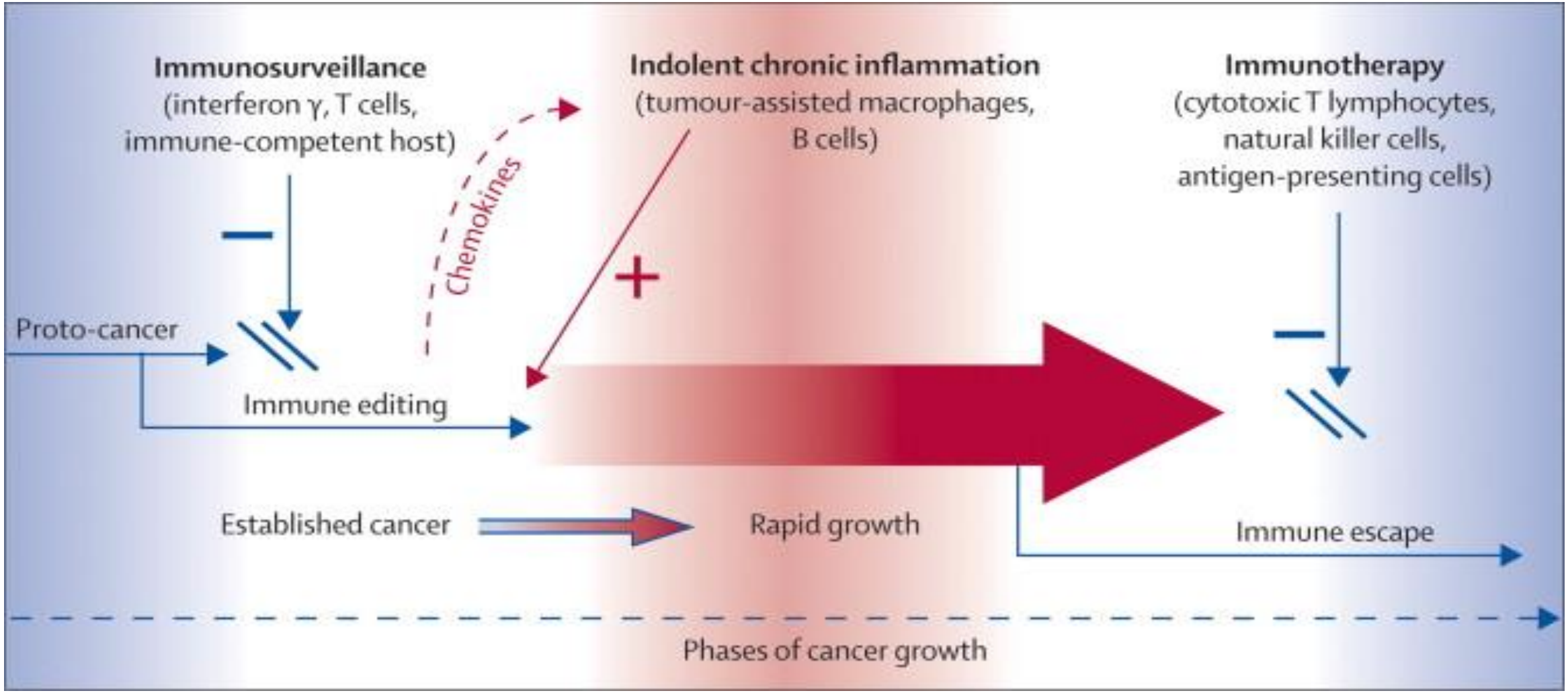
Transcriptome analysis in Immune oncology

Ena Wang

Transcriptome or Gene expression profiling

- **Gene expression profiling** is the measurement of the expression of thousands of genes at once, to create a global picture of cellular function.
- These profiles can distinguish between cells that are actively dividing, or show how the cells react to a particular treatment.
- Technologies such as RT-PCR (Fluidigm), NanoString, **cDNA microarray, RNAseq**, can measure an entire genome simultaneously in a given tissue or cell.

Cancer is a Process, not a State

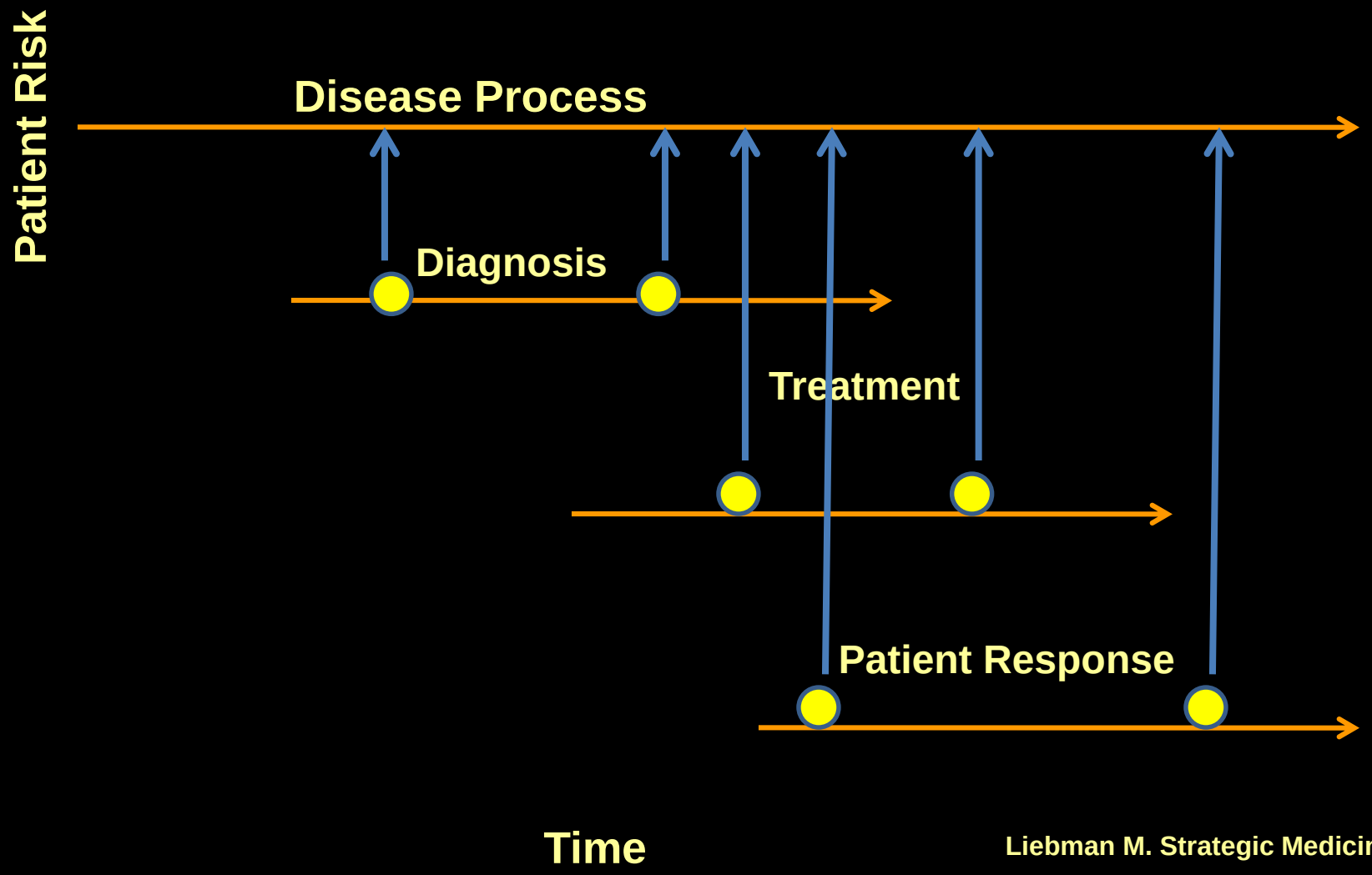


Tumour immunity: effector response to tumour and role of the microenvironment

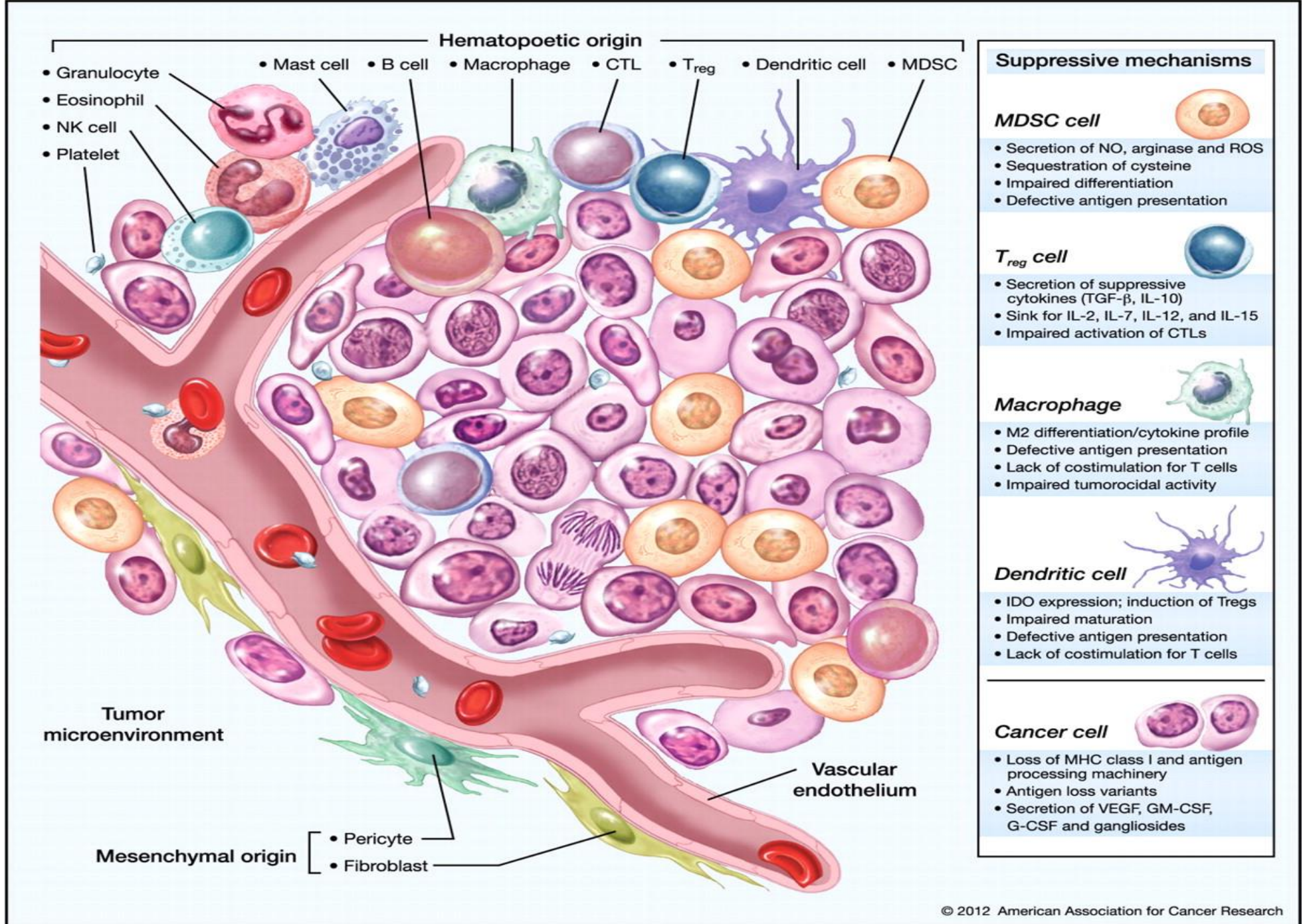
Alberto Mantovani, Pedro Romero, A Karolina Palucka, Francesco M Marincola

Lancet 2008; 371: 771–83

Disease is a Process, not a State



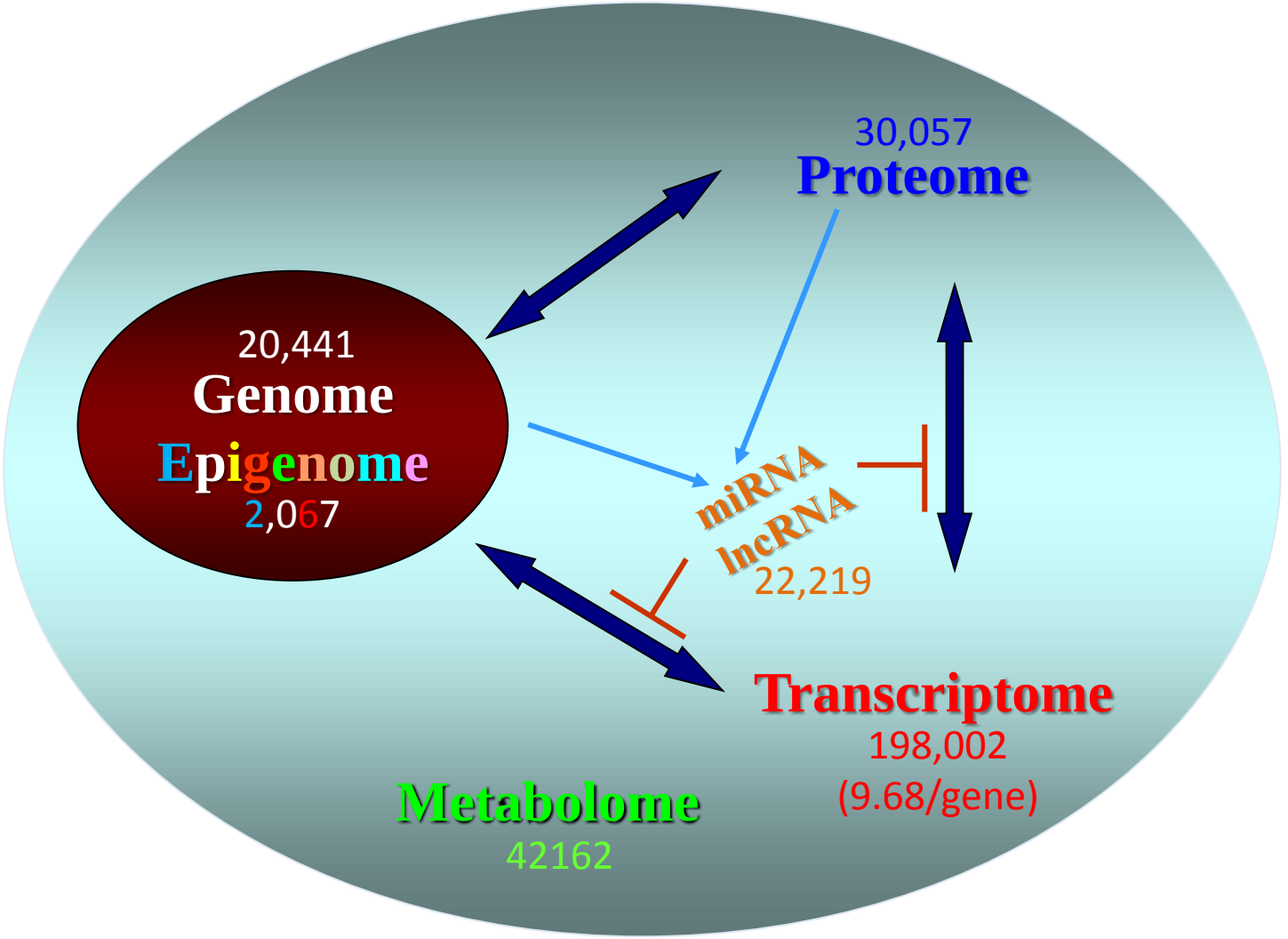
Liebman M. Strategic Medicine, 2010



The immune system is too complicated to proceed in the usual one-variable-at-a-time manner

- **Cells type**
  - Tumor cells
  - Immune cells
    - Dendritic cells
    - CD8 T cells
    - CD4 T cells
    - Regulatory T cells
  - Stromal cells
- **Cytokines and soluble factors**

- **Cell states**
  - Tumor cells
    - MHC expression
    - Neo-antigen repertoire
    - Mutation load
    - ϒ-IFN pathway status
  - CD8 T cells
    - Location
    - Maturity
    - CD25 signaling
    - CTLA signaling
    - PD1 signaling


30,057
Proteome
20,441
Genome
Epigenome
2,067
miRNA
lncRNA
22,219
Transcriptome
198,002
(9.68/gene)
Metabolome
42162

Lecture content:

- Technologies for gene expression analysis
- State of art RNAseq and Single Cell gene expression
- Sample collection and handling impact in data quality
- Utility and limitations of gene expression analysis.
- Peripheral vs tissue specific gene expression
- Bulk sample vs single cell vs immune repertoire gene expression
- Challenge in data throughput, analysis and interpretation
- Clinical applications in MOA, prediction of clinical outcome and prediction of treatment response

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History of micro array technology in gene expression analysis

1953
- Watson and Crick described double helix DNA

1975
- Grunstein and Hogness applied the process of molecular hybridization to DNA

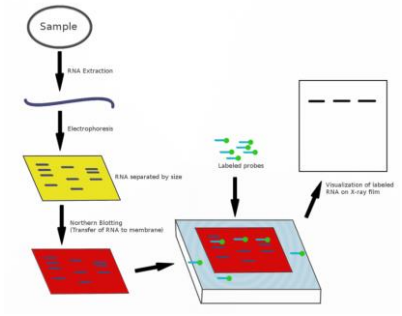
1979
- Gergan et al. adapted this methodology to produce arrays in 1979.

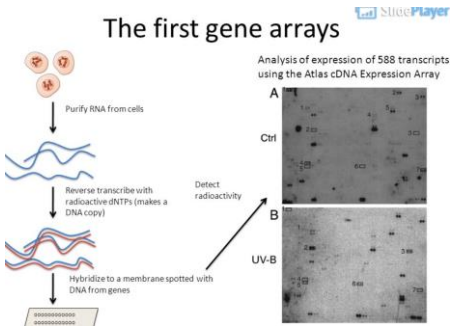
1980-1990
- Filter array

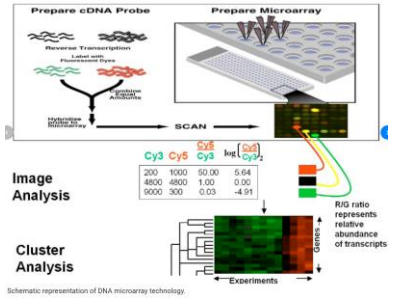
1995
- cDNA array on glass

1997
- Stanford first whole genome array of yeast in publication

1998
- Commercial arrays on Market
  - cDNA
  - Oligo
  - gDNA
  - miRNA

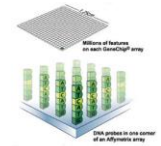




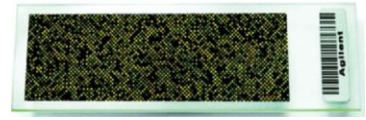


Gene expression array --- affymetrix

The Affymetrix platform is one of the most widely used.

