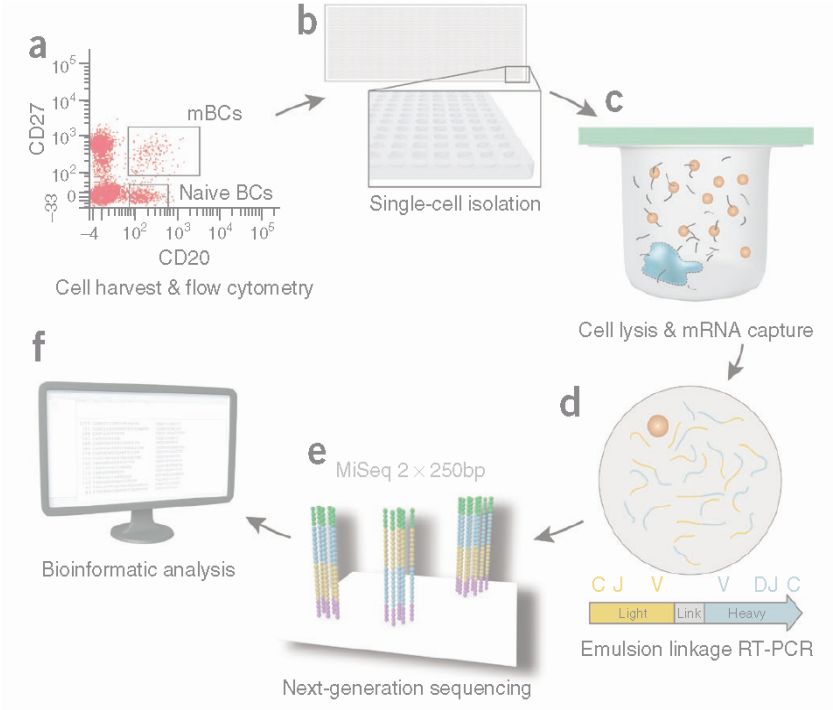
Experimental design

Source of B cells

- Lymph nodes (28%), Spleen and mucosal surface (23%), Red bone marrow (17%)
- Peripheral blood (only 2% of the 1-2 x 10^{11} B cells in the human body)

! Ig transcription varies up to 100 fold between naive B cells and plasma cells **!**

→ using unsorted bulk B cells will make it difficult to deduce cellular clonal frequencies



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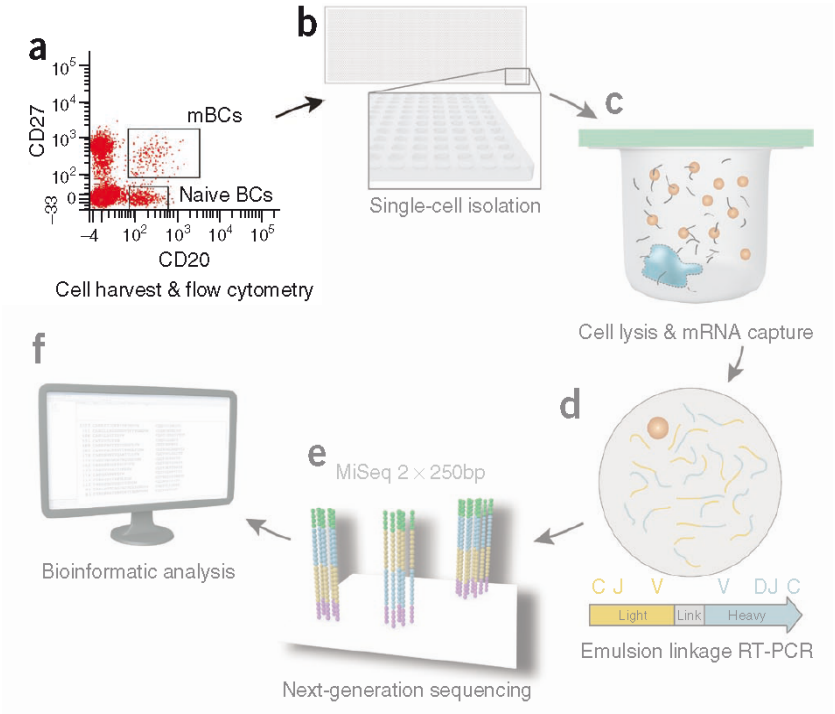
a. FACS sorting

- PBMCs sorted for $CD19^{+}CD3^{-}CD27^{+}CD38^{int}$ memory B cells



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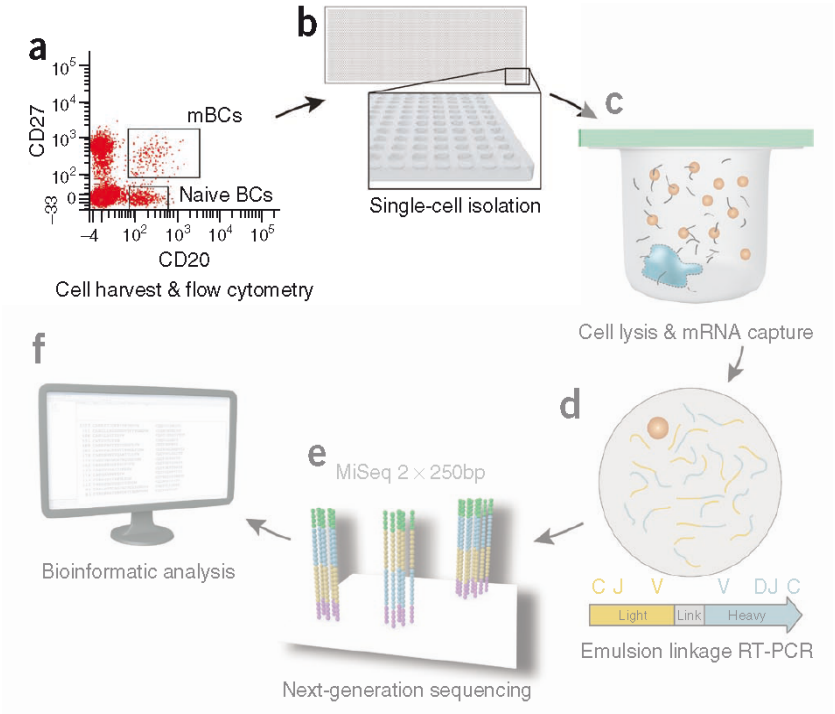
b. Single-cell isolation

- 125-pl wells molded in polydimethylsildoxan (PDMS) slides
- 1.7 x 10^{5} wells per slide



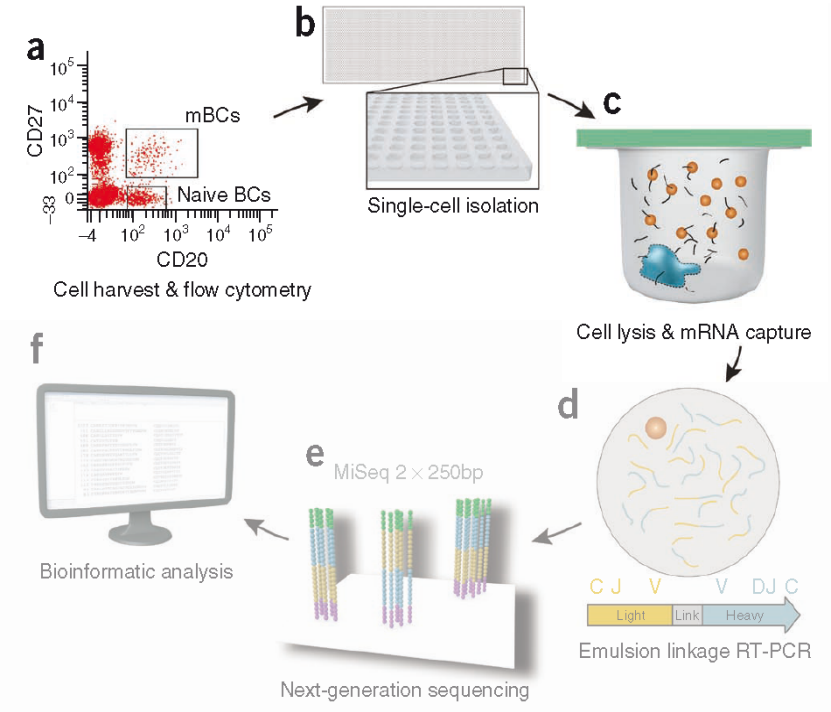
DeKosky et al. 2013

Experimental design

c. Cell lysis & mRNA capture

- poly(dT) magnetic beads added at an average 55 beads/well
- slides were incubated with optimized cell lysis solution (1% lithium dodecyl sulfate)