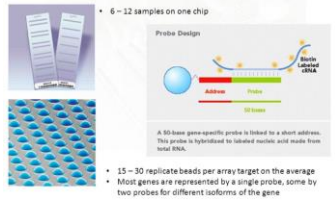


http://www.affymetrix.com/



Illumina Expression BeadChips

Probes are bound to magnetic beads randomly distributed across arrays



The fundamental technology of hybridization

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Gene expression profiling can:

- Identify predictors (pre-treatment samples) of immune or treatment induced responsiveness
- Uncover MOAs of therapeutics (on treatment samples)
- Understand and predict mechanism(s) of adverse events
- Understand the cause of recurrence and the underlying mechanisms adopted by cancer cells to escape from immune recognition (post treatment biopsies)

Next generation sequencing

(NGS)

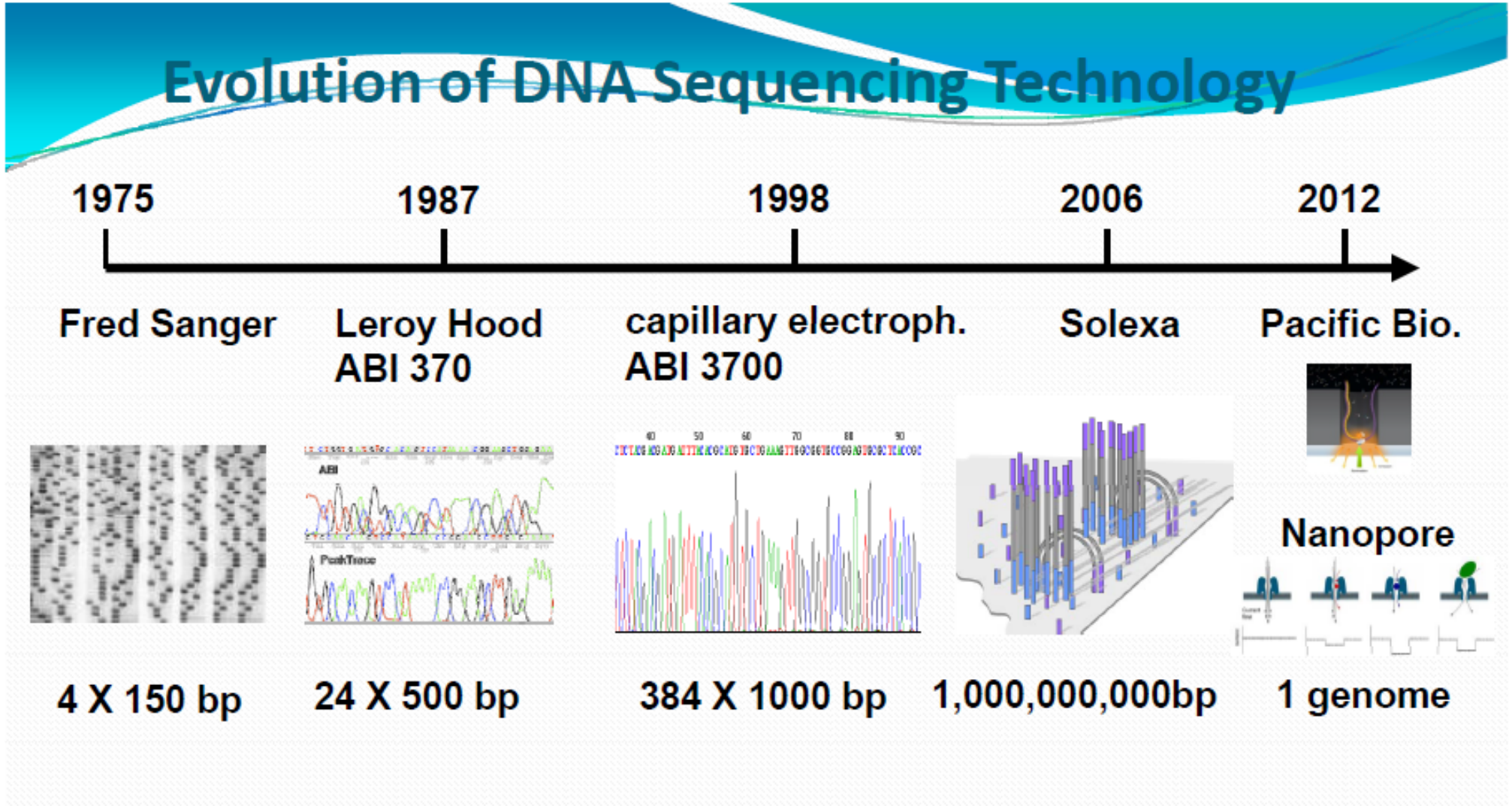
NextGen

Deepseq

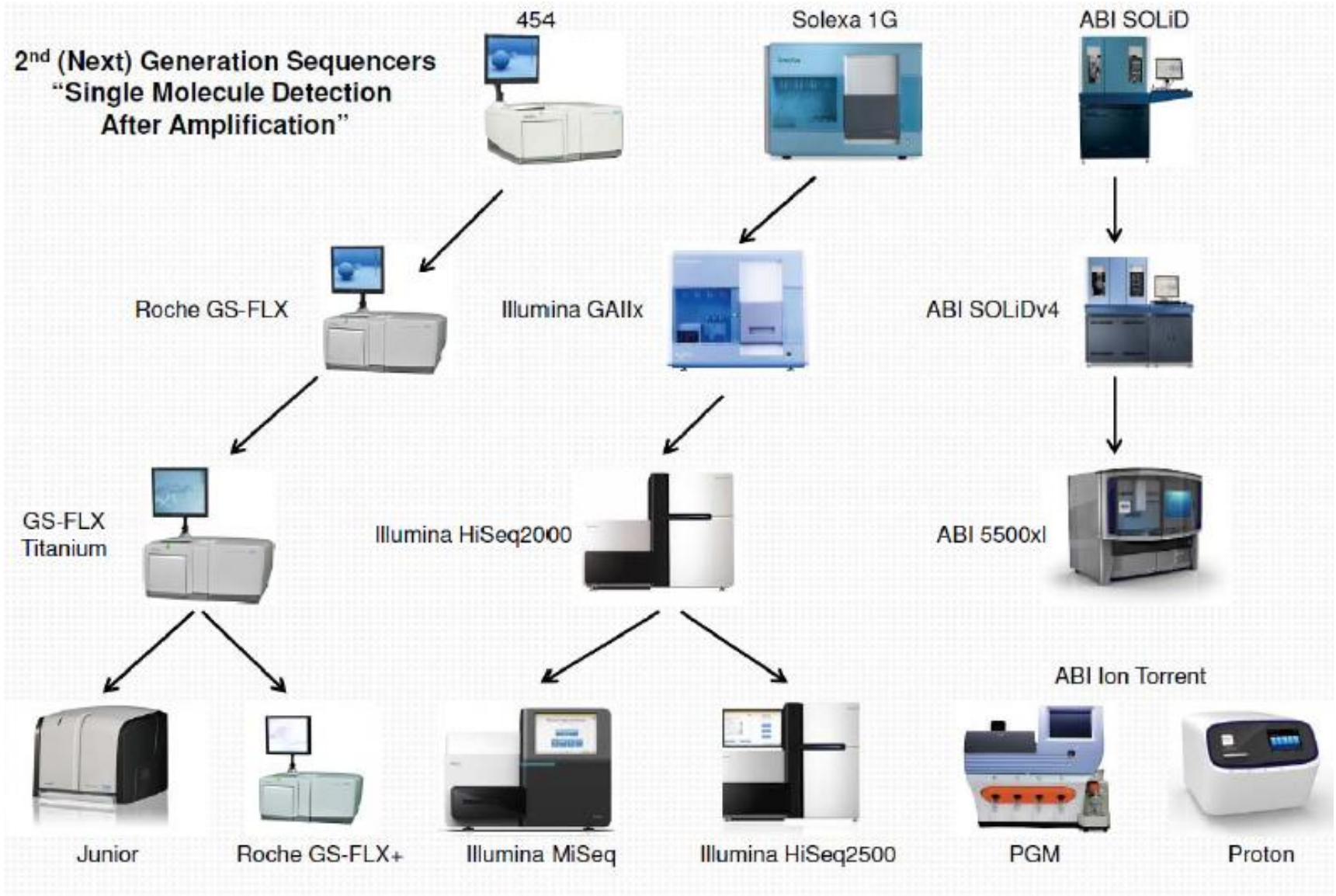
cDNA Array vs RNAseq

cDNA micro array	RNAseq
Profiling mRNA and some ESTs and miRs	Survey the entire Transcriptome, including novel un-annotated regions and non protein coding RNA (miRNA, snRNA, snoRNA, piRNA)
Profiling the prob region only	Can determine gene structure by mapping read density across transcript or gene
Probe are transcript specific not including splicing junction in design	Can use reads to map splice junctions to confirm isoform variants
Do not detect SNP variants	Can determine variation at nucleotide level and therefore can detect SNP and indels
Could not differential allelic or direction of transcription	Allele specific seq and directional seq
Cost low (reagent and chips)	cost relative high
instrument cost low	Insturment cost high
Data analysis relative traight forward	Need bioinformatics support
Data storage no challenge	much higher throughput
Anotation easy	Anotation challenge when explore beyound expression
Can detect low anoundance transcripts	When reads low, lower abundant transcripts could be missed or filtered out.

History of sequencing technology for gene expression analysis



Evolution of NGS platforms



PacBio

Nanopore

Sequencer comparison

Platforms	Primary advantage	Primary disvantage
Illumina MiniSeq	Low instrument cost; should be simple to use; much lower cost data than MiSeq Nano & Micro kits	Limited to PE150; High output PE150 kits are relatively expensive; newness creates unknowns
Illumina MiSeq	Moderate cost instrument and runs; Low cost per Mb for a small platform; Fastest Illumina run times and longest Illumina read lengths	Relatively few reads and Higher cost per Mb compared to NextSEq or HiSeq
Illumina NextSeq 500	Easy to use; Moderate instrument and run costs; Flow cells with room to grow	Version 2 of new chemistry still not as good as older chemistry (used on MiSeq & HiSeq)
Illumina HiSeq 2500	Low cost per MB of data; can run high output (8 lane) and rapid run (2 lane) flow cells with several possible read-length configurations	High instrument cost; high cost per run; requires highly trained personnel; ~20% downtime; can't run one rapid run and one high output flow cell at the same time
Illumina HiSeq 4000	Patterned flow cells; low cost per read & per MB	Similar to HiSeq 2500, but even more expensive; not as flexible as the HiSeq 2500
Illumina HiSeq X Five (or Ten)	Current lowest cost per read or per MB	Requires ≥$6M plus more data storage than imaginable; only authorized for ≥30x genome resequencing
Illumina NovaSeq	Lowest cost and highest throuput	Need large volume of sample per run. More for service center use
Ion Torrent – PGM	Low cost instrument upgraded through disposable chips (the chip is the machine); very simple machine with few moving parts; three chips available with varying numbers of reads	More hands-on time and fewer reads at higher cost per Mb relative to MiSeq; smaller user community; libraries made for 400 base reads don't work well on Proton
Ion Torrent – Proton	Moderate cost instrument for medium throughput applications; similar cost to NextSeq, but PII to actually deliver similar reads as NextSeq is years overdue	More hands on time, and fewer overall bases of data than Illumina; Higher cost per MB of data than most Illumina instruments; smaller user community
Oxford Nanopore minION	SMALL PORTABLE Instrument; It is a USB device; extremely low-cost instrument; extremely long reads feasible (multiple kb)	Weird quasi-commercial release; error-rates only now approaching what was promised in 2012; biased errors; High cost per read
Oxford Nanopore PromethION * (forecast)	Should be similar to an array of MinIONs to increase throughput & decrease costs	Similar to or same as MinION; Not yet available
PacBio – RS II	Single molecule real-time sequencing; Longest available read length among well established platforms; Ability to detect base modifications; Short instrument run time; Random error profile; Modest cost per sample; Excellent for full genome assembly with sufficient coverage	High error rates so high coverage is necessary; Low total number of reads per run; High cost per Mb; High capital cost; Many methods still in development; past company performance not very good (though markedly improved recently)
PacBio – Sequel	Lower cost for instrument, data & service contract relative to RS II; otherwise should be similar to RSII	New, thus limited info from non-company sources; High cost per Mb and per read relative to other platforms; should be similar to RS II

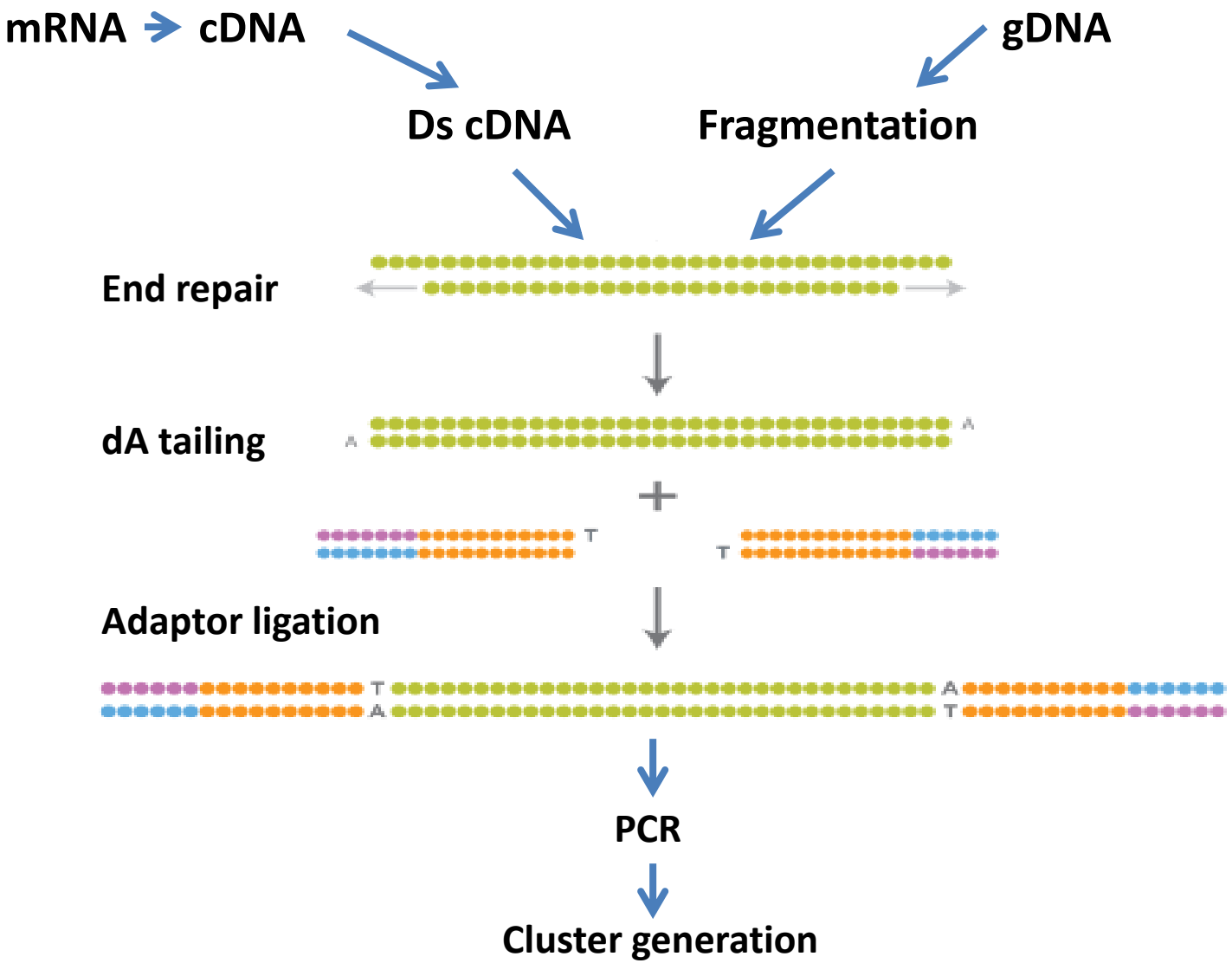
Illumina-GAII, Hiseq, Miseq, NextSeq, NovaSeq

library+cluster generation

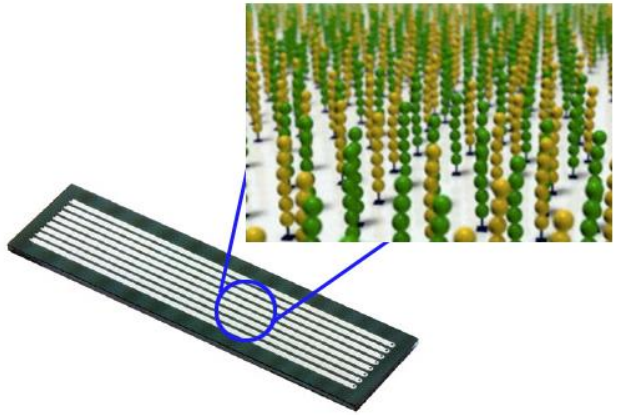
CRT (cyclic reversible termination)

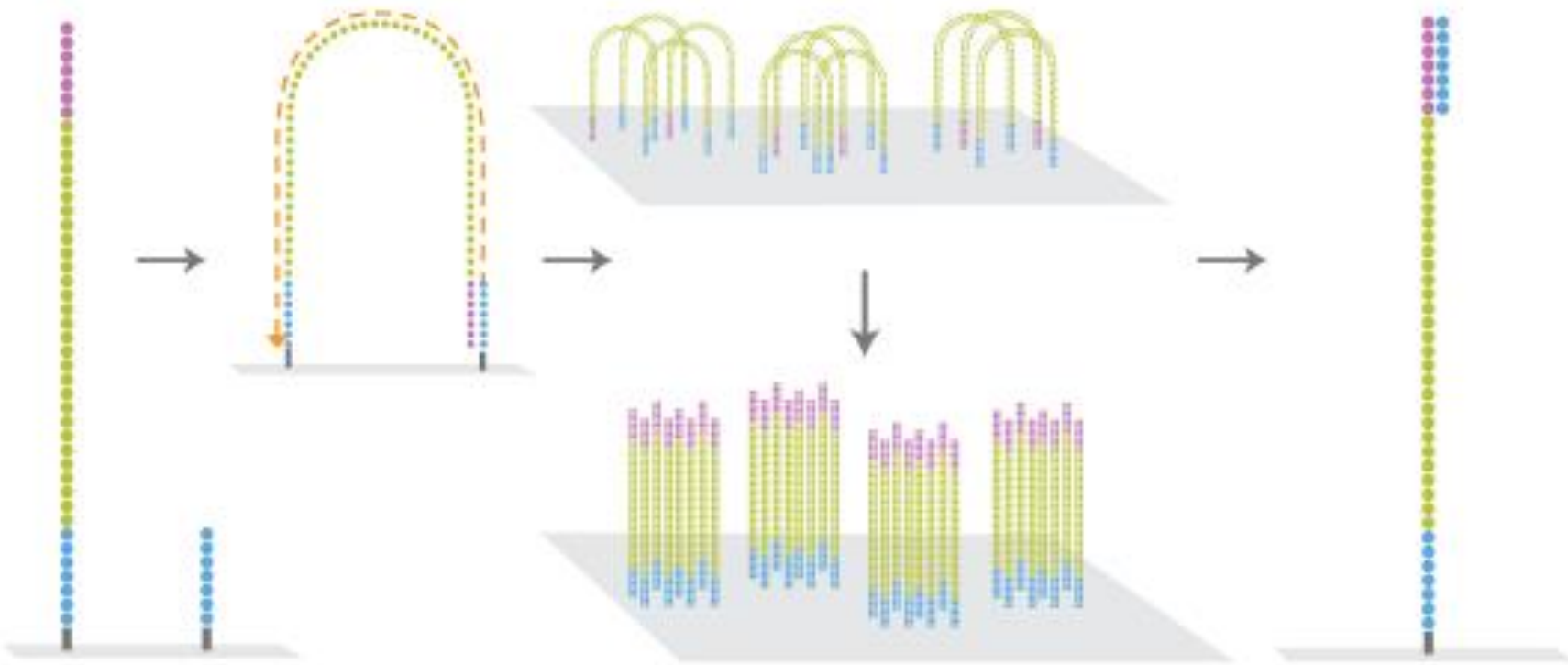
Image capture

Library preparation

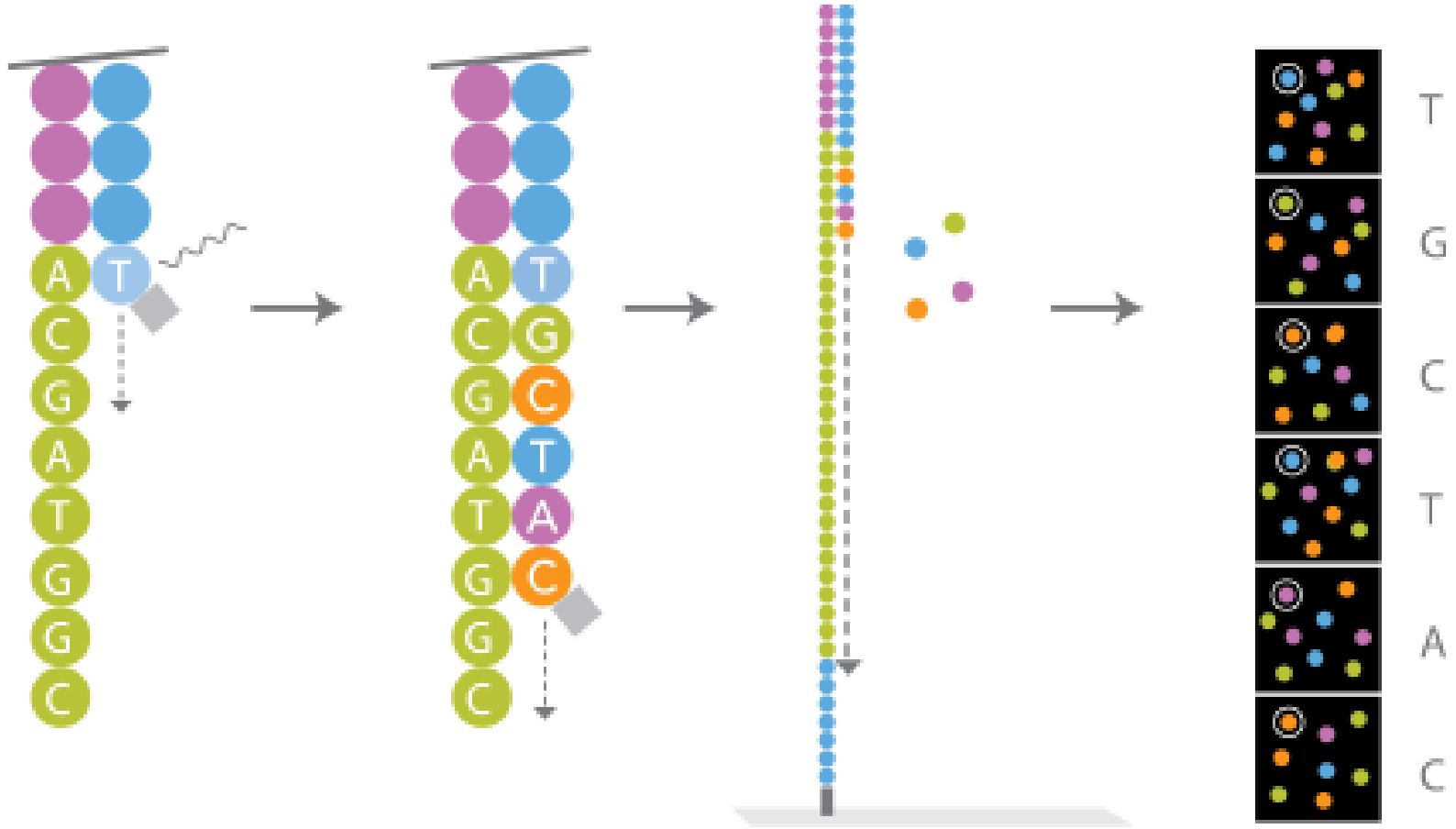


Cluster generation





Cyclic reversible terminator sequencing and image capture



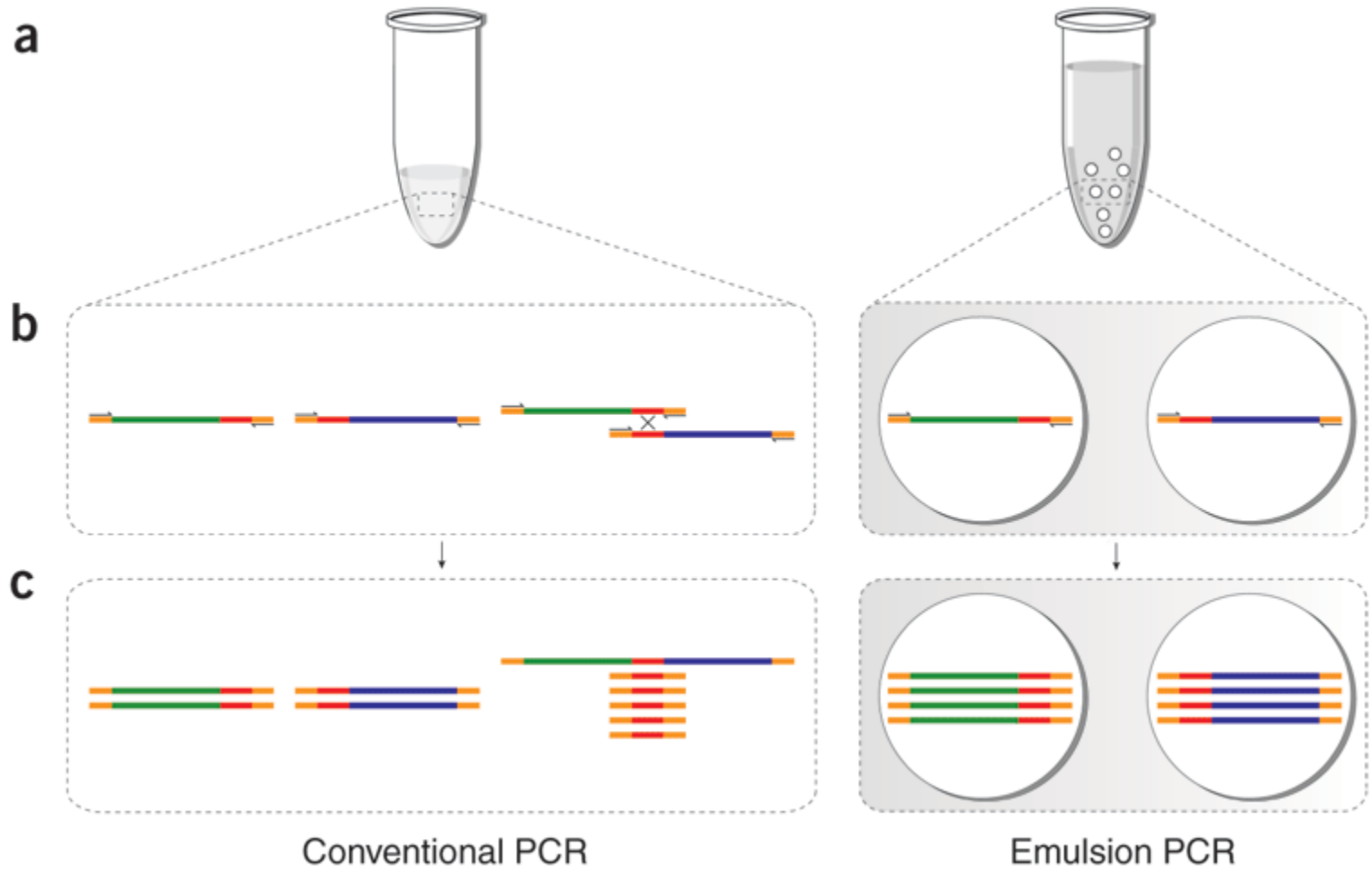
ABI-Ion Torrent, Ion Proton

ePCR

Pyrosequencing

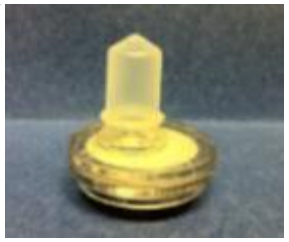
Semiconductor

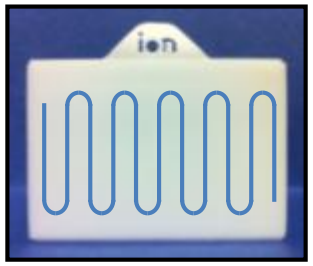
Library generation by Emulsion PCR

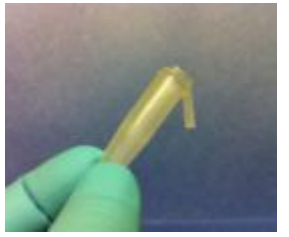


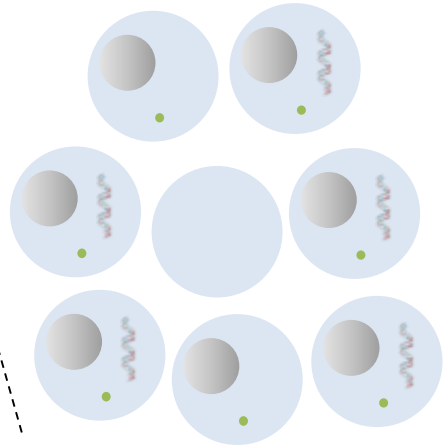
R Williams, et al. Nature methods

Revolutionary In-Line PCR Technology



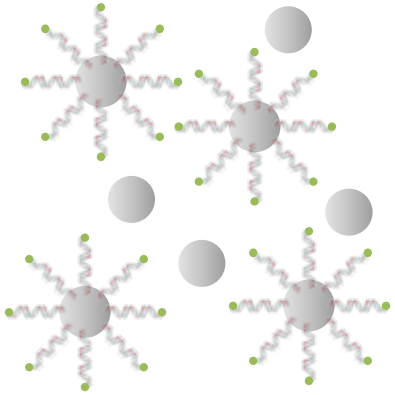






Denature

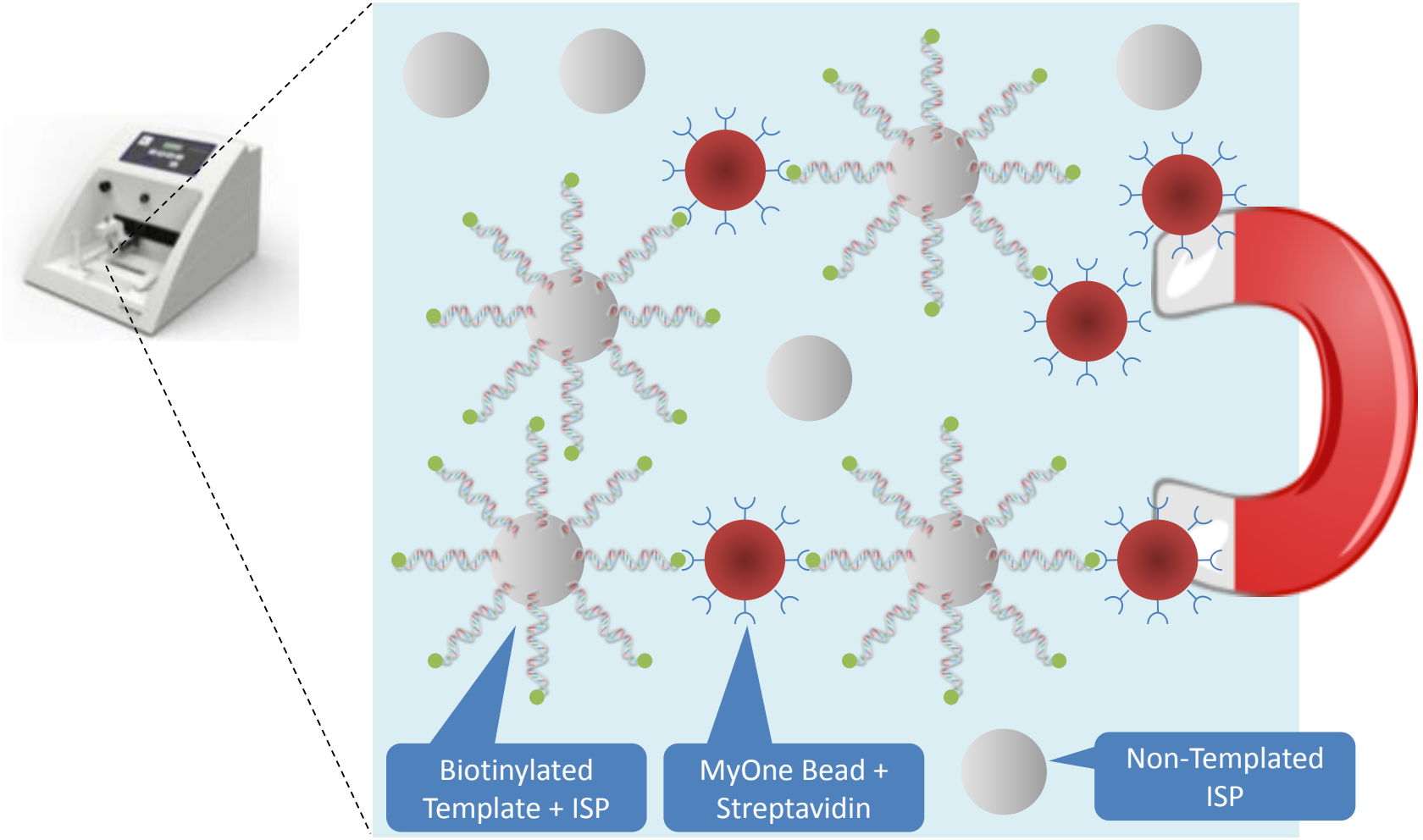
Prime and Extend



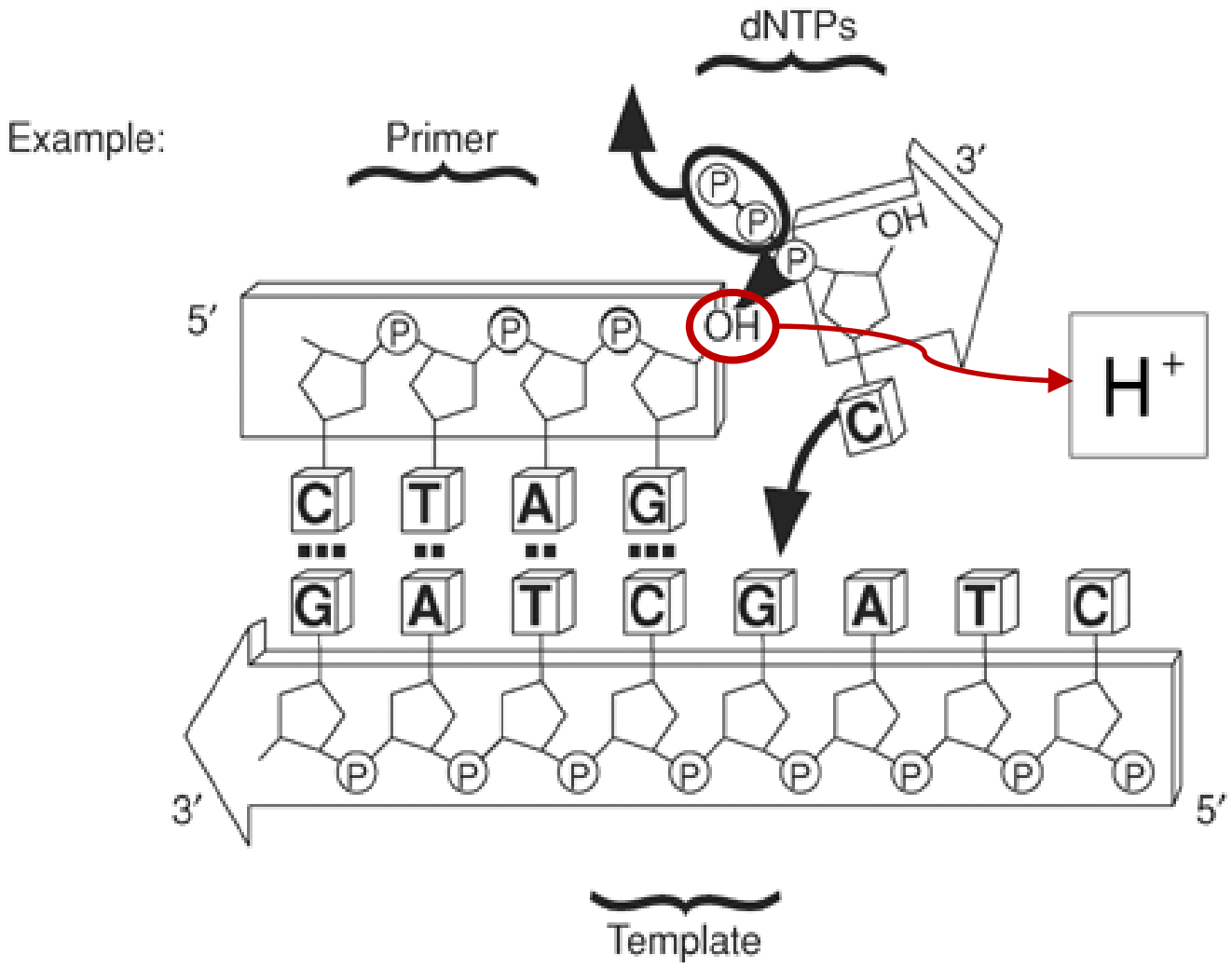
Ion Sphere Particles with 5’ primer
Template
Biotinilate 3’ primer

PCR cycle

Automated template Enrichment

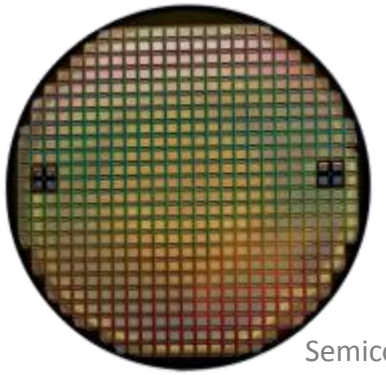


Same chemistry but no enzymes involved in signal generation



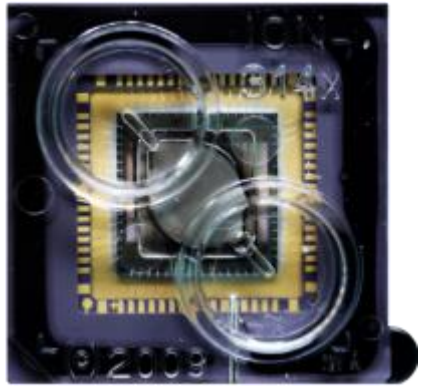
Transformative Technology, Unprecedented Progress

Scalable Semiconductor Technology plus Simple Chemistry



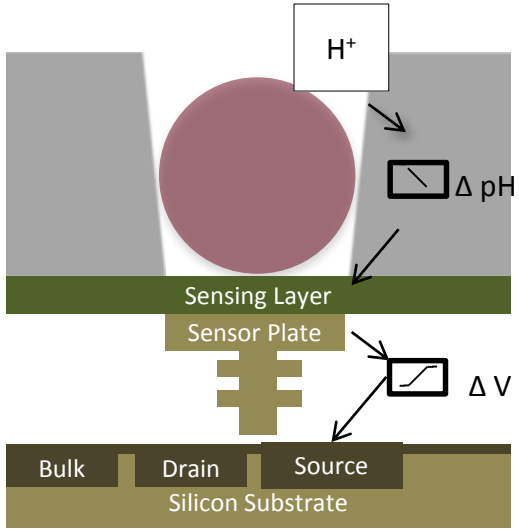
Wafer

Semiconductor Manufacturing



Chip

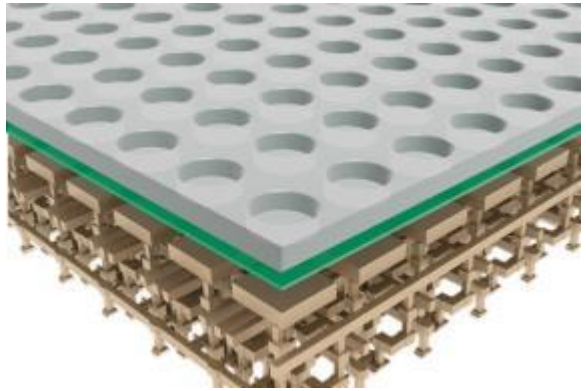
Semiconductor Packaging



TCGTACC…

Single Sensor

Chemical to Digital Sequence

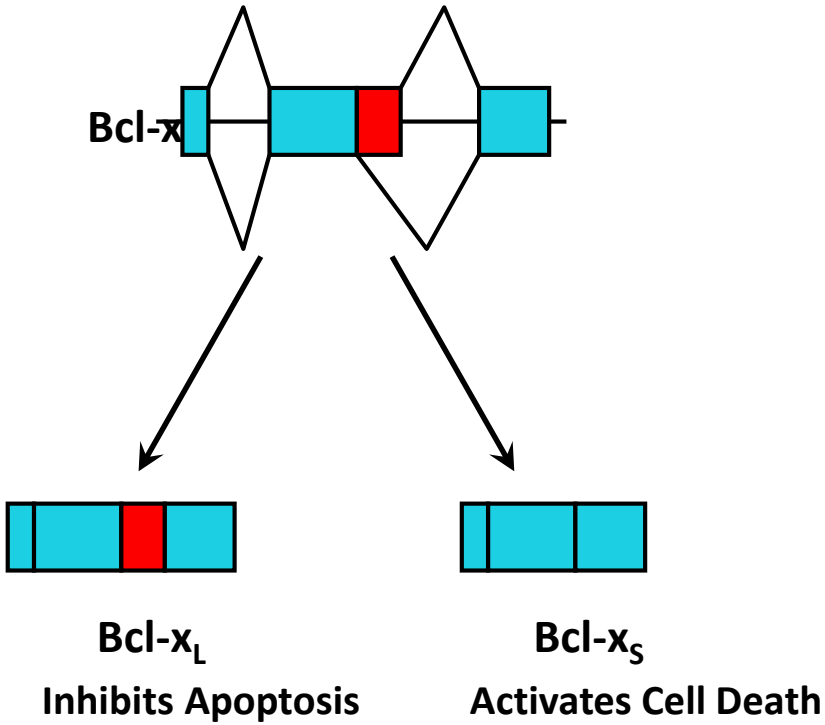


Millions of Sensors

Semiconductor Design

Importance of alternative splicing

- **Approximately 25,000 protein coding genes in the human genome, but there are >100,000s of transcripts**
- **~75% of human genes are thought to be involved in alternative splicing**
- **ENCyclopedia Of DNA Elements (ENCODE) regions contain an average of 5.4 alternative transcripts/locus**
- **20 – 30% of disease causing mutations are believed to affect pre-mRNA splicing**