  → complete cell lysis within < 1min



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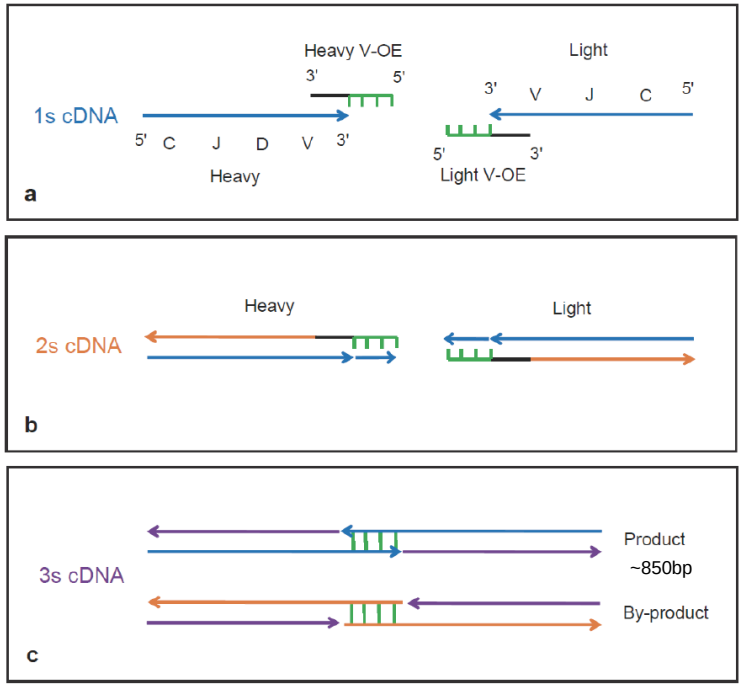
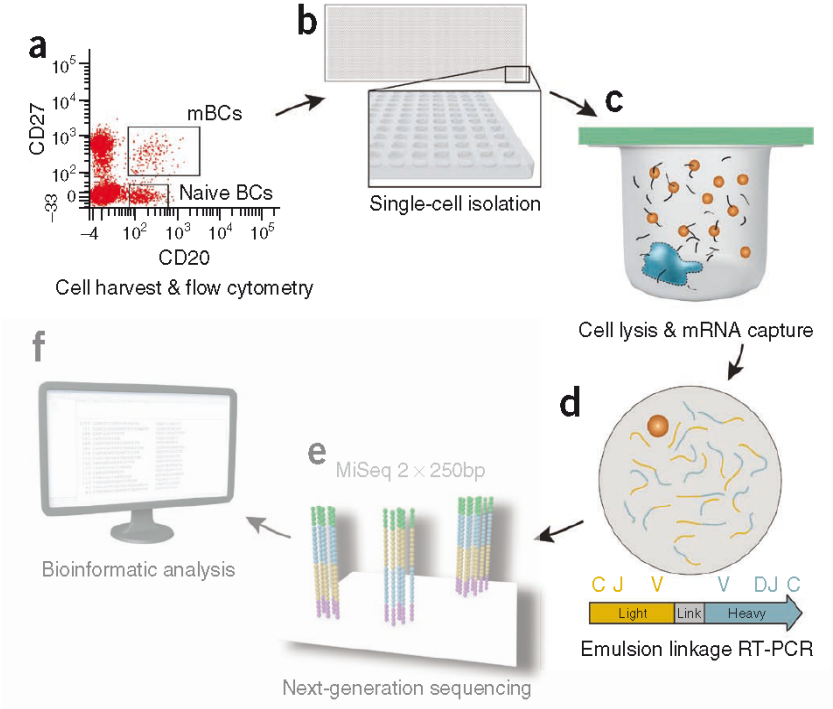
- captured mRNA was emulsified with

  primers, RTase and thermostable DNA polymerase

  → RT-PCR & linkage PCR (Meijer et al. 2006)





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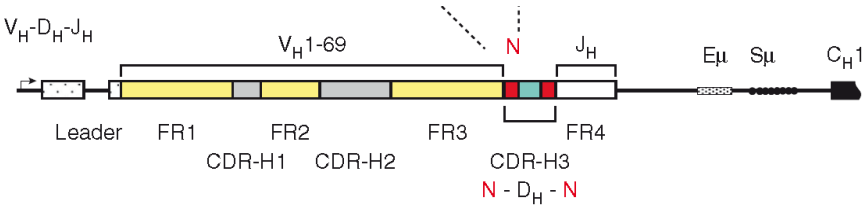
e. NGS using Illumina MiSeq 2 x250pb

- sequencing of CDR-H3 and CDR-L3



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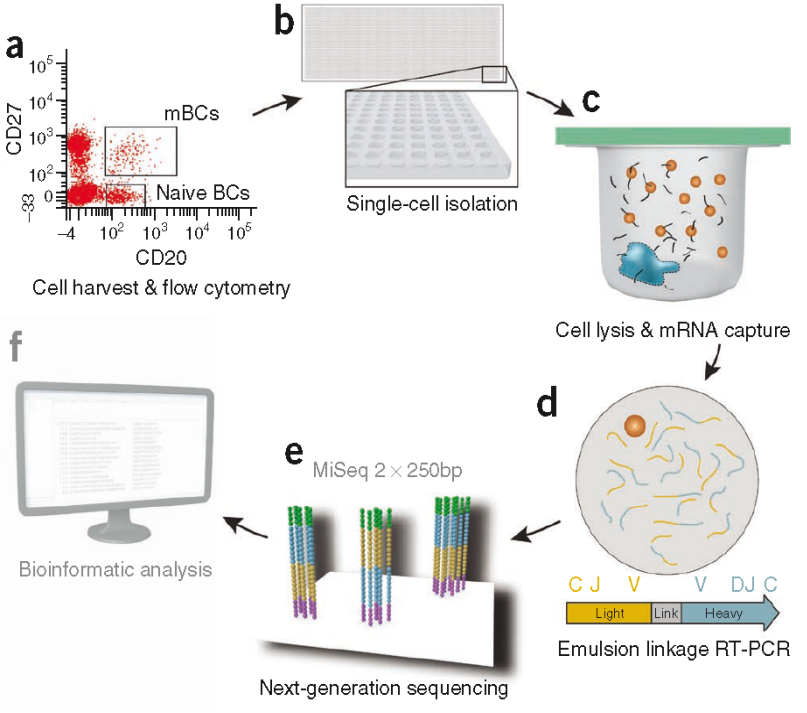

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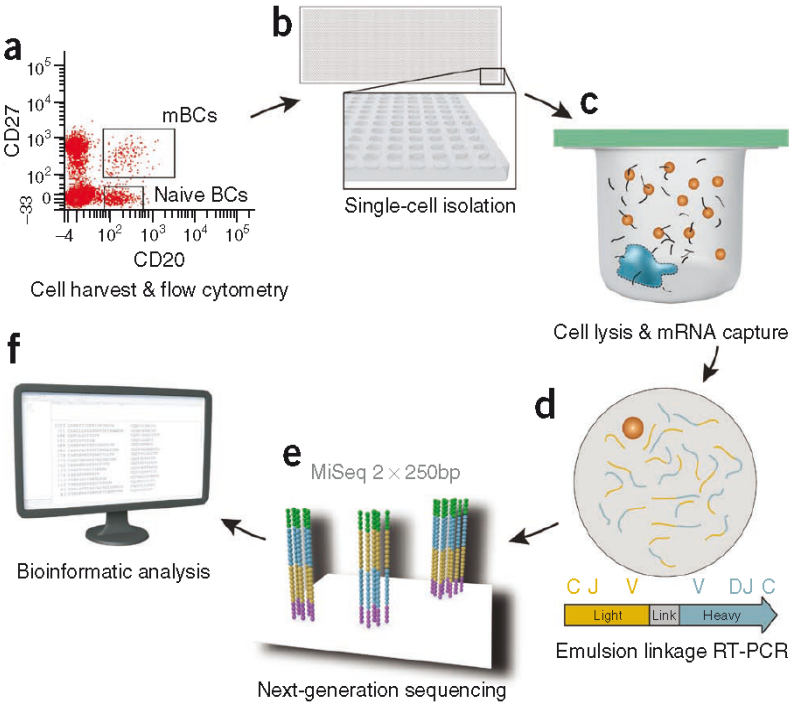

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Evaluation of Methodology

IgG$^+$ B cells from two healthy individuals

Plasmablasts from a healthy individual 7d after tetanus toxin immunization

Memory B cells from a healthy individual 14d after influenza vaccination

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B cells were spiked with immortalized IM-9 lymphoblasts (~4% of total mixture)

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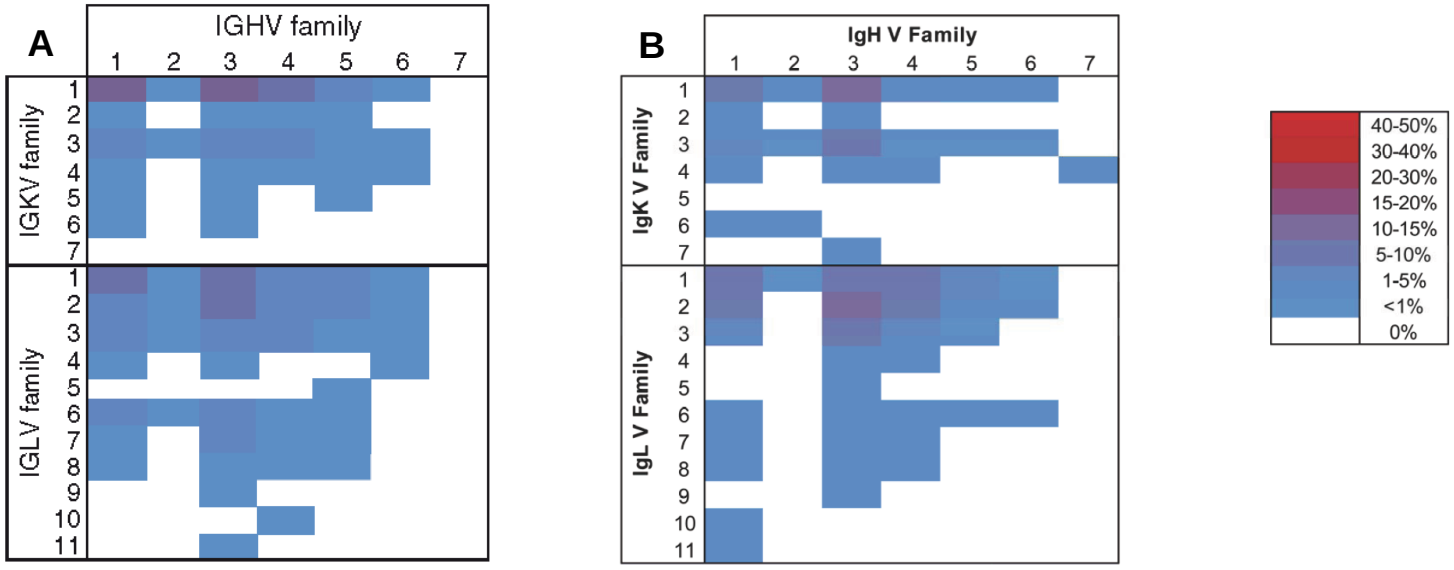

- 61,000 B cells
- 2,716 unique pairs
- correct pairing of IM-9 V_H and V_L 78 fold above background

- 47,000 B cells
- 2,248 unique pairs
- correct pairing of IM-9 V_H and V_L 125 fold above background

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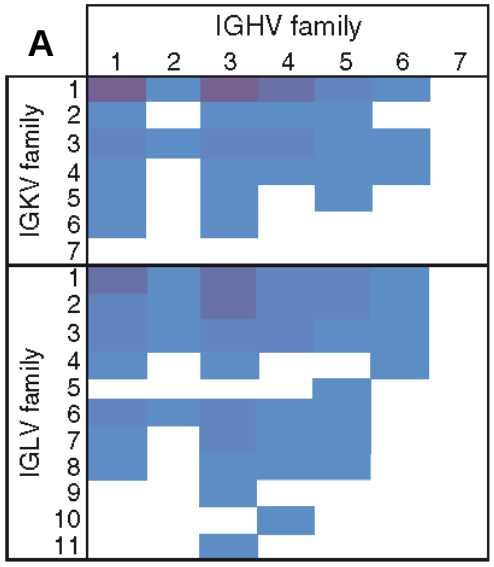

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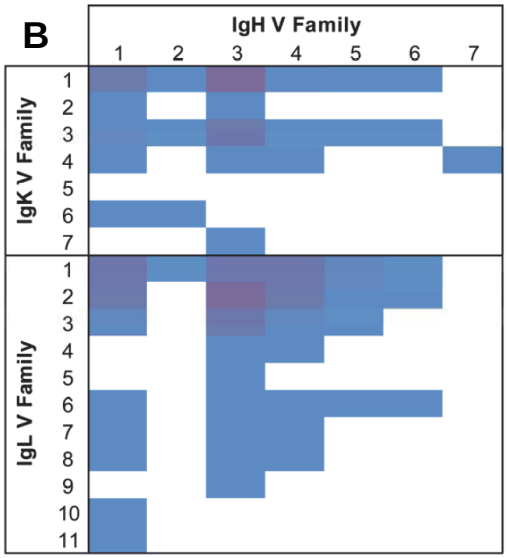

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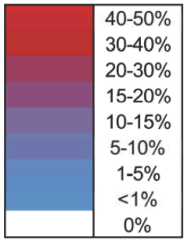

Spearman rank correlation coefficient= 0.804; $P < 10^{-29}$

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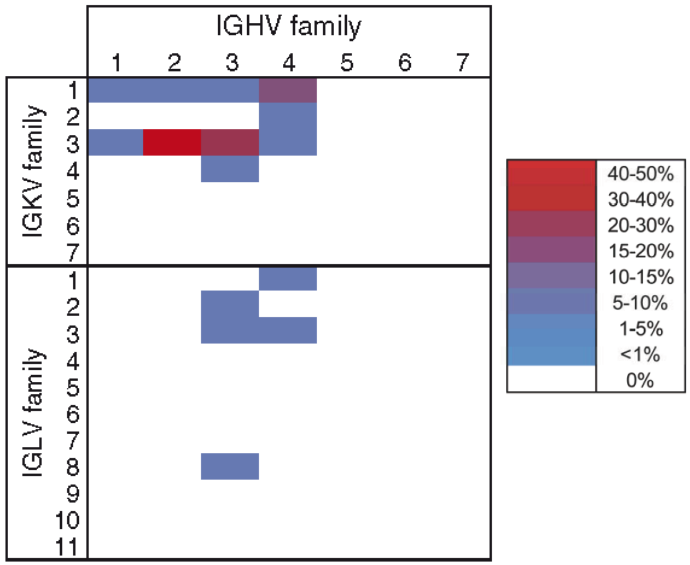

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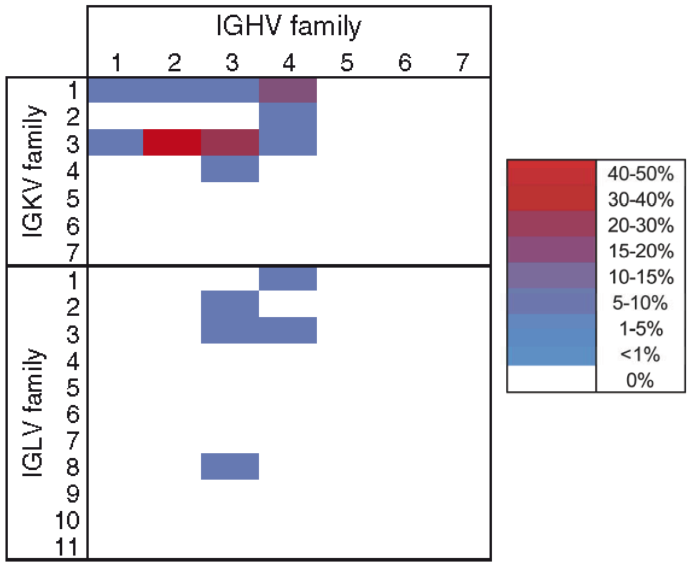