Deregulation of apoptosis contributes to the development and progression of Cancer

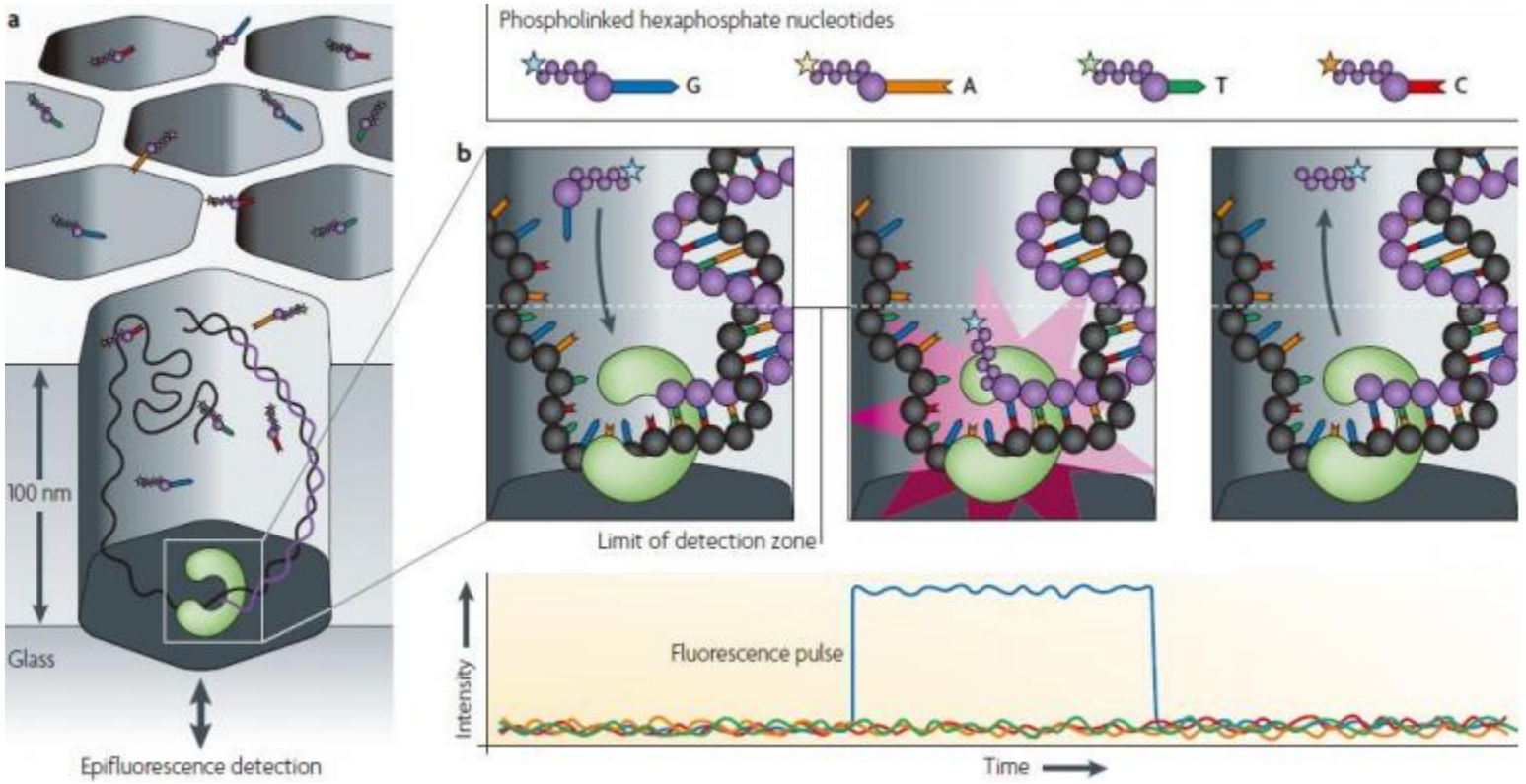
Disease modification and susceptibility mediated through splicing events

Splice event linked with disease modification	
•CFTR	Cystic fibrosis
•MCAD	Medium-chain acyl-CoA dehydrogenase deficiency
•SCN1A	Susceptibility to anti-epileptics
•IKBKAP	Familial dysautonomia
•Scn8a	Mouse Neurological disorder

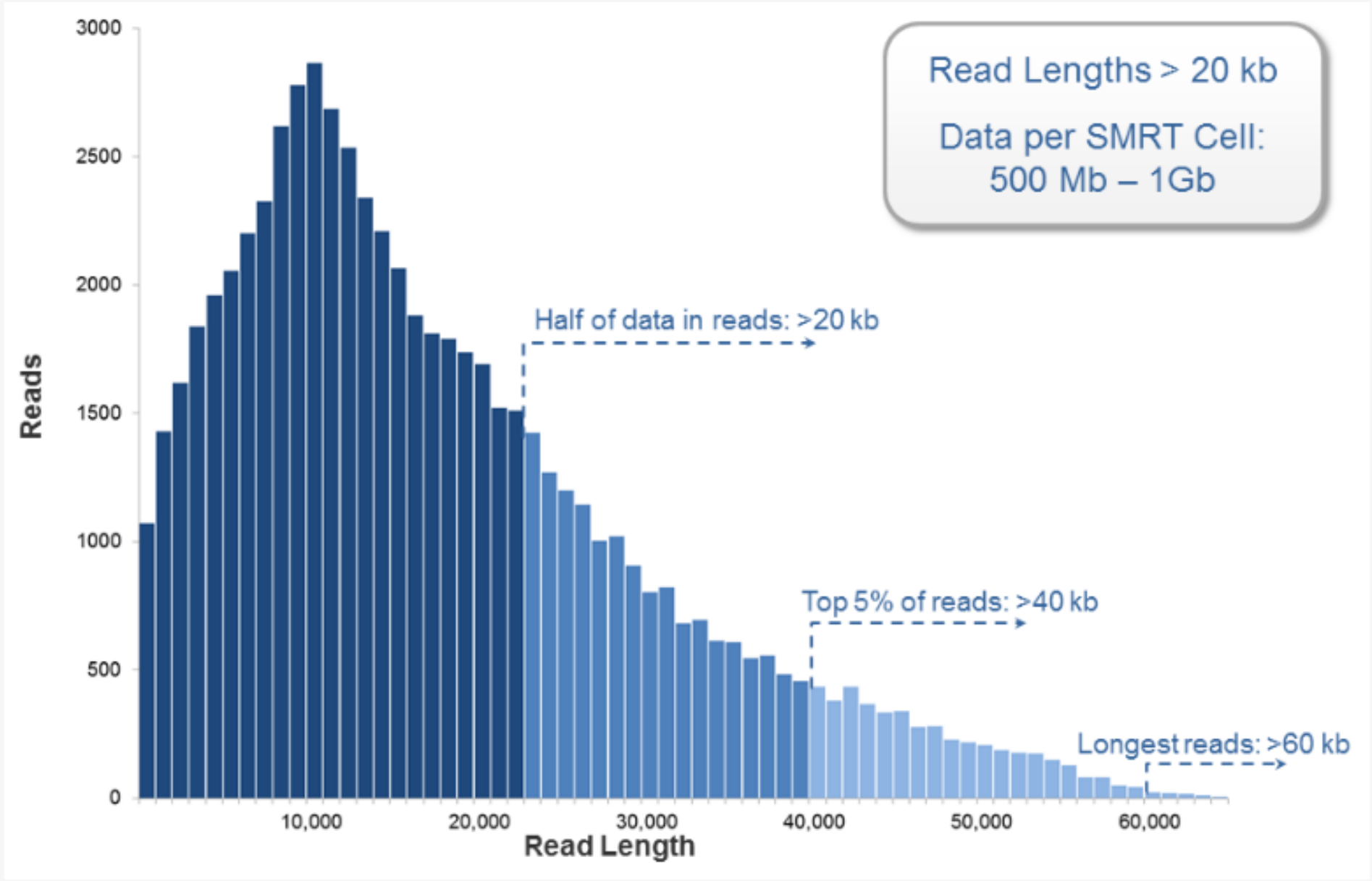
Splice event linked with disease susceptibility	
•IRF5	Systemic lupus erythematosus
•CTLA4	Autoimmune diseases
•NCAM1	Bipolar disorder
•ERBB4	Schizophrenia
•OLR1	Myocardial infarction
•OAS1	Type I diabetes
•TNNT2	Cardiac hypertrophy
•GPRA	Asthma
•MAPT	Tauopathies
•PTPRC	Altered immune function & Multiple sclerosis
•LDLR	Elevated cholesterol
•SFRS8	Asthma

Wang and Cooper, Nature Review Genetics (2007)

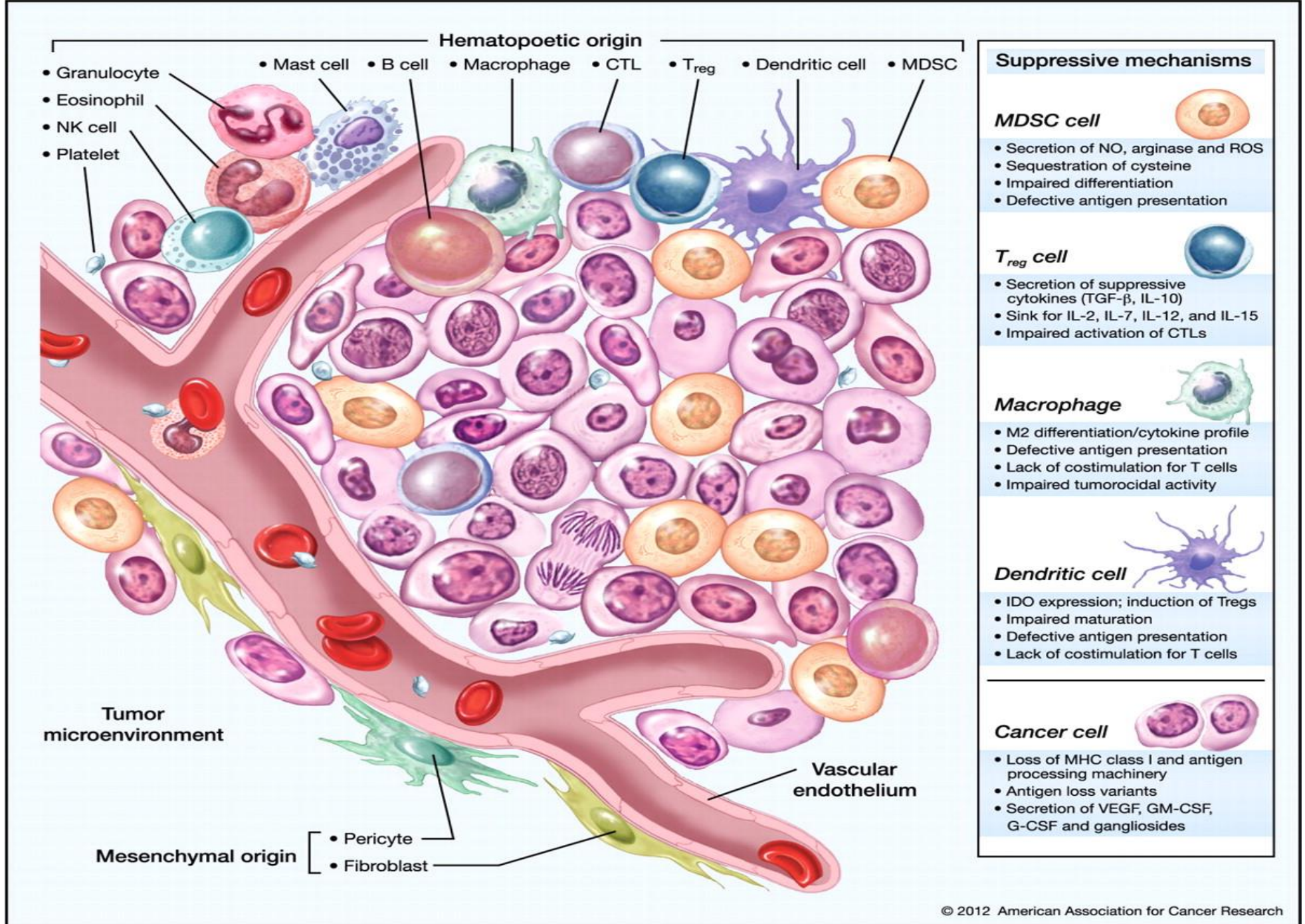
Third Generation Sequencers



10bp / second
Directly sequence RNA without the need to RT to cDNA
Methylation of the DNA sequence can be determined due to the delayed signal detection



Based on data from a 20 kb size-selected human library using 4-hour movies. Average read lengths for data set shown are 18 kb. Each SMRT Cell generates ~55,000 reads.



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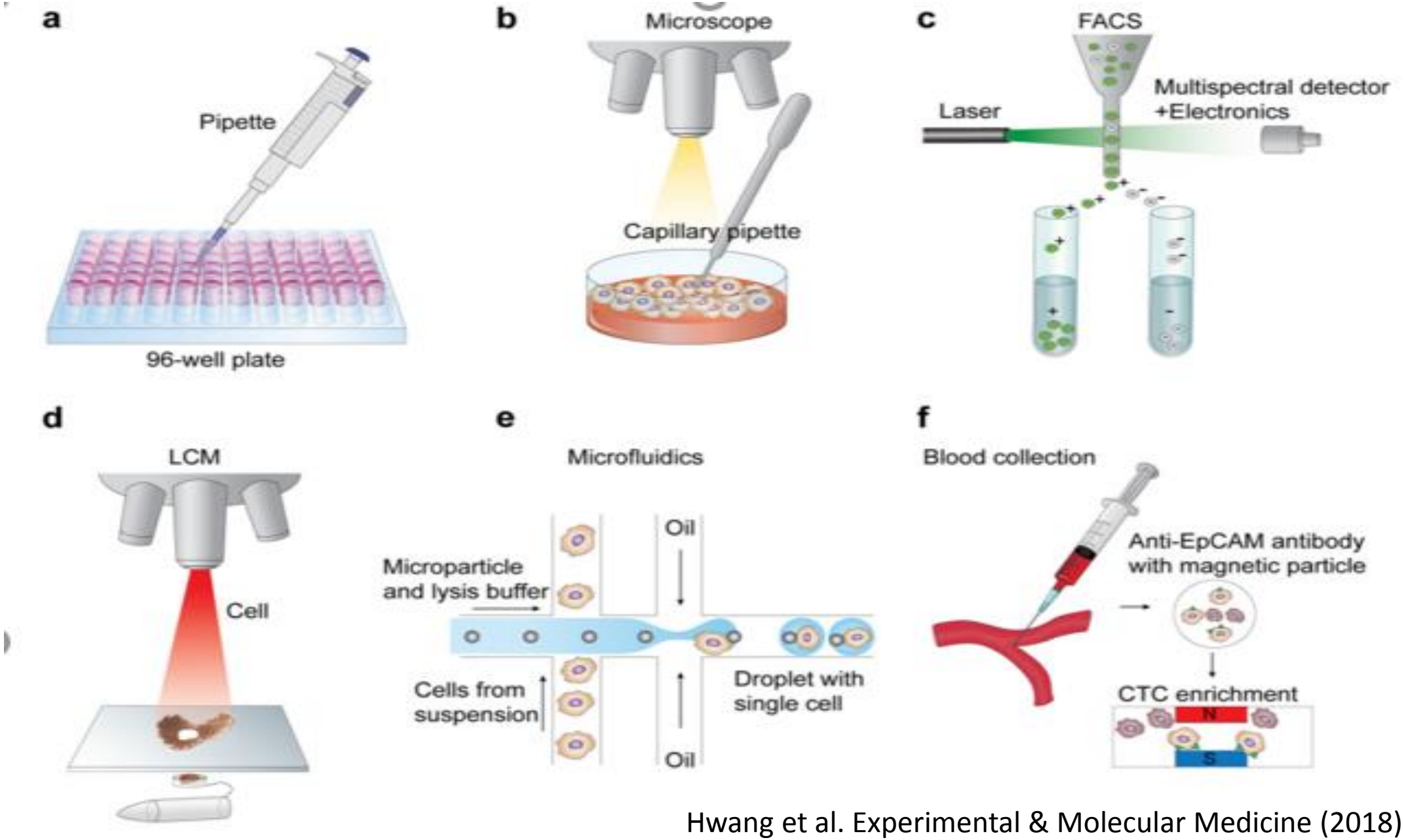
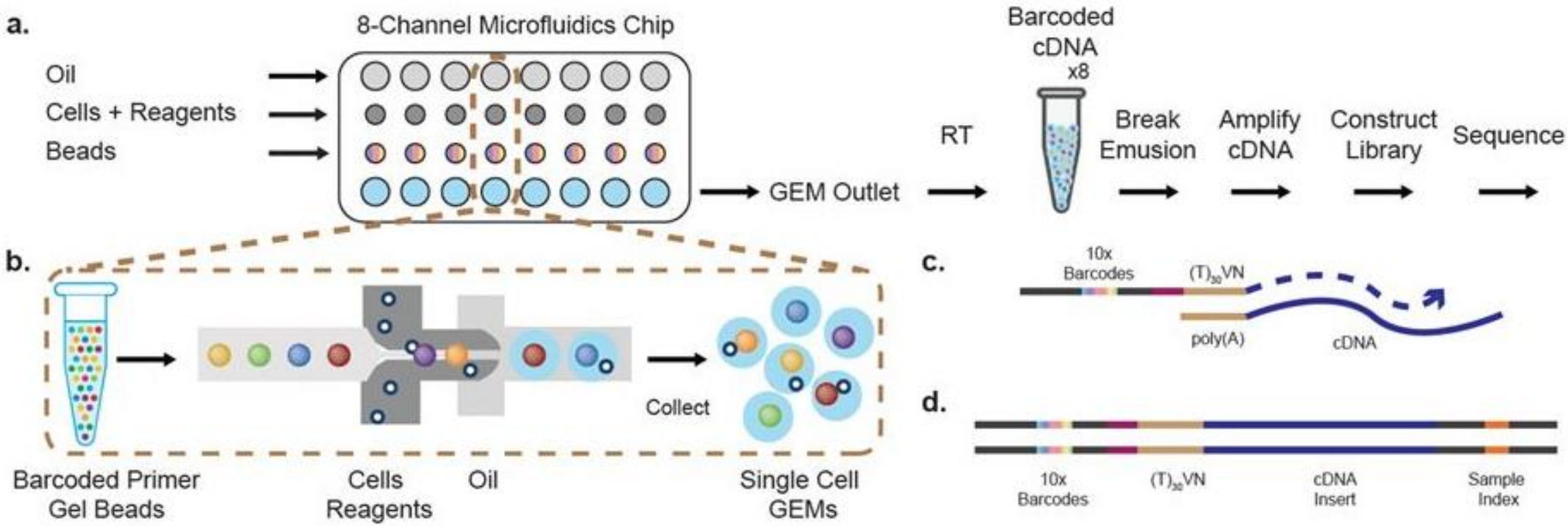
Single-cell isolation techniques