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expression of ten of the identified V_H:V_L pairs as IgG proteins in HEK293 cells

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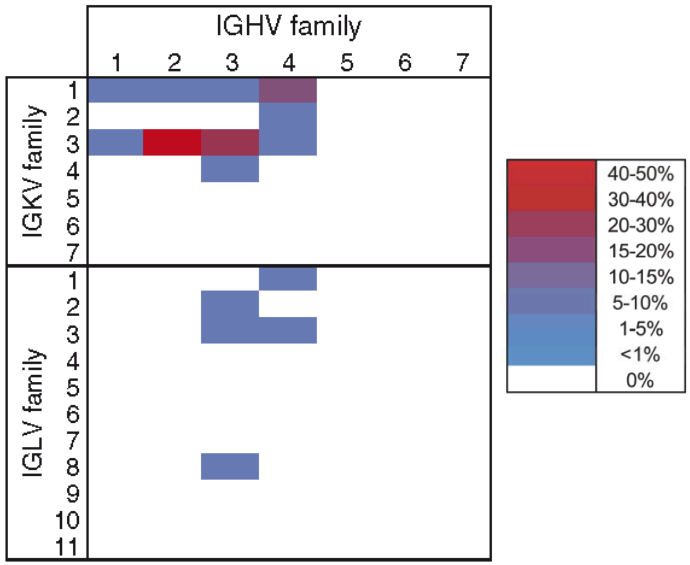

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ELISA

Table 1 TT-binding affinities of IgG antibodies sequenced from TT⁺ peripheral plasmablasts

Antibody ID	Gene family assignment[a]	Affinity (K_D)
TT1	HV3-HD1-HJ6: KV3-KJ5	1.6 ± 0.1 nM
TT2	HV3-HD3-HJ4: LV3-LJ1	14 ± 3 nM
TT3	HV1-HD2-HJ4: KV3-KJ5	3.6 ± 1.8 nM
TT4	HV2-HD2-HJ4: KV1-KJ1	2.7 ± 0.3 nM
TT5	HV4-HD2-HJ6: KV2-KJ3	18 ± 4 nM
TT6	HV1-HD3-HJ4: KV1-KJ2	0.57 ± 0.03 nM
TT7	HV4-HD3-HJ4: KV1-KJ2	0.46 ± 0.01 nM
TT8	HV3-HD3-HJ4: LV8-LJ3	2.8 ± 0.3 nM
TT9	HV4-HD2-HJ4: KV1-KJ1	0.10 ± 0.01 nM
TT10	HV1-HD3-HJ5: KV3-KJ5	1.6 ± 0.1 nM

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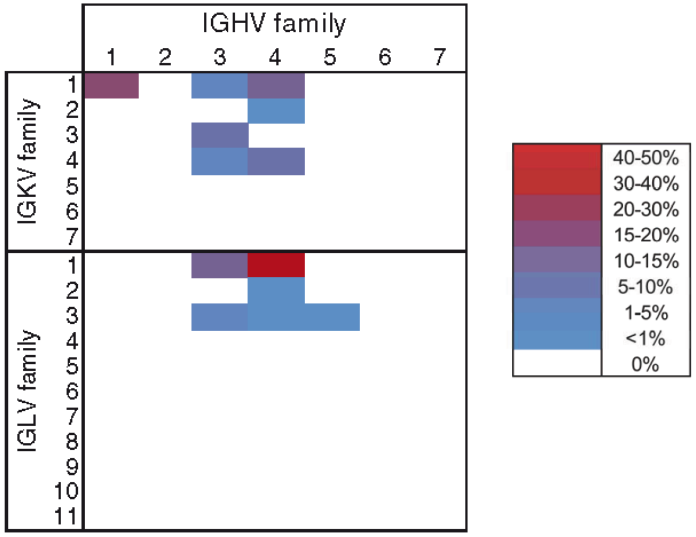
Identification of V_H:V_L pairs by high-throughput approach vs scRT-PCR:

Sanger scRT-PCR	High-throughput approach
168 single memory B cells	8,000 single memory B cells
168 RT- & 504 nested PCR reactions → Sanger Sequencing	Workflow as described → MiSeq
50 unique V_H:V_L pairs	240 unique V_H:V_L pairs



- 8000 recovered B cells
- 240 unique pairs

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Seq ID	Isotype	CDR-H3	Paired CDR-L3[1]	Source
2D02	IgM	gcgagaggcggaaatgggcgacccttgacaac	gcagcatgggatgacagcctgaatggttgggtg	Sanger scRT-PCR
2D02	IgM	gcgagaggcggaaatgggcgacccttgacaac	gcagcatgggatgacagcctgaatggttgggtg	MiSeq VH:VL
3D05	IgM	gcgagaaggtactttgactac	gnagcatgggatgacagcctgaatgtttggntg	Sanger scRT-PCR
3D05	IgM	gcgagaaggtactttgactac	gcagcatgggatgacagcctgaatgtttggctg	MiSeq VH:VL
1E02	IgG1	gcgcgacatggccctgcgggaaaaagcgcgtatggttttgatatc	cagtcctatgacagcggactgaatggttatgtggtc	Sanger scRT-PCR
1E02	IgG	gcgcgacatggccctgcgggaaaaagcgcgtatggttttgatatc	cagtcctatgacaacagactgaatggttatgtggtg	MiSeq VH:VL
3A01	IgG3	gcgagagtaatagcagctcgcgaccgccggatcactcctaactactaccgccctatggacgtc	caggtgtgggatagtagtagtgaccatcaggtg	Sanger scRT-PCR
3A01	IgG	gcgagagtaatagcagctcgcgaccgccggatcactcctaattactaccgccctatggacgtc	caggtgtgggacagtagtagtgatcatcaggtg	MiSeq VH:VL

[1] The 2D02 and 3D05 CDR-L3 sequences are highly similar but differ by two bases

Conclusion Paper 1

Workflow for high-throughput sequencing of the paired heavy and light chain repertoire

- entire process can be completed by a single investigator in 10 working hours over 4days

  → identification of 2,716 unique V_H:V_L to a cost of $550

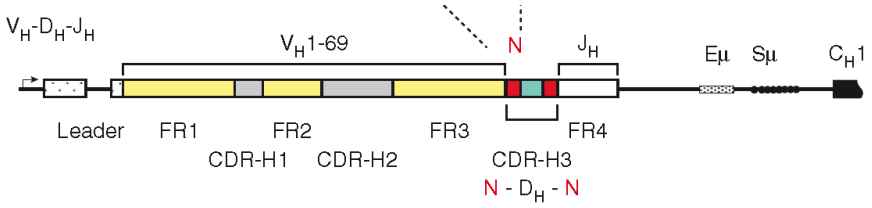
  vs. > $25,000 using scRT-PCR protocol

- identification of TT specific antibodies with high affinity

  → can be applied to investigate vaccine efficacy

- high CDR-H3:CDR-L3 pairing accuracy

  → longer sequencing reads are needed to distinguish somatic variants based on mutations between the FR1 and CDR2 region



Georgiou et al. 2014

- capacity > 5 x 10^4 single cells per experiment

  → greater depth still needed (10ml blood draw contains ~0.7 x 10^6 to 4 x10^6 B cells)