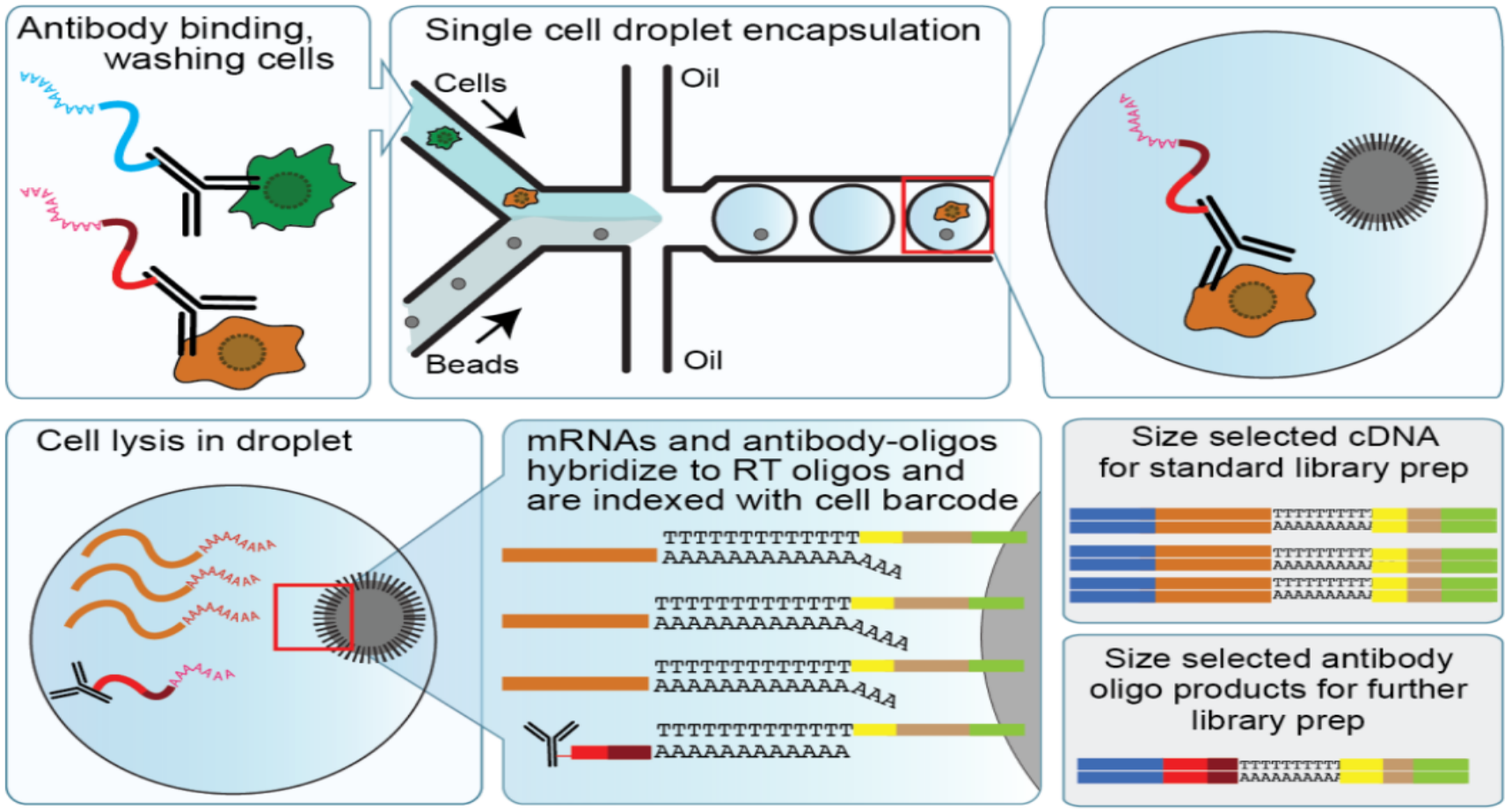
Hwang et al. Experimental & Molecular Medicine (2018) 50:96

Single cell RNAseq – 10X genomics



PCR HANDLE ANTIBODY BARCODE AAAAAAAAAAAAAAAAAAAAA

CITE-seq uses DNA-barcoded antibodies to convert detection of proteins into a quantitative, sequenceable readout. Antibody-bound oligos act as synthetic transcripts that are captured during most large-scale oligodT-based scRNA-seq library preparation protocols (e.g. 10x Genomics, Drop-seq, ddSeq).



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Clinical samples

- Collections relevant to disease process
- Collections at relevant time points
- Proper controls
- Sufficient number of samples
- Storage and handling
- High quality and sufficient quantity

Schematic of clinical studies

- Biopsy for disease characterization and marker screen
- Baseline sample collection for prediction
- Sample for MOA
  - Pre Tx
  - Day1
  - Day 4
  - Day7
  - Day 14
  - Mon 1
- Corelative analysis with Clinical endpoints
- Efficacy prediction model
- AE prediction
- Correlative analysis with survival
- Survival prediction

Sample collection for gene expression analysis

- Tempus or PaxGene Blood RNA Tube
- CPT tube for PBMC isolation and cryopreservation
- RNAlater RNA stabilization for tissues biopsy
- Fresh tumor digests

- OCT embedded samples
- FFPE

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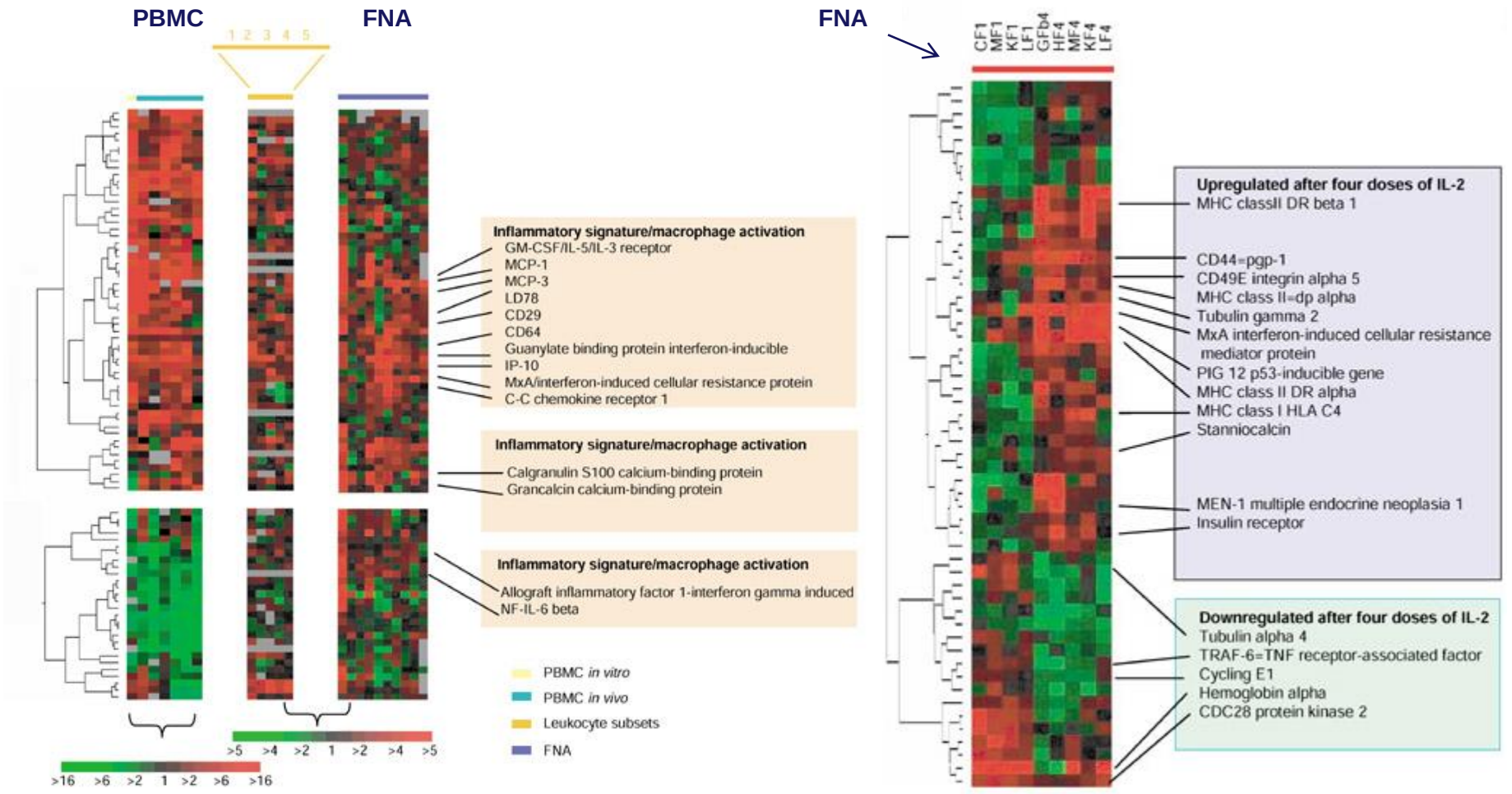
Research

Gene-expression profiling of the response of peripheral blood mononuclear cells and melanoma metastases to systemic IL-2 administration

Published: 25 June 2002
Genome Biology 2002, 3(7):research0035.1–0035.17

Monica C Panelli*, Ena Wang*, Giao Phan†, Markus Puhlmann†, Lance Miller†, Galen A Ohnmacht†, Harvey G Klein* and Francesco M Marincola*



Lecture content:

- Technologies for gene expression analysis
- State of art RNAseq and Single Cell gene expression
- Sample collection and handling impact in data quality
- Utility and limitations of gene expression analysis.
- Peripheral vs tissue specific gene expression
- Bulk sample vs single cell vs immune repertoire gene expression
- **Challenge in data throughput, analysis and interpretation**
- Clinical applications in MOA, prediction of clinical outcome and prediction of treatment response

Where to star?

Hurdles – Choice of analytical software

- Valvet
- BFAST
- BLAT
- BWT
- Bowtie
- Tophat
- CLC Bio
- ELAND (CASAVA)
- GMAP
- MAQ
- NOVAALIGN
- SOAP….

NGS aligner tools

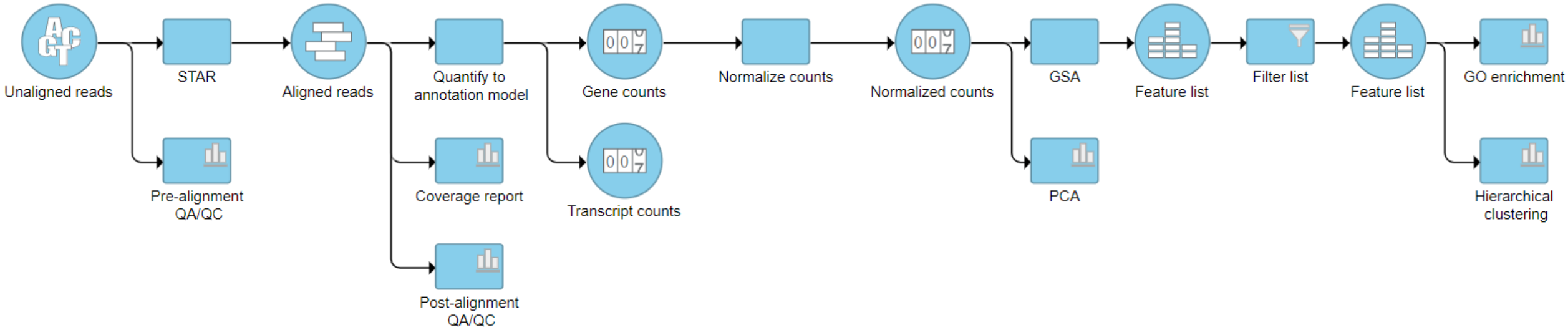
No one has all the needed features!