TECHNICAL REPORTS

nature medicine

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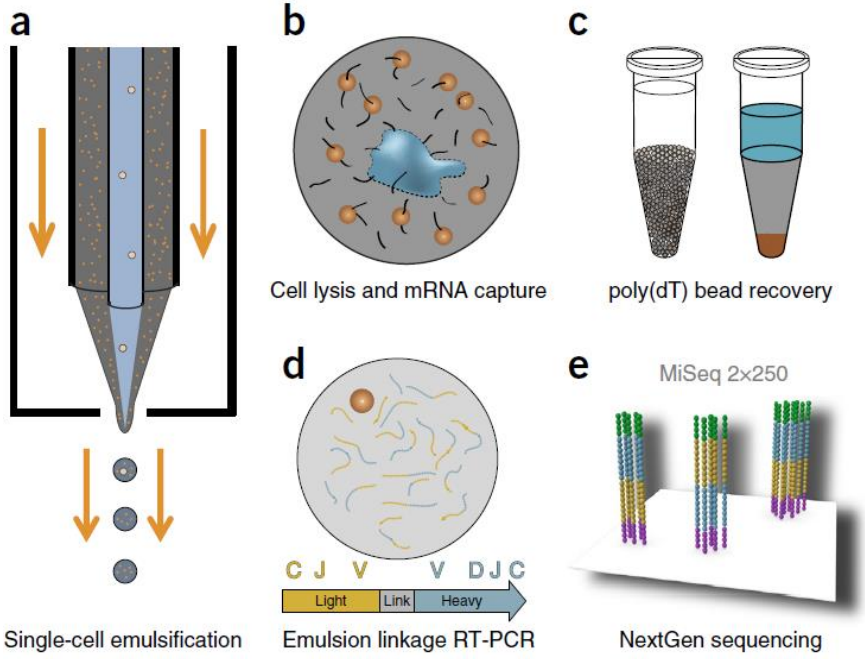
Greater depth by using an axissymmetric flow-focusing devices



DeKosky et al. 2015

Experimental design

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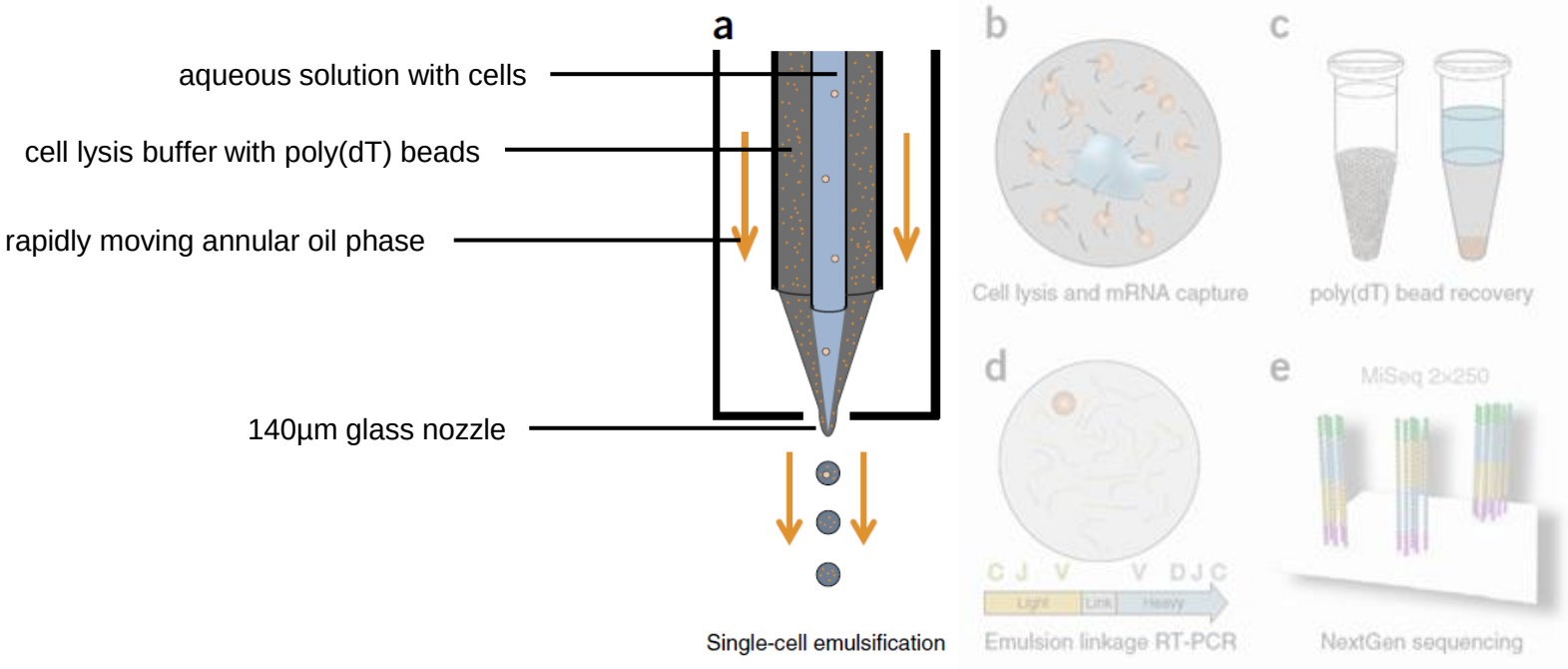

DeKosky et al. 2015

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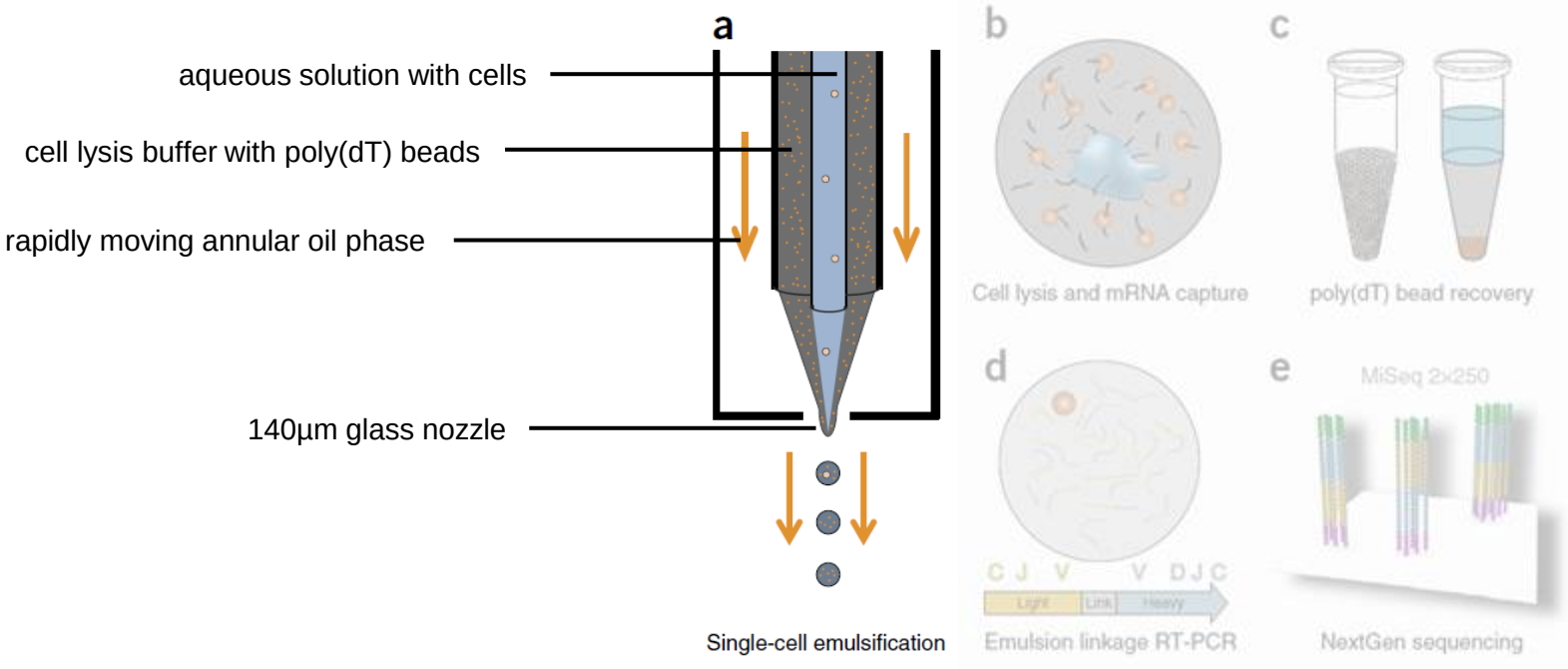

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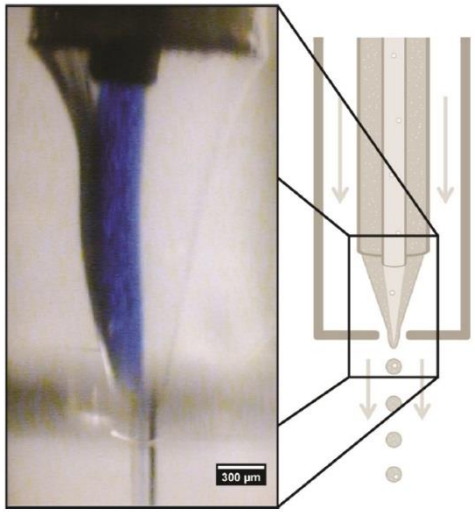
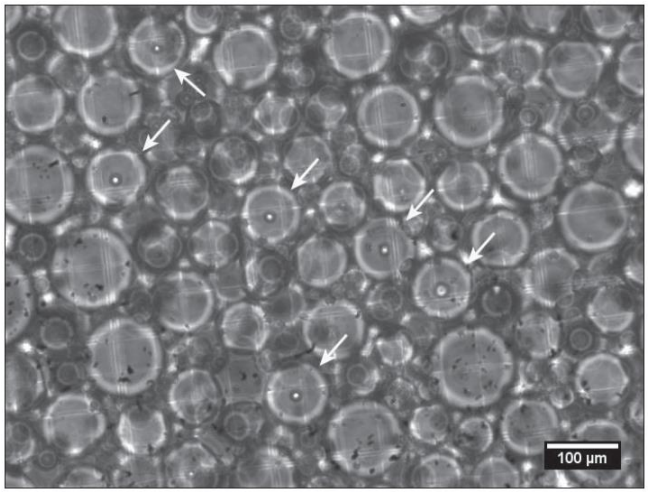
droplet formation with predictable size distribution

Evaluation of Methodology

Evaluation of encapsulation and droplet size distribution

- middle tubing containing PBS and Trypan blue (0,4% v/v)
- 250,000 cells per minute





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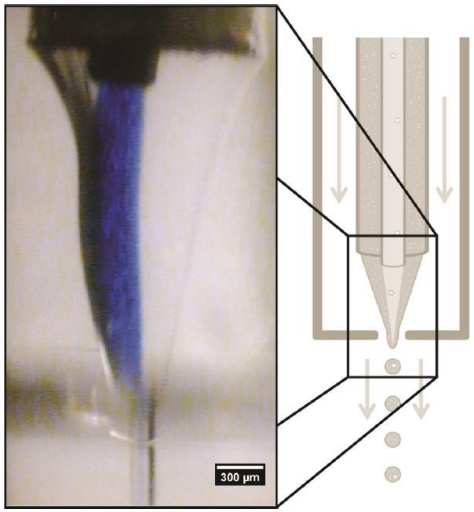
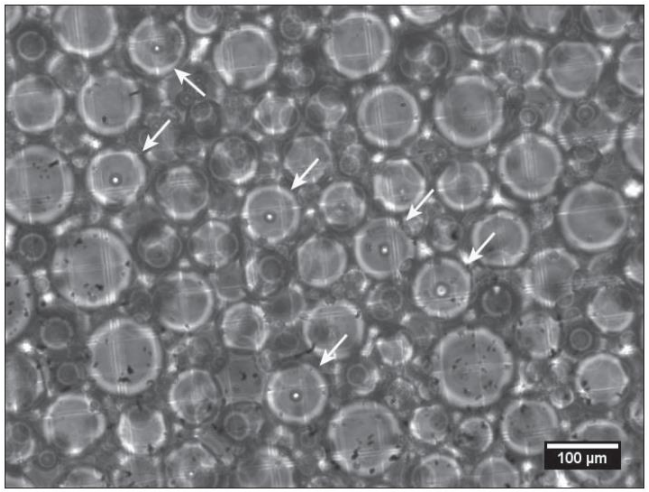




- Trypan blue exclusion → cells remained viable
- droplet diameter 73 ± 20µm

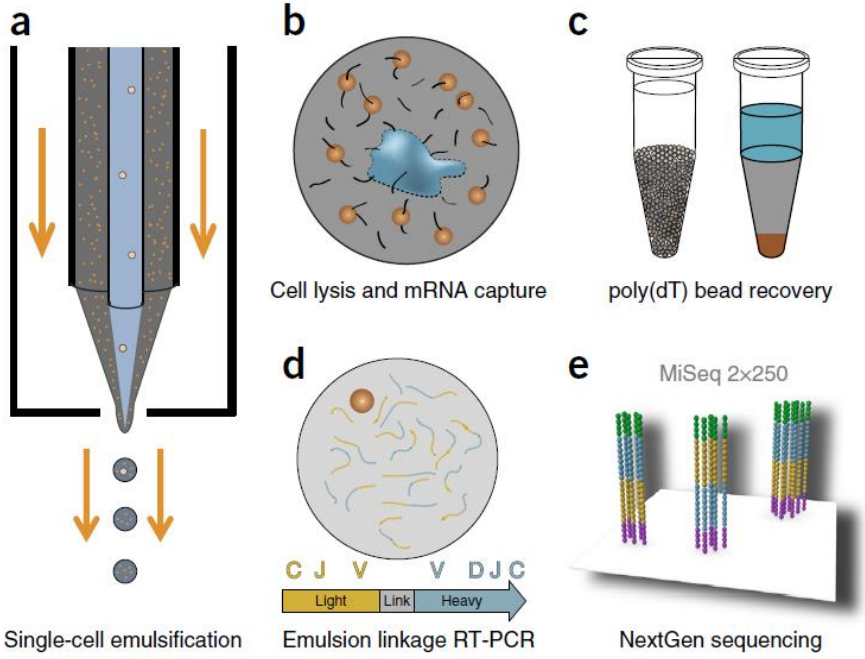
Experimental design

b. **Cell lysis & mRNA capture**
   - emulsion maintained for 3min

c. **poly(dT) bead recovery**
   - chemical breakage of emulsion

d. **Emulsion linkage RT-PCR**

e. **NGS using Illumina MiSeq 2 x 250pb**
   - sequencing of CDR-H3 and CDR-L3



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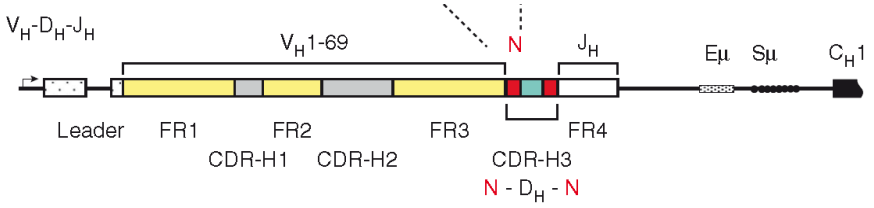