BaseSpace-Illumina Miseq, Hiseq
Genome Modeling tools-http://genomie.
Wustl.edu

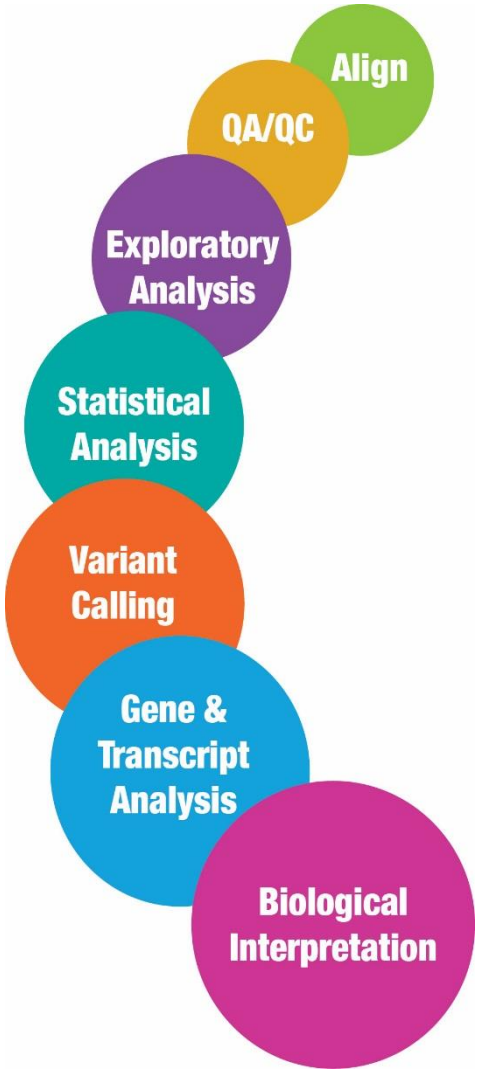
Data analysis and visualization tools

- **Partek Genomic Suite, Partek Flow**

Point and Click to Build Analysis Pipelines



Gene expression analysis



Data input

Data QC

Mapping to reference

Data conversion

Data normalization

Data analysis

Unsupervised

- PCA or T-SNE

Supervised statistic analysis

- Differential expression
- Signature gene selection

Fusion genes

Splicing transcripts

SNP/mutation

Functional analysis

Pathway analysis

GSEA analysis

Cross validation using MsigDB

Clinical outcome/response analysis

Survival analysis

Validation

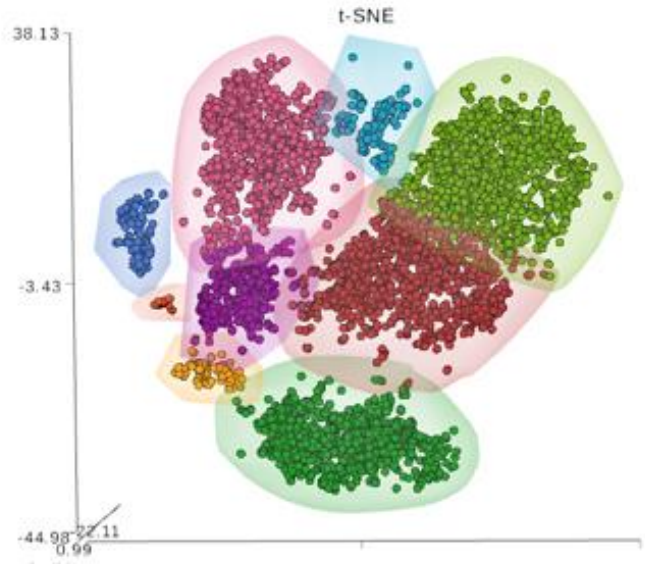
Technique validation

Biological validation

Animal model

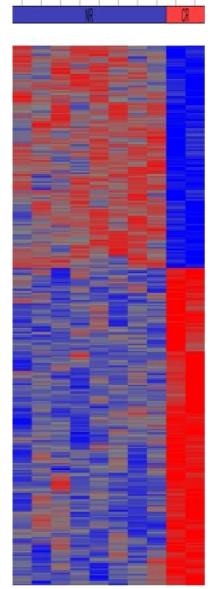
Independent clinical study

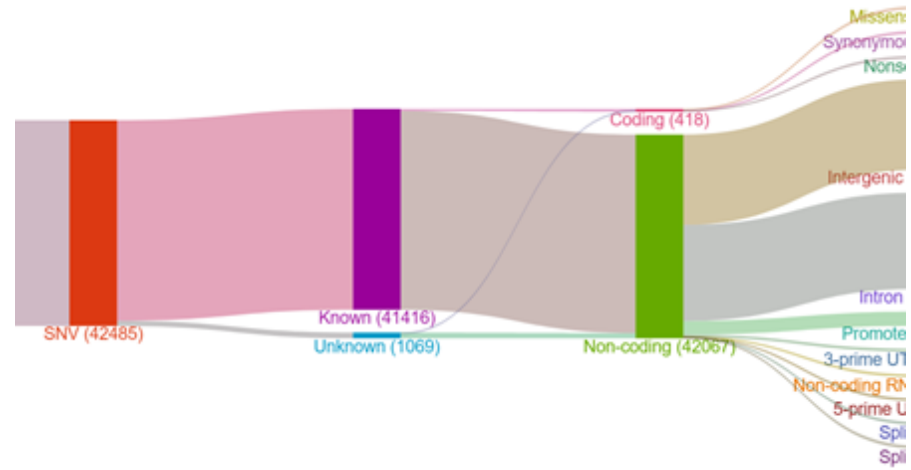
Optimization of the study

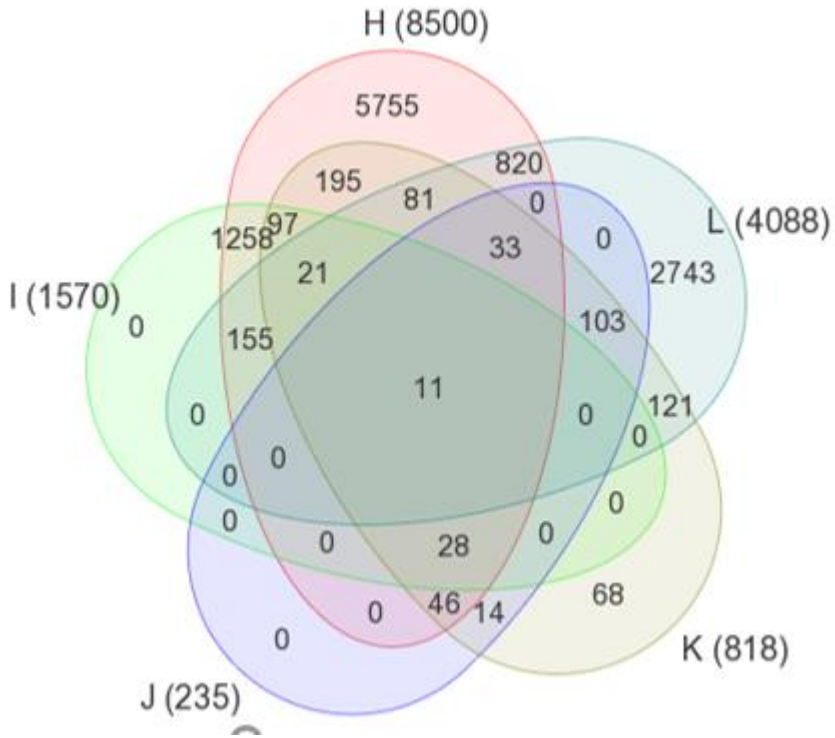


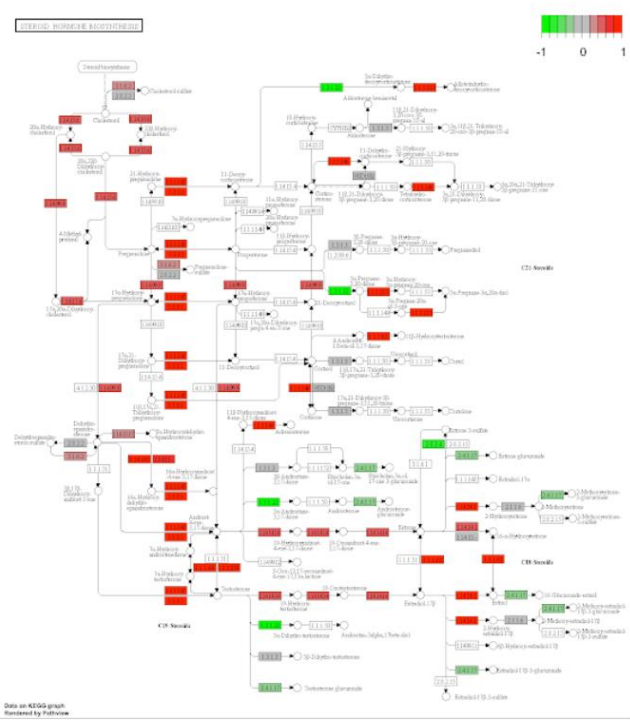
Classifications

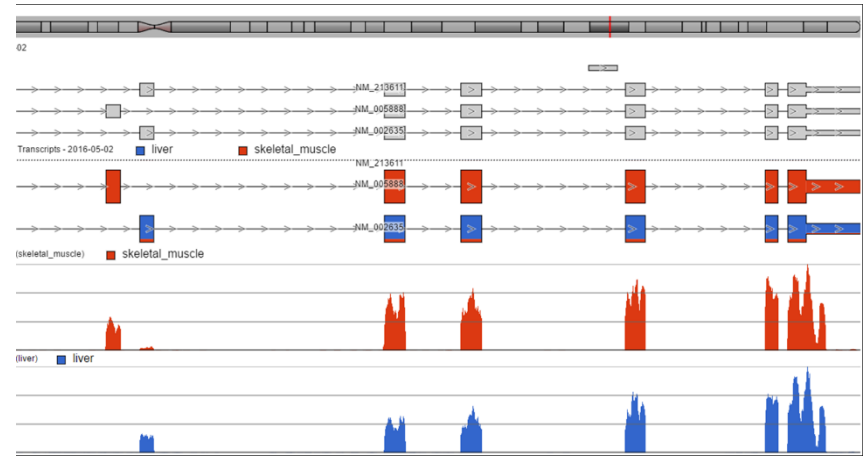
Color	Value
	Novel subtype
	Megakaryocytes
	Dendritic cells
	B-cells
	FCGR3A Monocytes
	NK cells
	CD14 Monocytes
	CD8 T-cells
	CD4 T-cells











Understanding the data

“We are close to having a $1,000 genome sequence, but this may be accompanied by a $1,000,000 interpretation.”

-Bruce Korf, president American College of Medical Genetics

Not only is the cost of sequencing essentially free, but big computers and big storage are cheap, too. what will keep us busy for the next 50 years is understanding the data”

-Russ Altman, chair of Biomedical Engineering at Stanford

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Why some patient benefit from the treatment while the others do not?

Mechanism of Immunotherapy

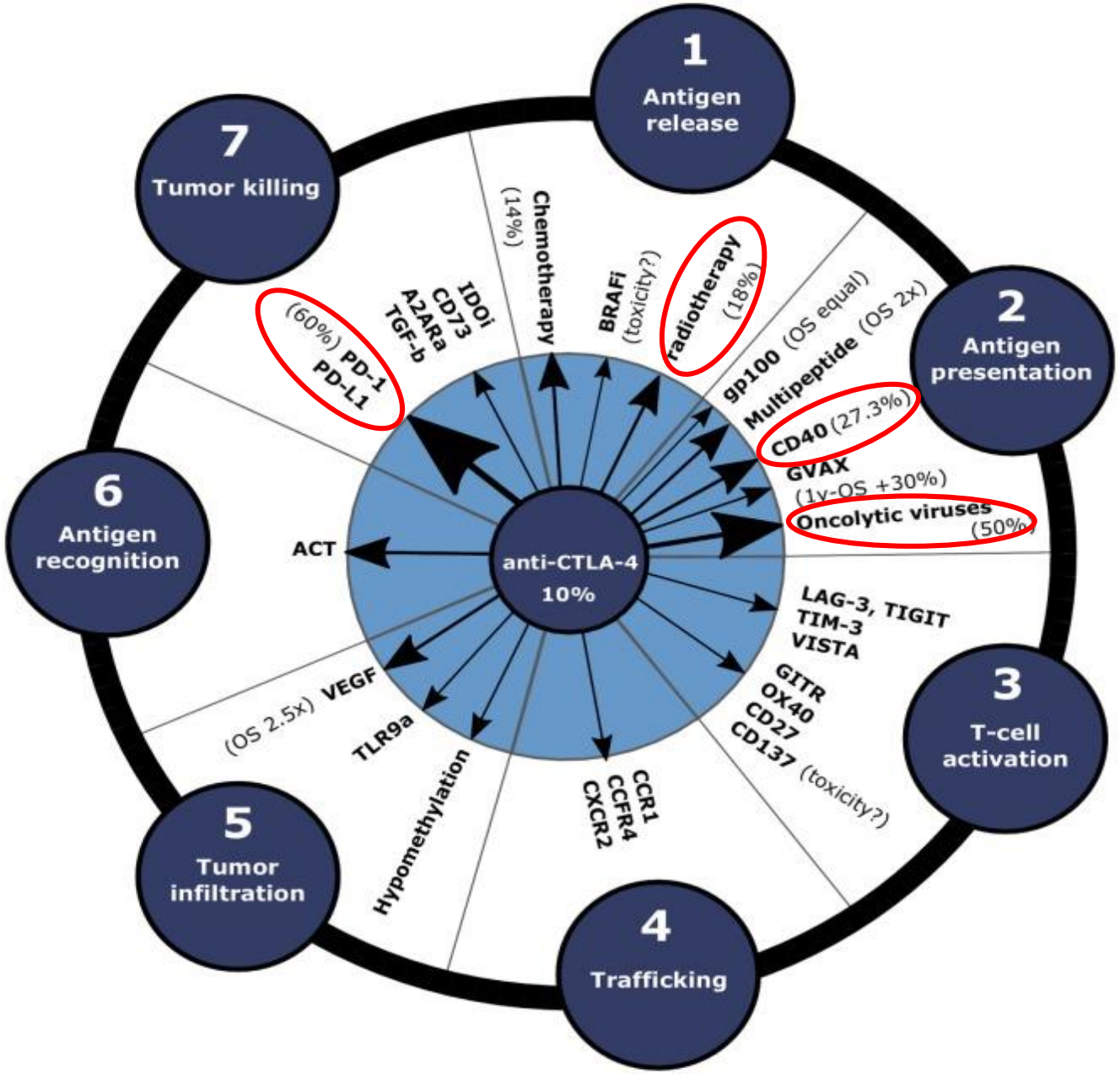
- **Cancer vaccine:** Directing the immune system to cancer cell by flagging cancer cells for destruction by the immune system
- **Cytokine therapy:** Increase the killing power of the immune system by enhancing T cell functions

- **Oncolytic viro-therapy:** Enhance the killing power of the immune system by introducing cancer tropical virus to kill cancer.

- **Checkpoint blockade:** Releasing the brakes on the immune system

- **Adoptive T cell therapy** and **CAR-T cell therapy:** Boosting the killing power of the immune system

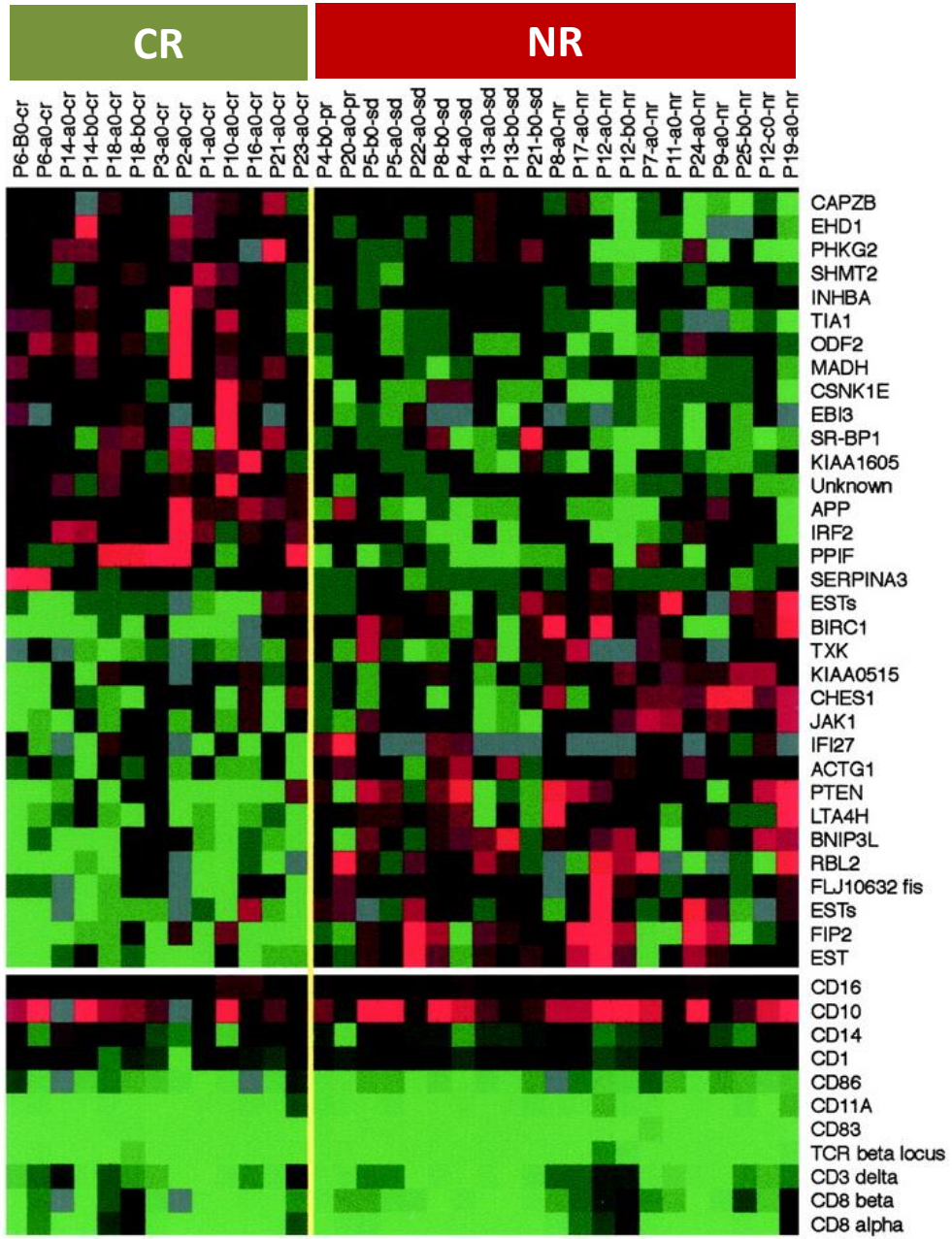
Anti PD1 or PD-L1 combine with CTLA-4 treatment in melanoma



Front Oncol. 2016; 6: 233.

Prediction of response to anti-cancer immunotherapy using gene signatures

Pre treatment biopsies of
Cancer vaccine + High dose IL2



Wang et al. Cancer Res. 2002

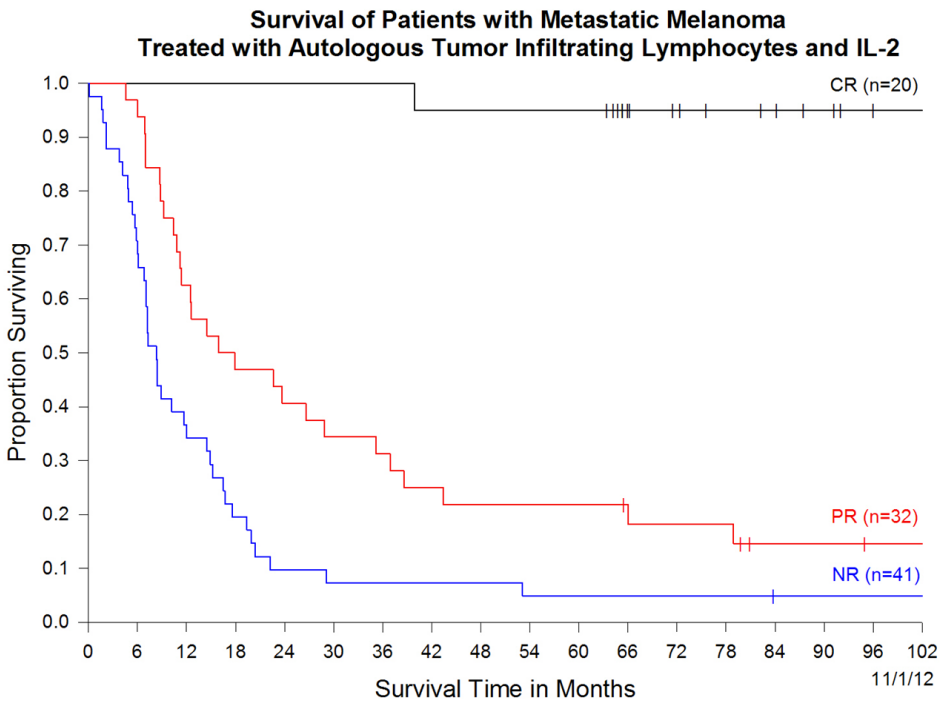
Project overview

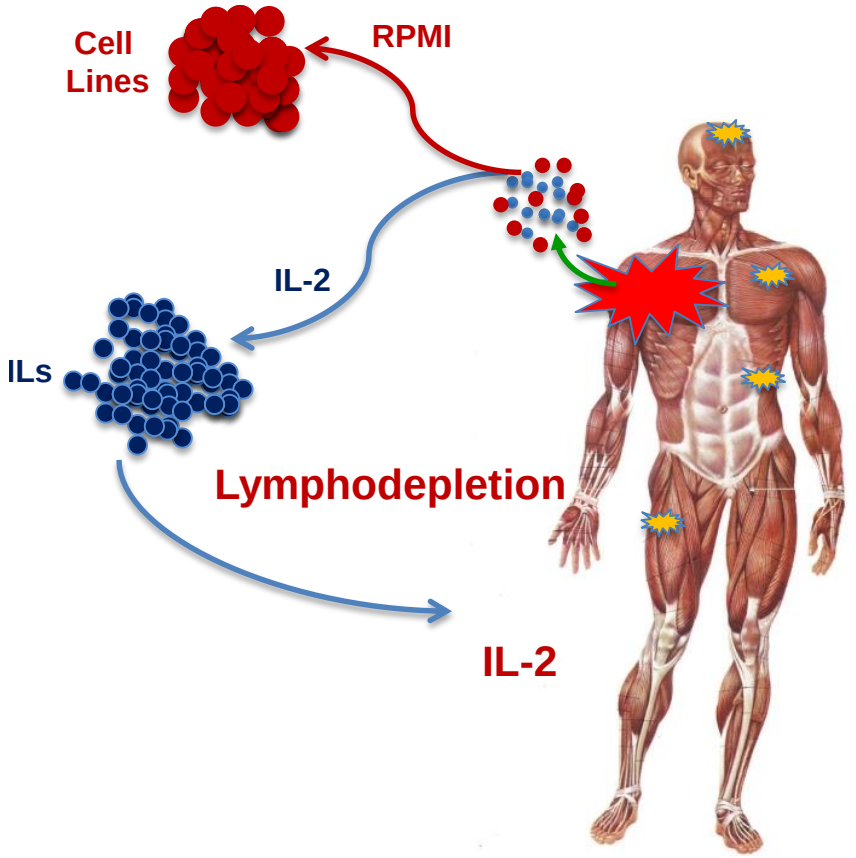
SAMPLES STUDIED

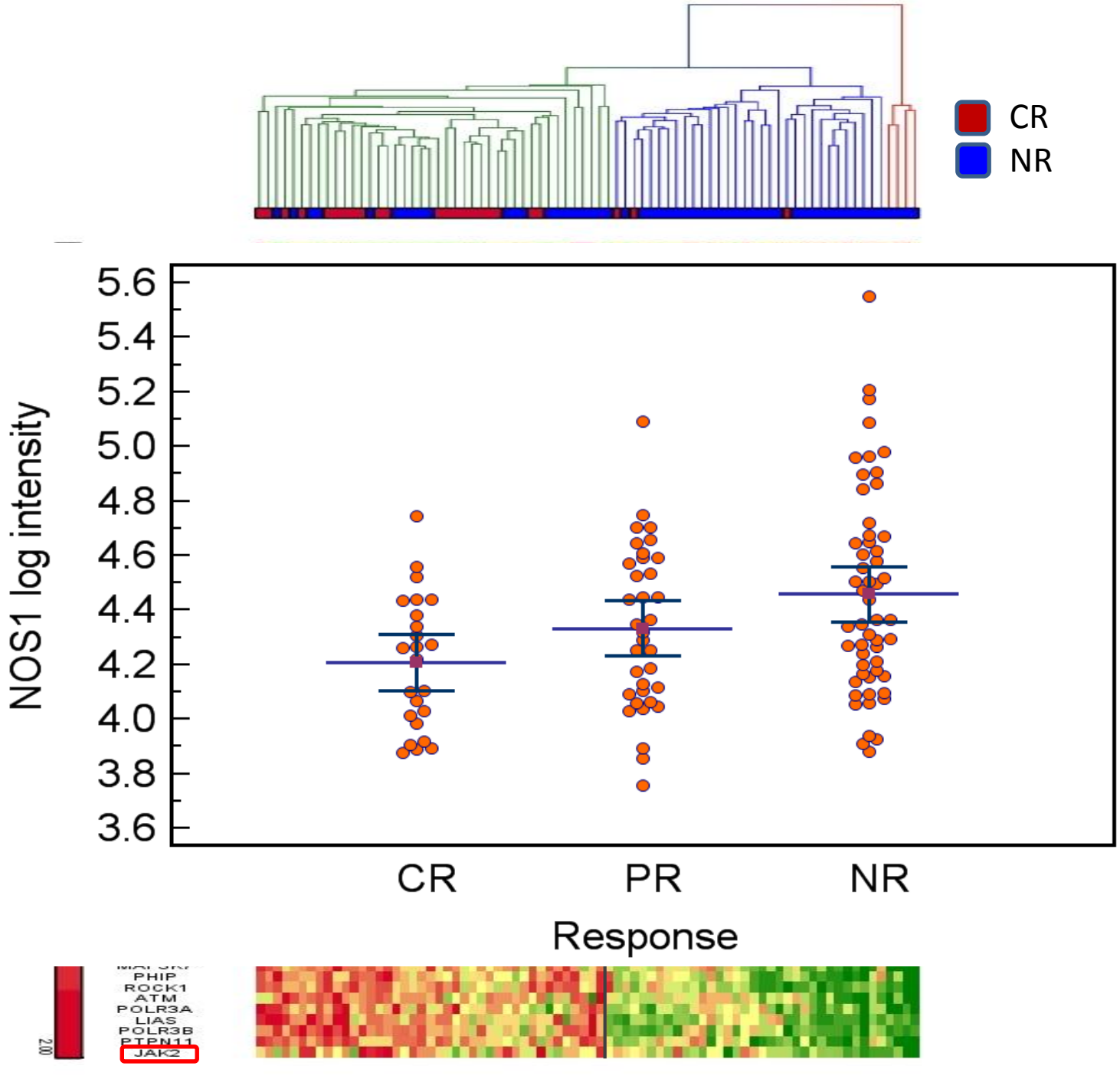
142 TILs from patients enrolled in five adoptive cell therapy trials

113 parental melanoma metastases

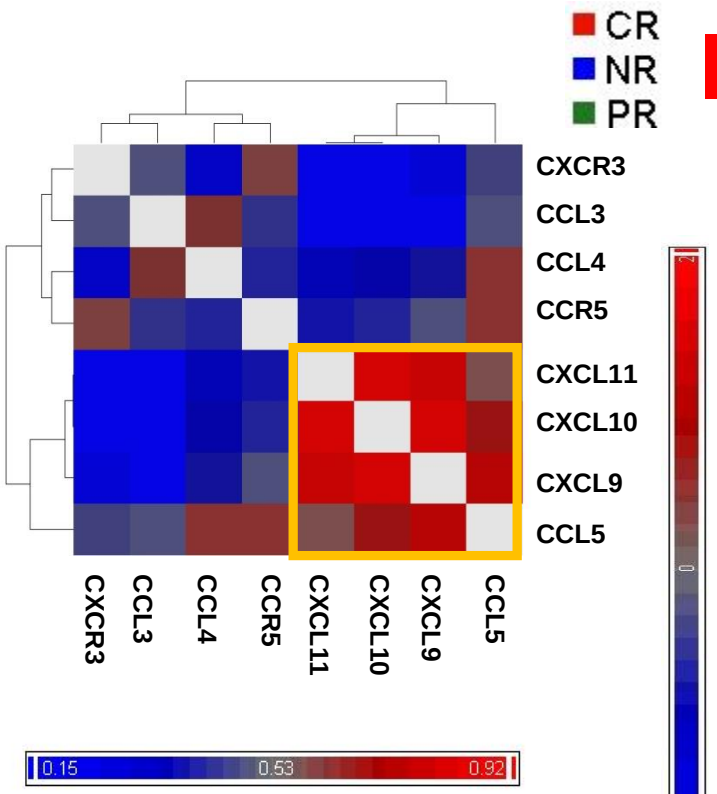
15 melanoma cell lines derived from the 15 melanoma metastases

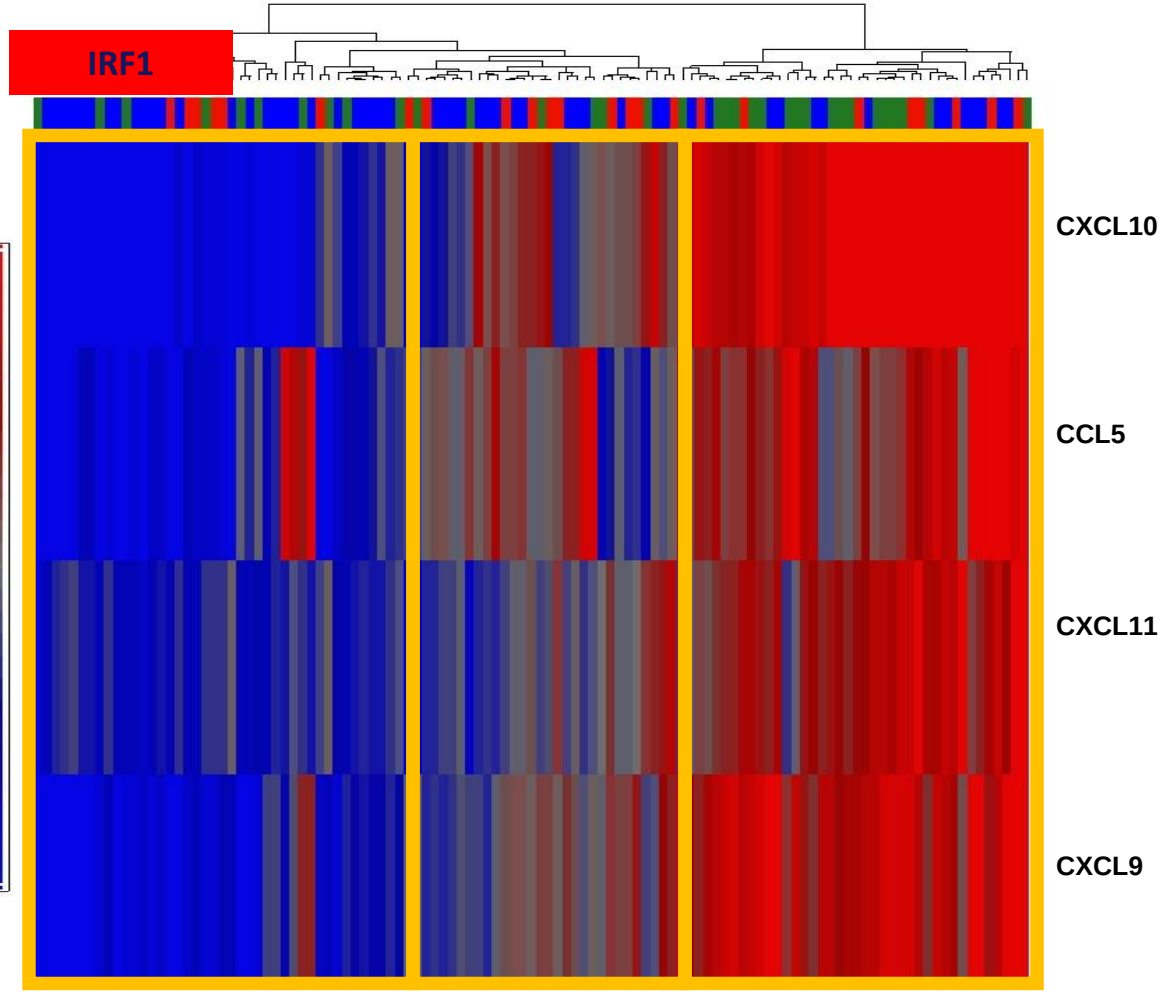





CR
NR
NOS1 log intensity
5.6
5.4
5.2
5.0
4.8
4.6
4.4
4.2
4.0
3.8
3.6
CR
PR
NR
Response
PHIP
ROCK1
ATM
POLR3A
LIAS
POLR3B
PTPN11
JAK2

Adoptive therapy + IL-2 (113 pre-treatment melanoma lesions)





113 pre-treatment melanoma biopsies

FULL PAPER

BJC

British Journal of Cancer (2013), 1–12 | doi: 10.1038/bjc.2013.557

Keywords: immunotherapy; interlukin-2; chemokines; melanoma; tumour microenvironment

CXCR3/CCR5 pathways in metastatic melanoma patients treated with adoptive therapy and interleukin-2

D Bedognetti[*,1,2,3], T L Spivey[1,4,5], Y Zhao[6], L Uccellini[1,7], S Tomei[1], M E Dudley[8], M L Ascierto[1,3,9], V De Giorgi[1], Q Liu[1], L G Delogu[10], M Sommariva[1,11], M R Sertoli[2,3], R Simon[6], E Wang[1], S A Rosenberg[8] and F M Marincola[*,1,12]

Cluster 1 (LOW):
OR: 38% (16/42)

Cluster 2 (MID)
OR: 52% (16/31)

Cluster 3 (HIGH)
OR: 65% (26/40)

enrich P = 0.03

CXCL9/10/11, CCL5

OR Rate: Cluster 1 < Cluster 2 < Cluster 3

Tumor components: Tumor intrinsic property

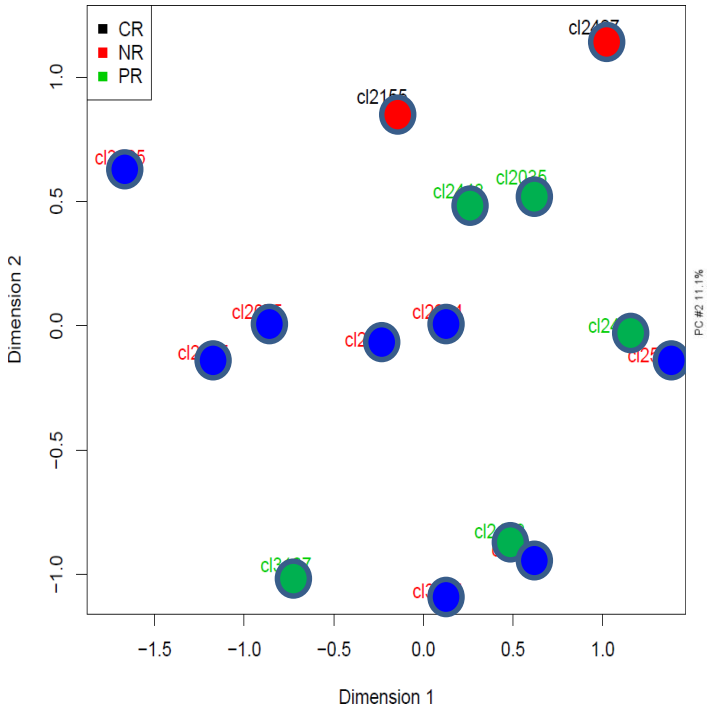
RNAseq analysis of melanoma cell lines based on RPKM

- NR
- CR
- PR

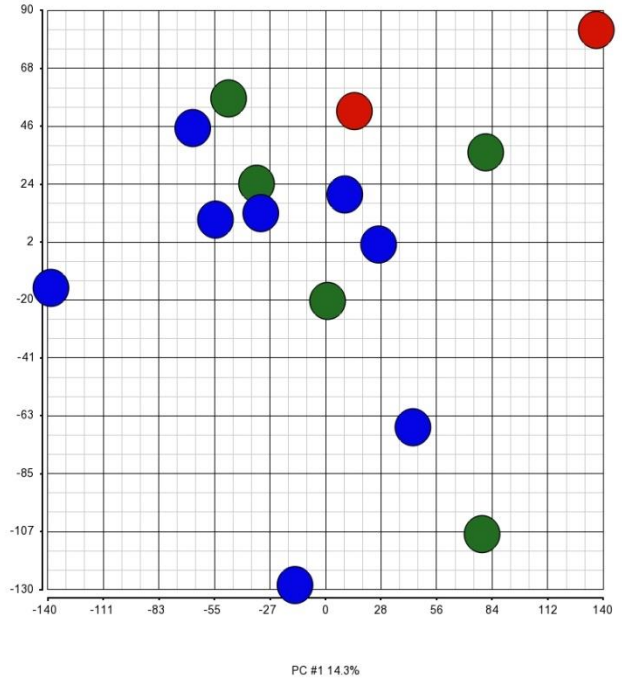
PCA analysis

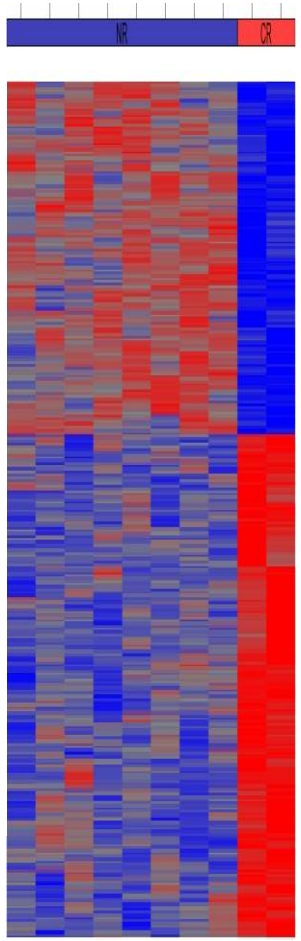
CR vs NR p<0.005 515 Genes

RNAseq



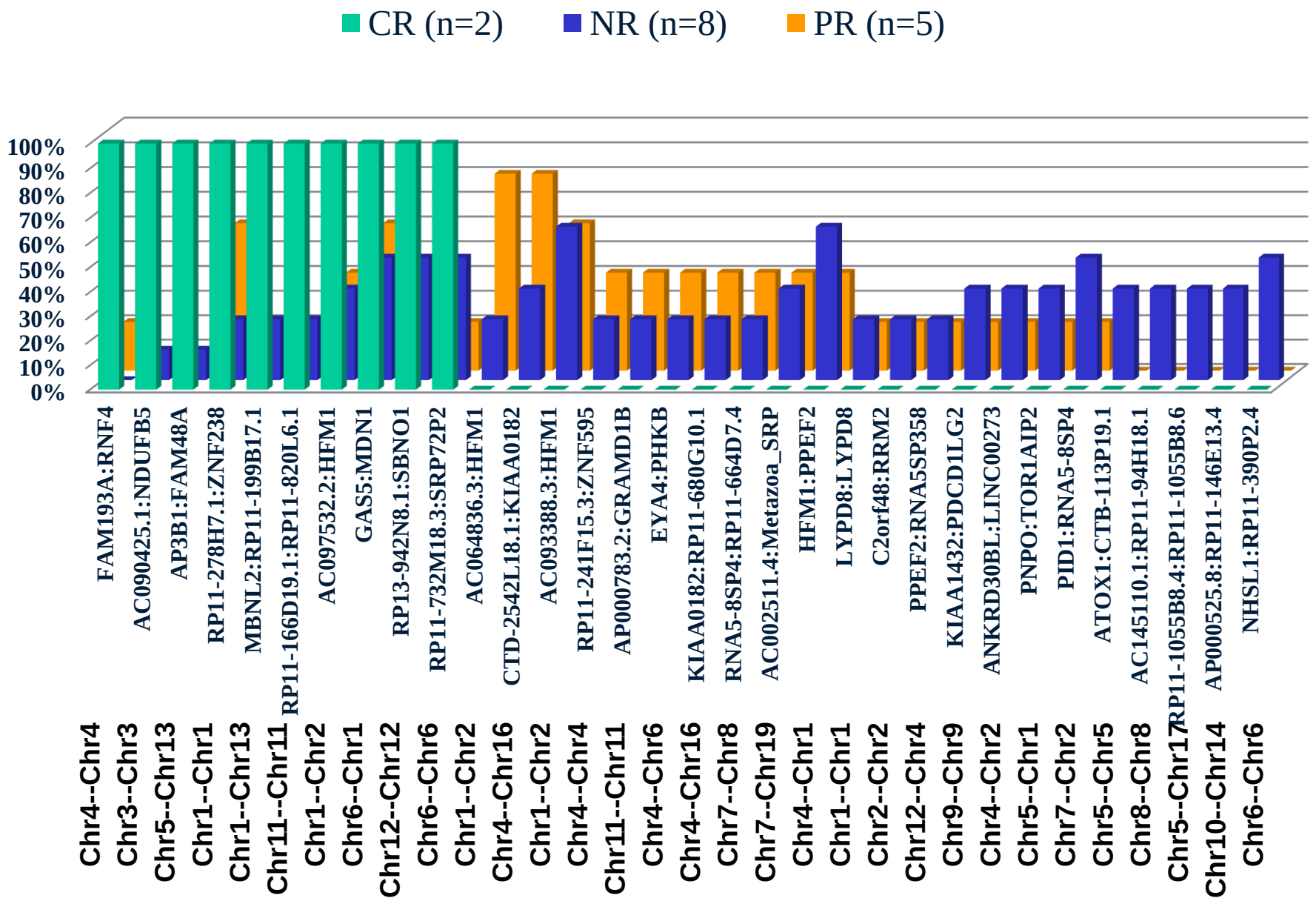
Gene expression





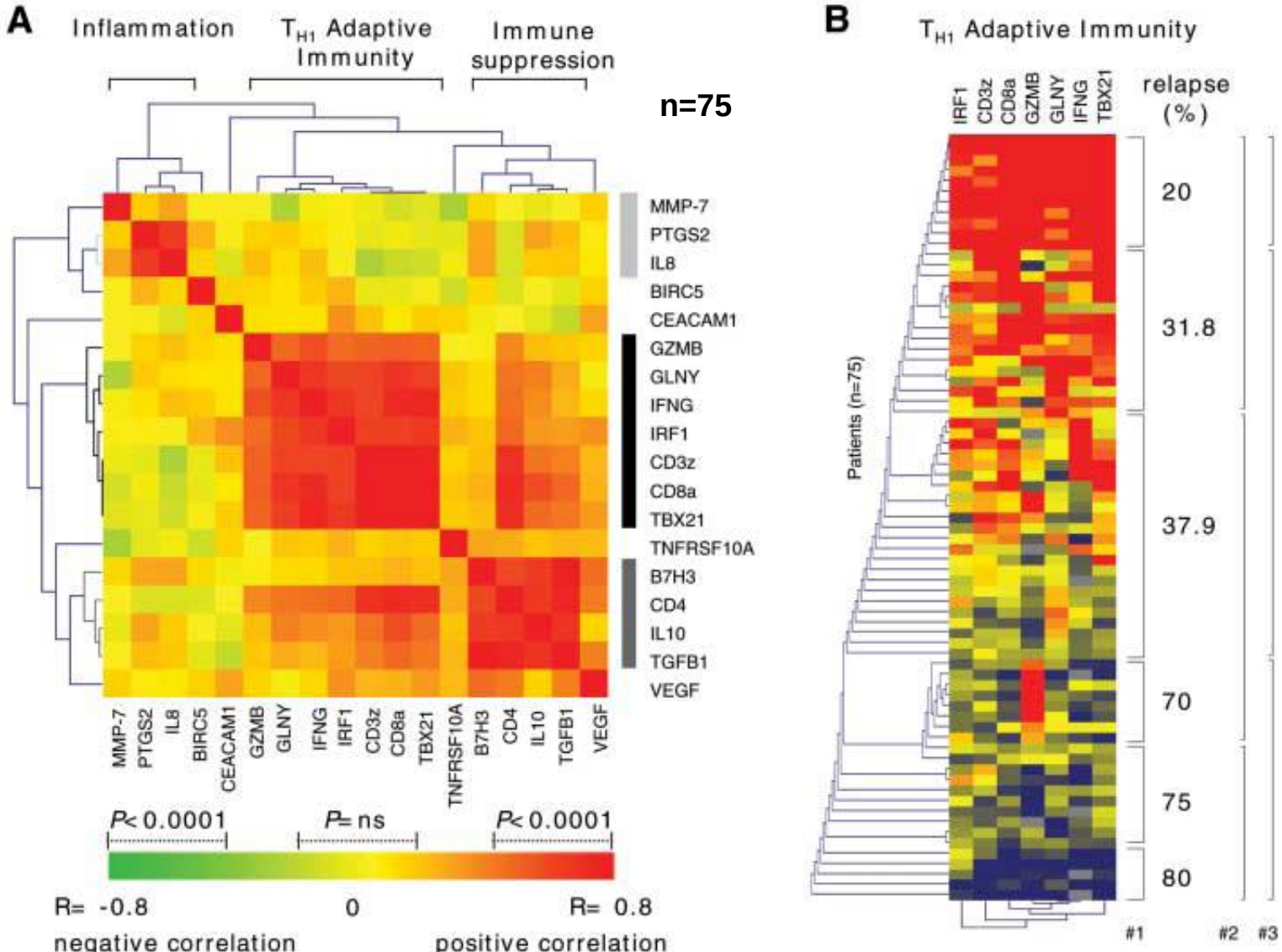
NR **CR**

Fusion trascripts identified by RNAseq



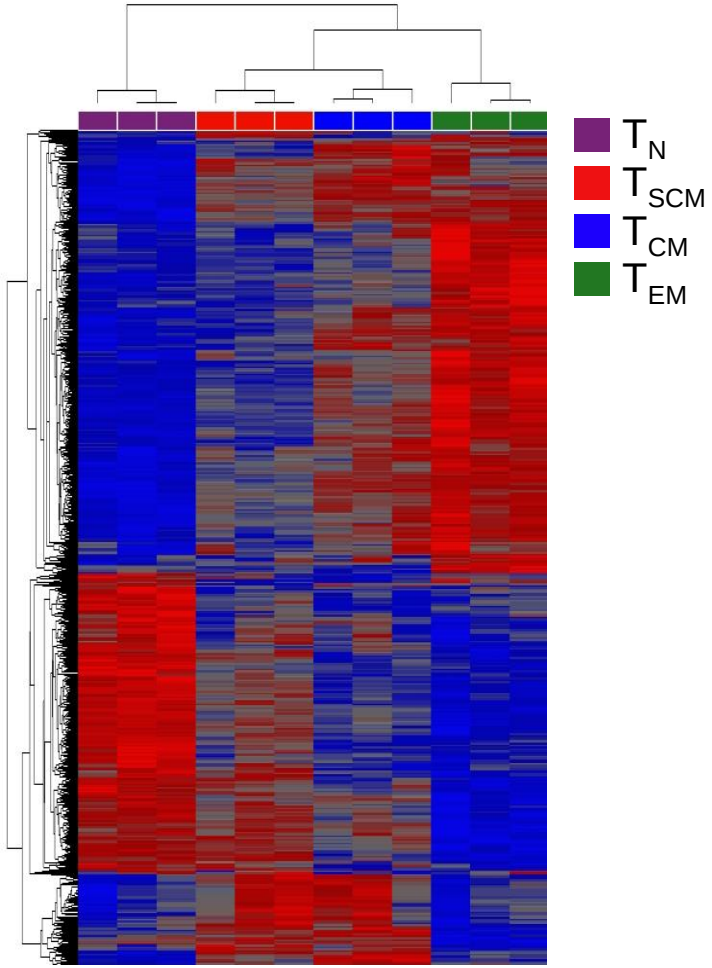
Type, Density, and Location of Immune Cells Within Human Colorectal Tumors Predict Clinical Outcome