Georgiou et al. 2014

Evaluation of Methodology

Memory B cells from healthy individuals in two technical replicates

B cells were expanded for 4 days

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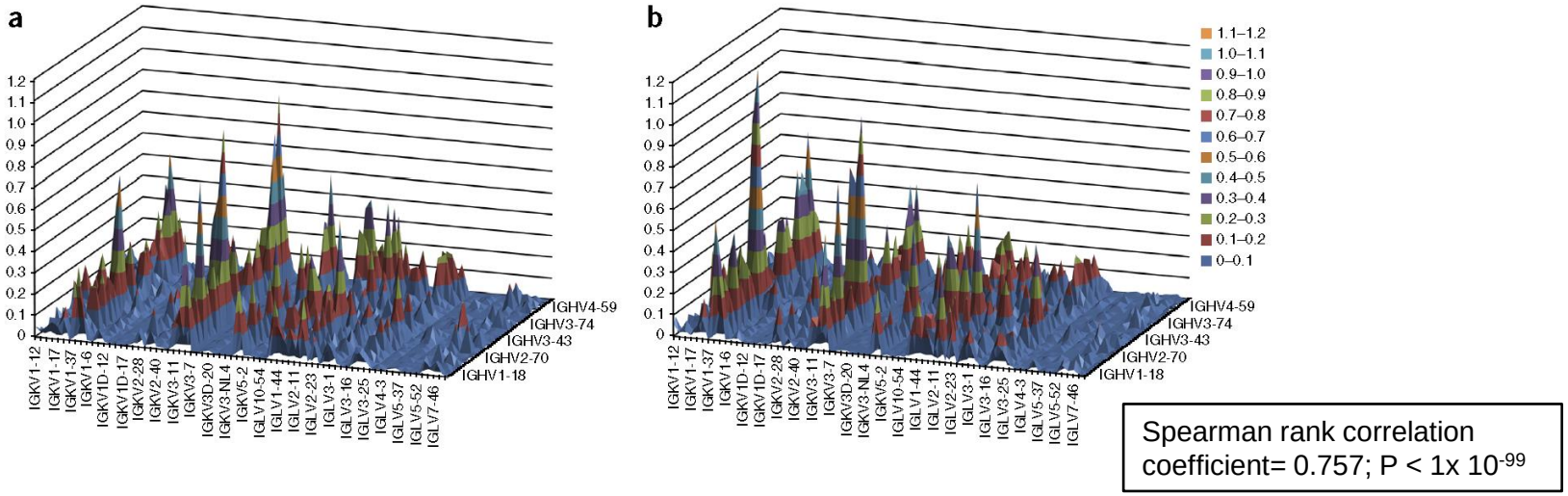

Spearman rank correlation coefficient= 0.757; $P < 1x\ 10^{-99}$

Table 1 High-throughput VH-VL sequence analysis of CD3⁻CD19⁺CD20⁺CD27⁺ *in vitro*–expanded human B cells

Human donor	V-region primer set	No. cells analyzed	Emulsification rate (cells per minute)	Observed VH-VL clusters	CDR-H3 detected in both replicates	CDR-H3–CDR-L3 clusters detected in both replicates	VH-VL pairing precision
Donor 1	Framework 1	1,600,000	50,000	129,097	37,995	36,468	98.0%
Donor 2	Framework 1	810,000	50,000	53,679	19,096	18,115	97.4%
Donor 3	Leader peptide	210,000	33,000	15,372	4,267	4,170	98.9%

Evaluation of Methodology

Identification and characterization of promiscuous V_L junctions

- light chains have a much lower theoretical diversity than heavy chains

→ light chain sequences pair with multiple heavy chains

(promiscuous light chains)

Functional gene segments in human IgG loci

Segment	Light chains		Heavy chain
	κ	λ	H
Variable (V)	34-38	29-33	38-46
Diversity (D)	-	-	27
Joining (J)	5	4-5	6
Constant (C)	1	4-5	9

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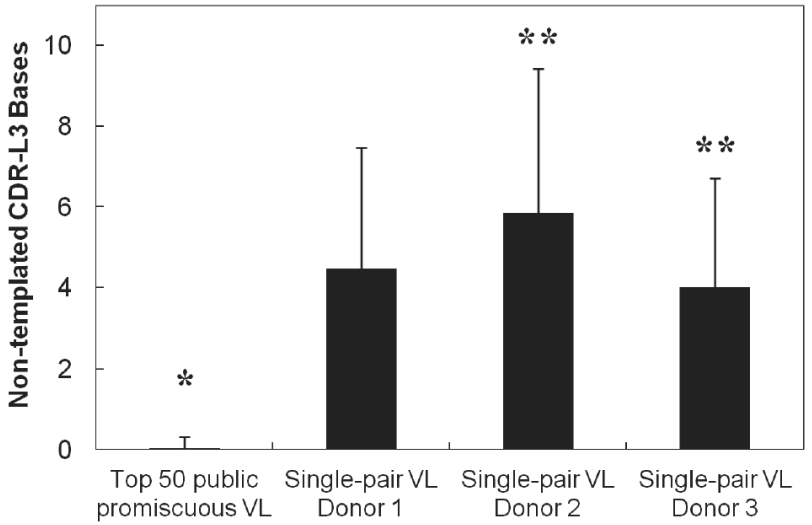
  Average of 0.04 (promiscuous)

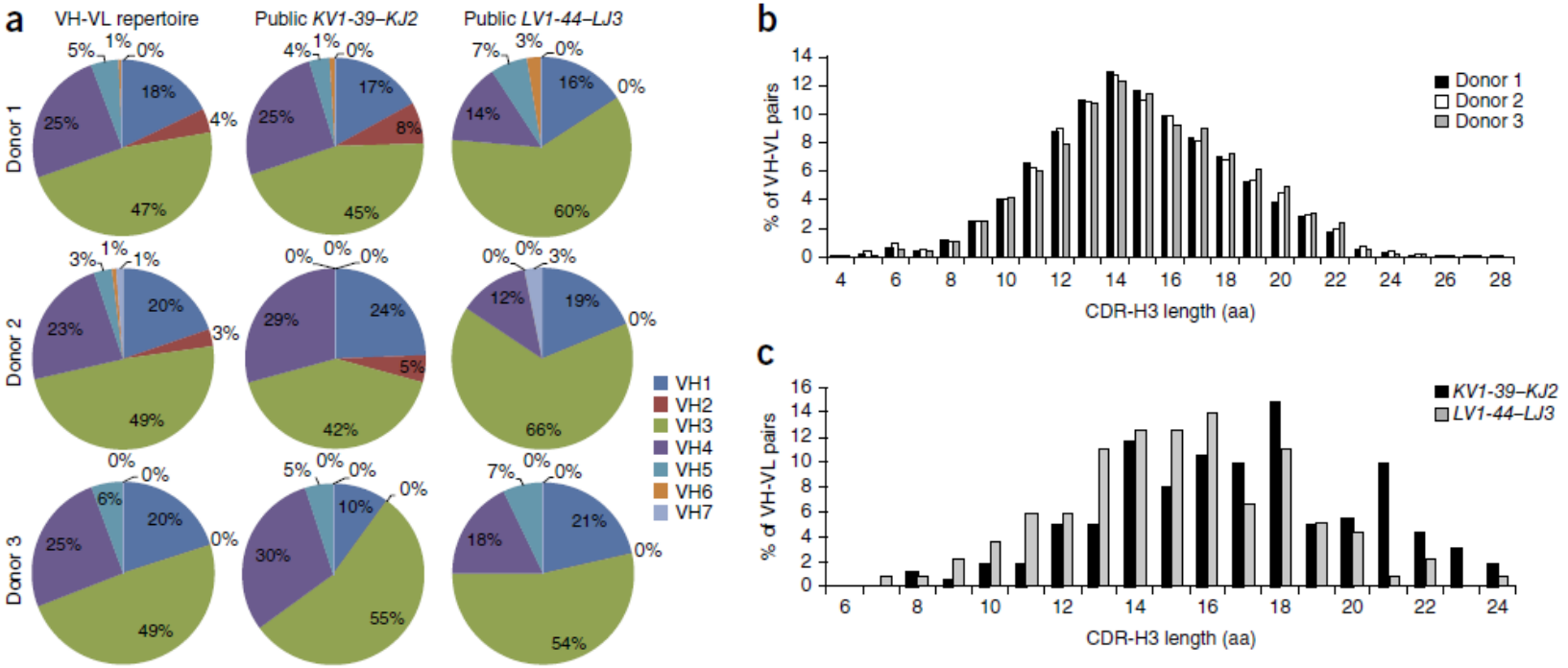
  vs. 5 (non-promiscuous)



Evaluation of Methodology

Identification and characterization of promiscuous V_L junctions

- ***KV1-39-KJ2*** (9aa CDR-L3) and ***LV1-44-LJ3*** (11aa CDR-L3) were examined in more detail (containing V and G genes with high prevalence in steady-state human immune repertoires)



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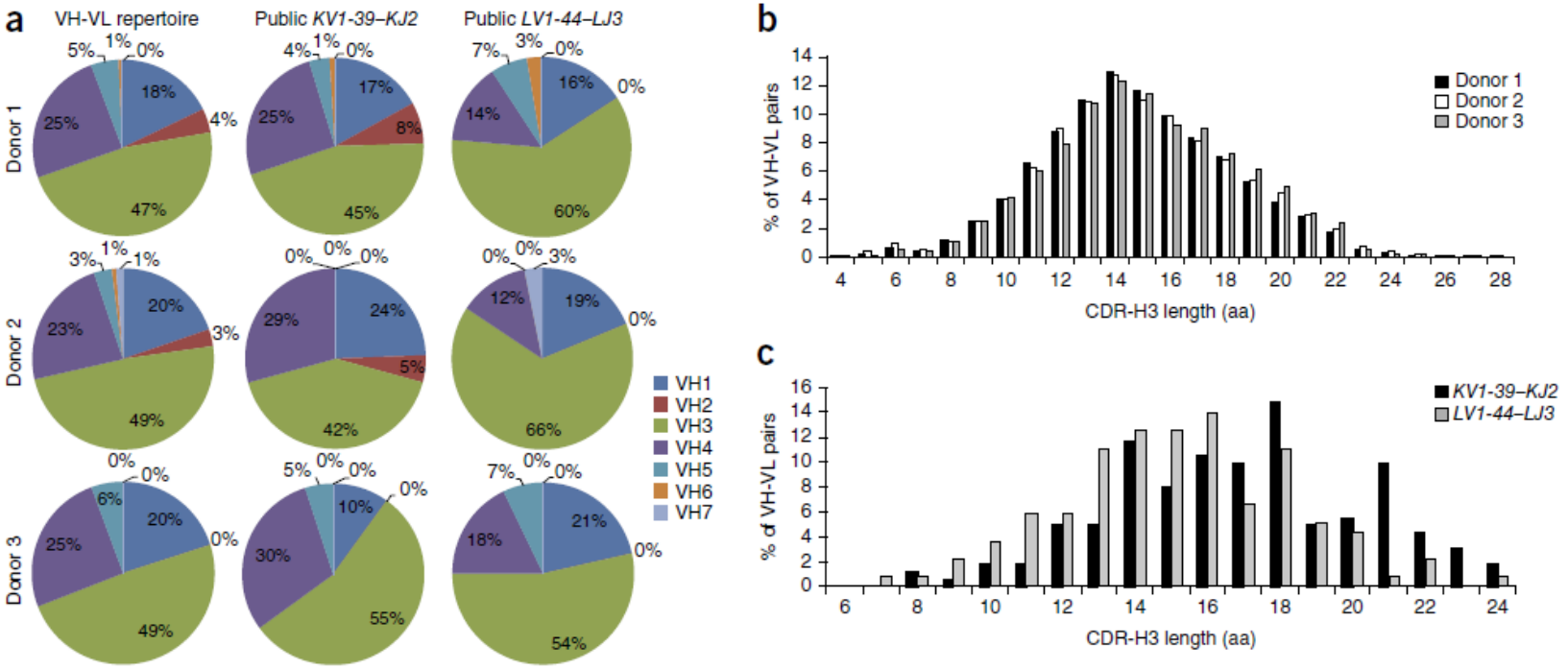

→ V_L nucleotide promiscuity due to VL recombination rather than due to B cell activation and clonal expansion

Evaluation of Methodology

Quantification of allelic inclusion

- one B cell expressing two B cell receptors
- involved in autoimmunity
- detected in 0.2-0.5% of human memory B cells (Giachino et al. 1995)

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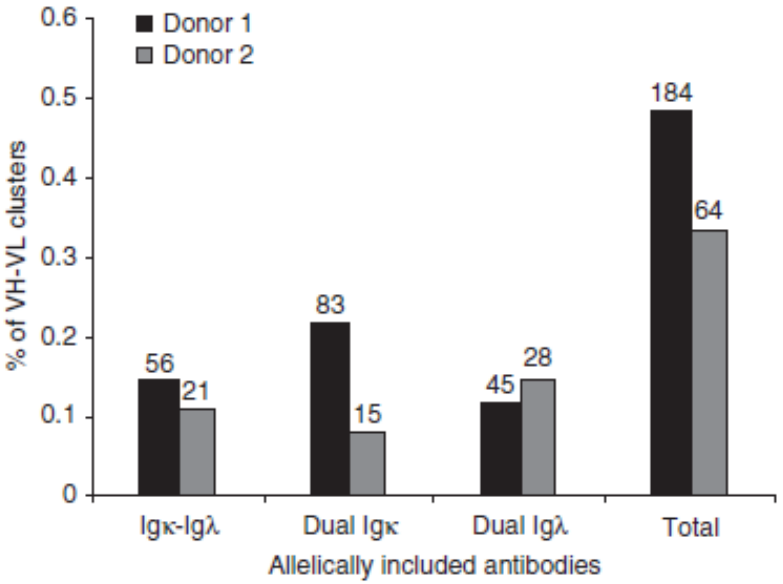

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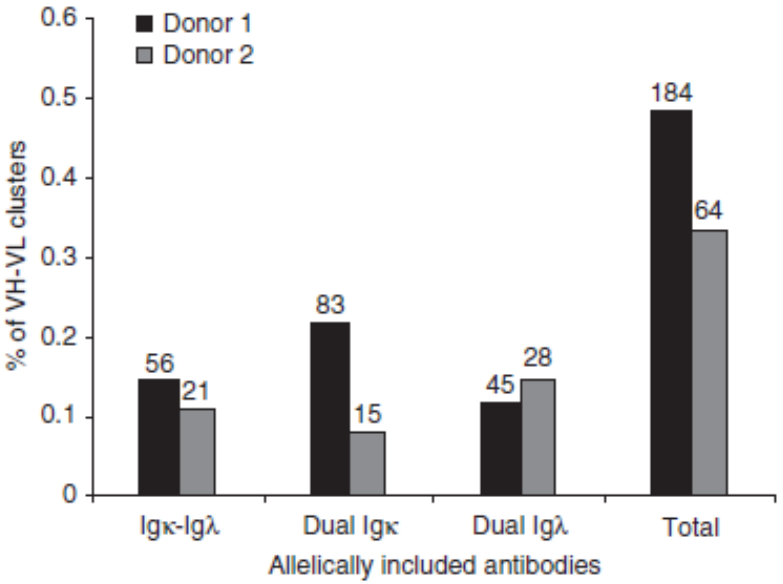
→ consistent with the study by Giachino

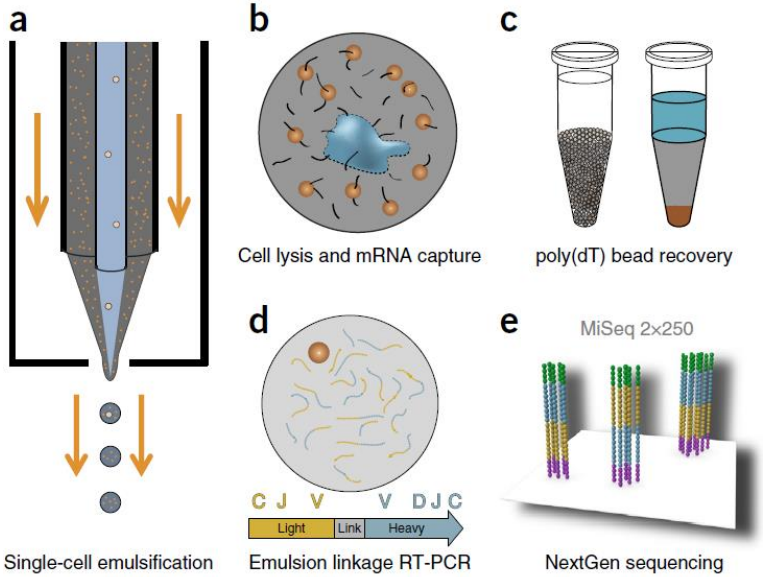
Allelic inclusion can be studied and quantified using this approach



Conclusion

High-throughput sequencing of the paired heavy and light chain repertoire

- great depth >2 x 10^6 per experiment (vs. capacity > 5 x 10^4 Paper 1)
- high accuracy of CDR-H3:CDR-L3 pairing
- fast and low costs
- can be used as tool to study allelic inclusion or vaccine efficacy



DeKosky et al. 2015

Thank you for your attention!!!