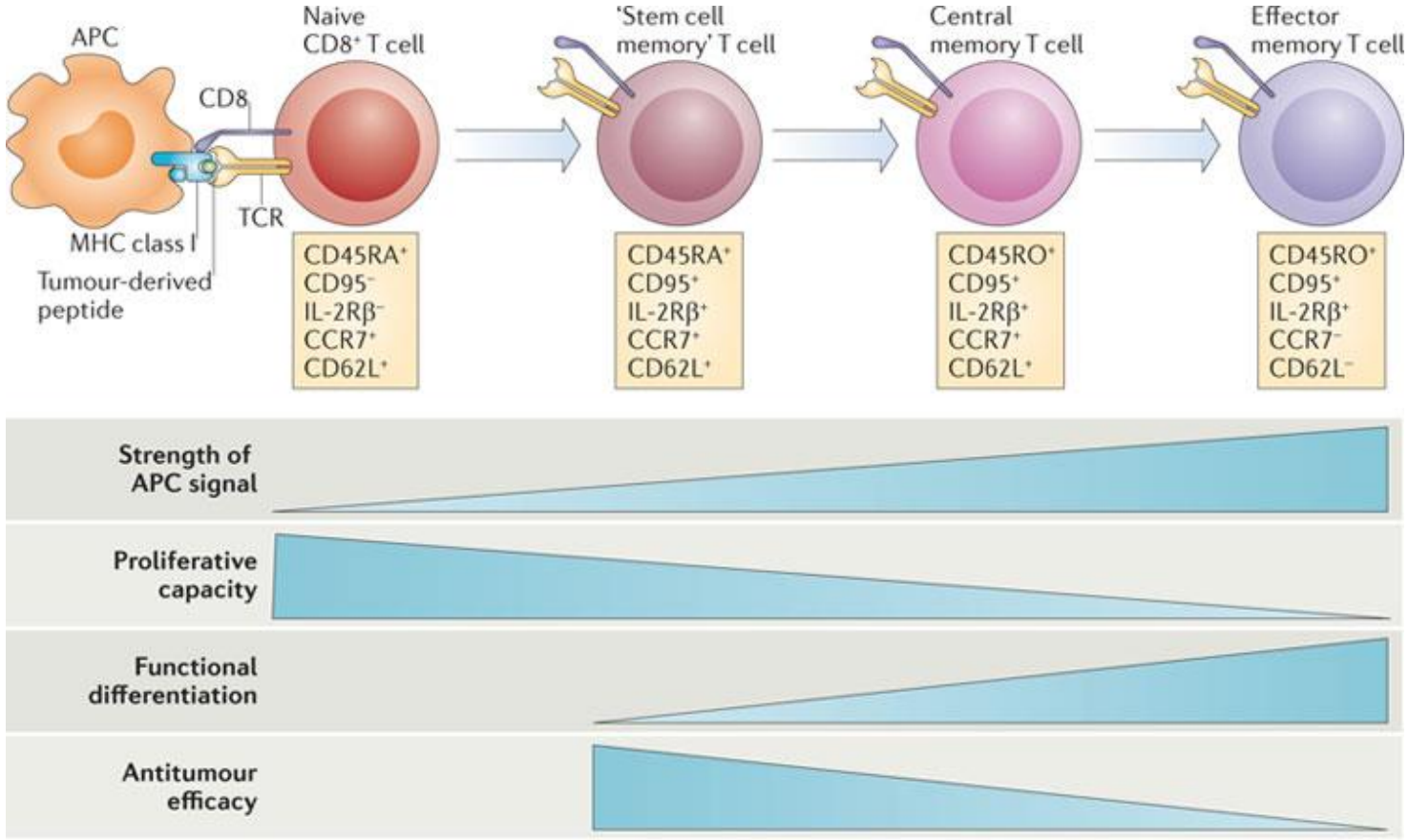
Jérôme Galon,[1*†] Anne Costes,[1] Fatima Sanchez-Cabo,[2] Amos Kirilovsky,[1] Bernhard Mlecnik,[2] Christine Lagorce-Pagès,[3] Marie Tosolini,[1] Matthieu Camus,[1] Anne Berger,[4] Philippe Wind,[4] Franck Zinzindohoué,[5] Patrick Bruneval,[6] Paul-Henri Cugnenc,[5] Zlatko Trajanoski,[2] Wolf-Herman Fridman,[1,7] Franck Pagès[1,7†]



Immune components: Immune cell differentiation status

T_{SCM} are a distinct early T cell memory subset

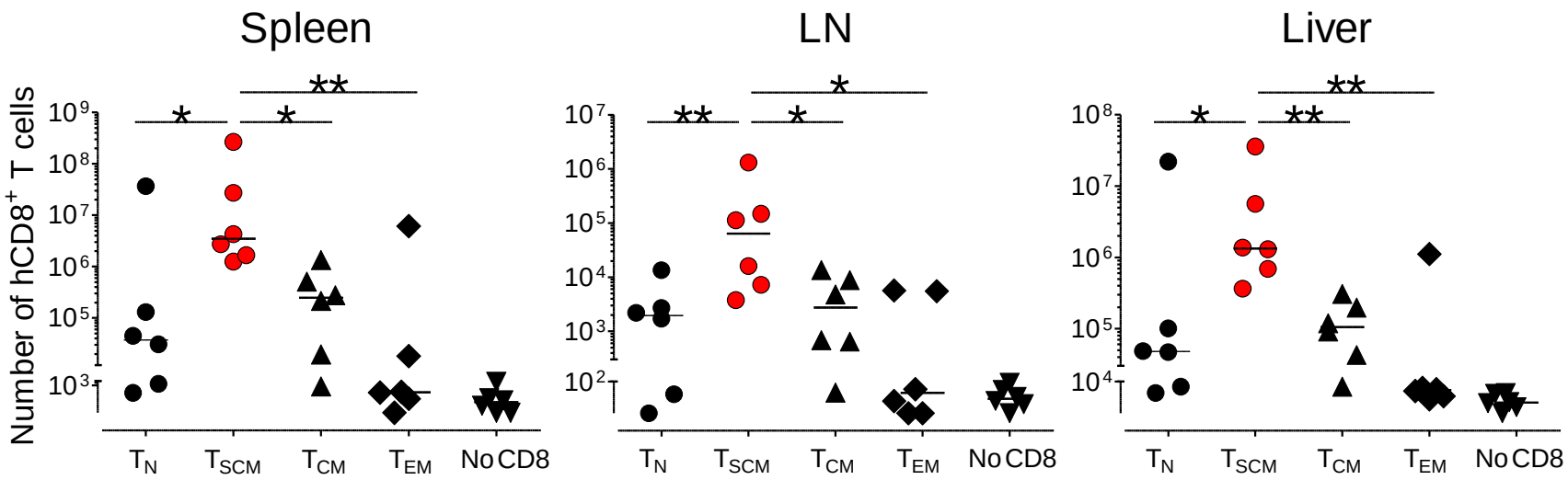




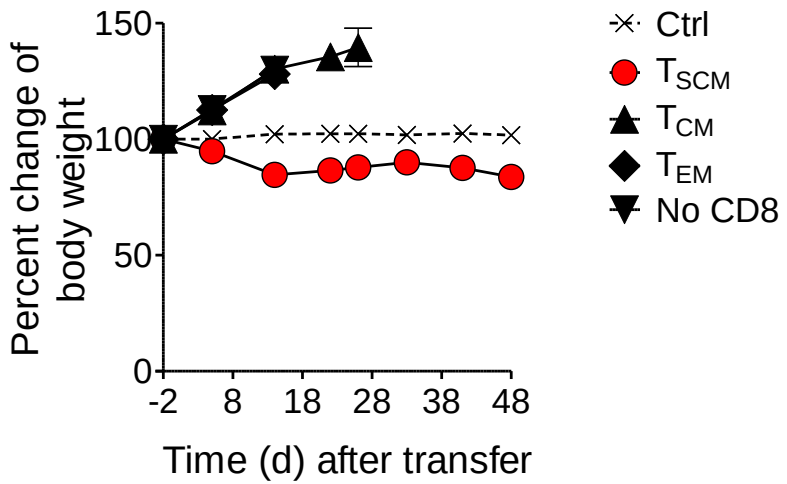
Luca Gattinoni, *Nature Med* 2011

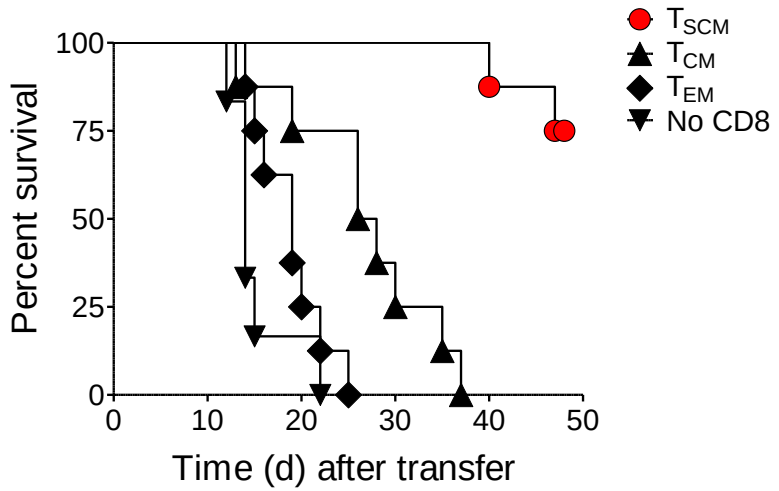
Nature Reviews | Immunology

T_{SCM} have enhanced proliferative and survival capacities



T_{SCM} have increased anti-tumor activities upon adoptive transfer

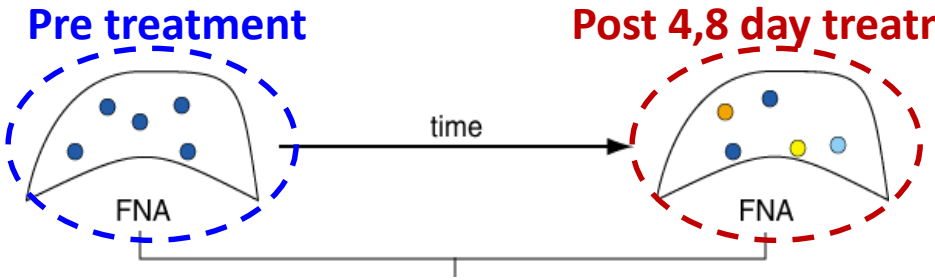




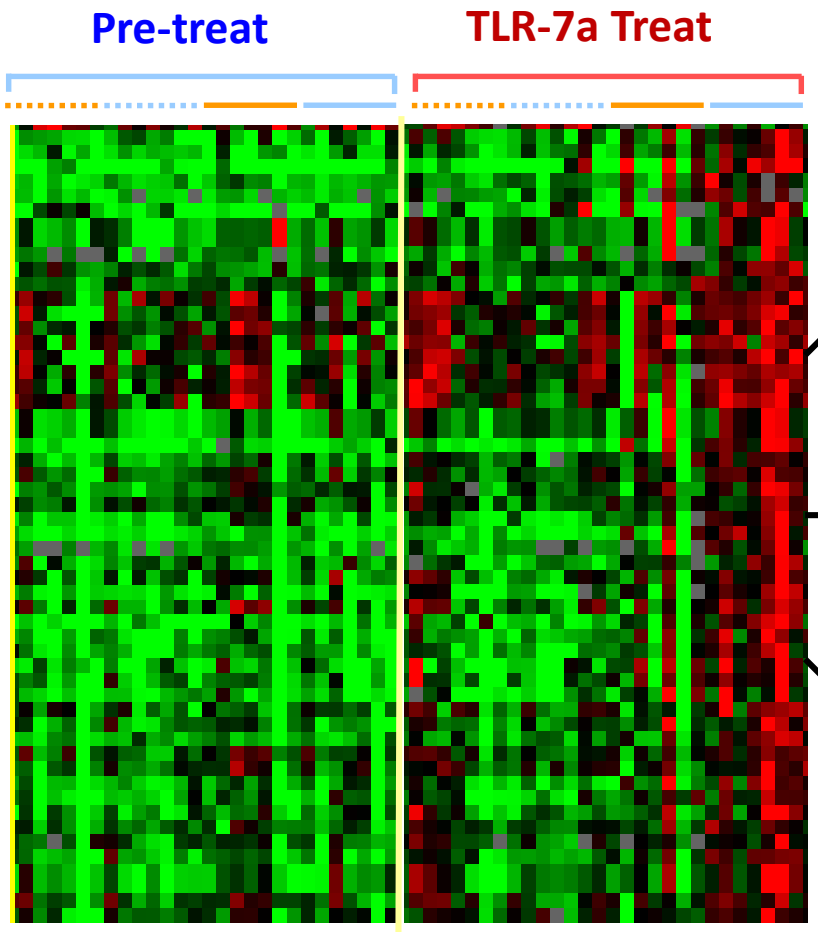
Luca Gattinoni, Nature Med 2011

Mechanism of action: pre vs post treatment

Imiquimod (TLR-7a)-Basal cell Carcinoma



Placebo X 4 days
Placebo X 8 days
Treat X 4 days
Treat X 8 days



Genome Biology 2007, 8:R8 (doi:10.1186/gb-2007-8-1-r8)

STAT 1/IRF1
Allograft inflammatory factor 1
IL-15, IL-6
IL-2/IL15 R b
IL-15 R a
IL-2/IL-4/IL-7/IL-9/IL-15 R g
HLA Class I, HLA Class II, TAP, LMP

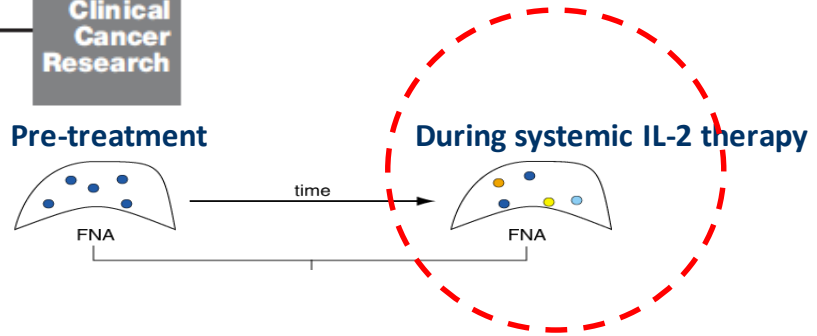
Granzyme A, B, K
Perforin
Natural killer-cell transcript 4 (IL-32)
Natural killer cell gene-5
Caspase 1, 5, 8

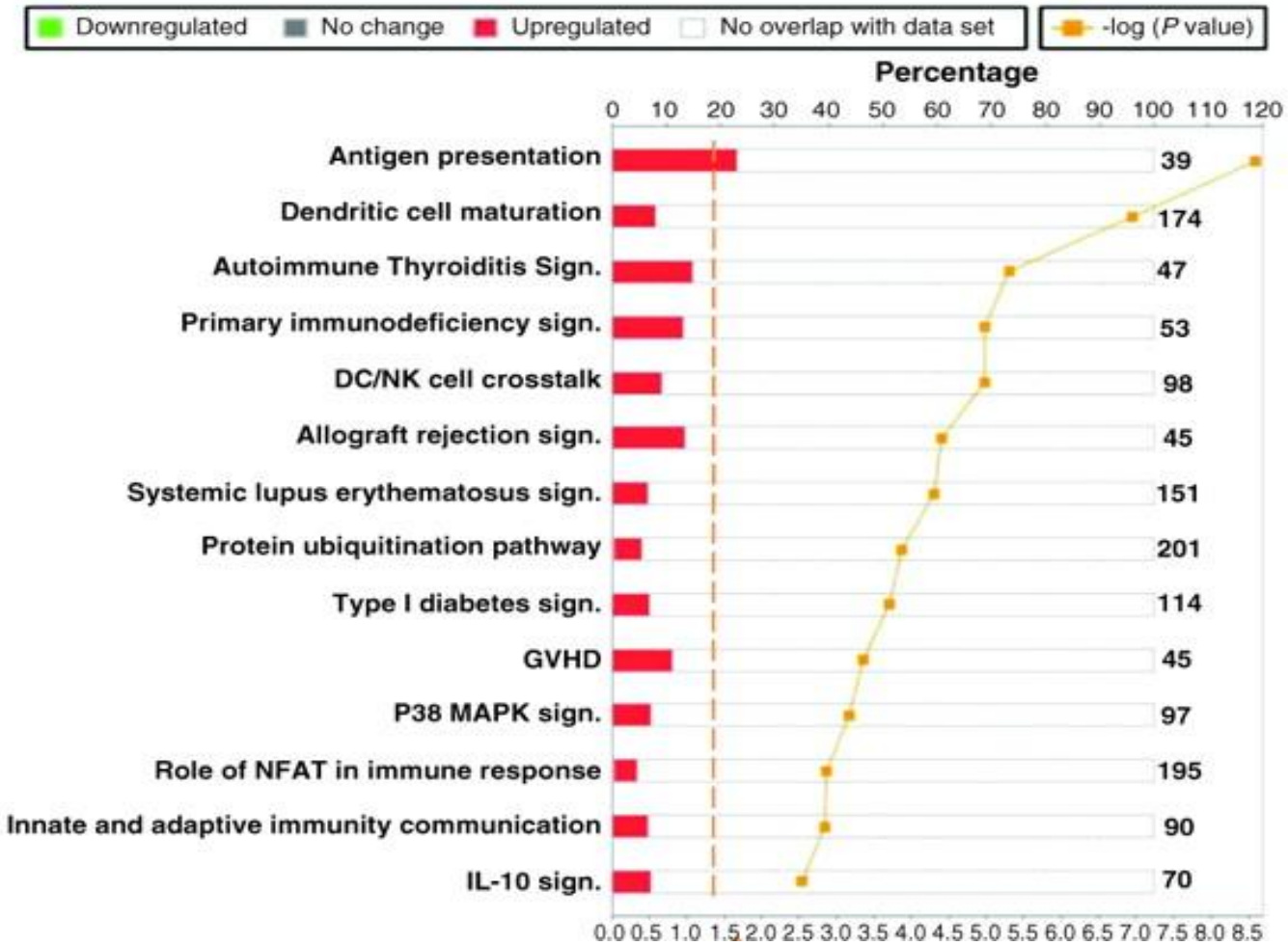
CXCL10
CXCL9
CCL5
CCL4

Clinical Cancer Research

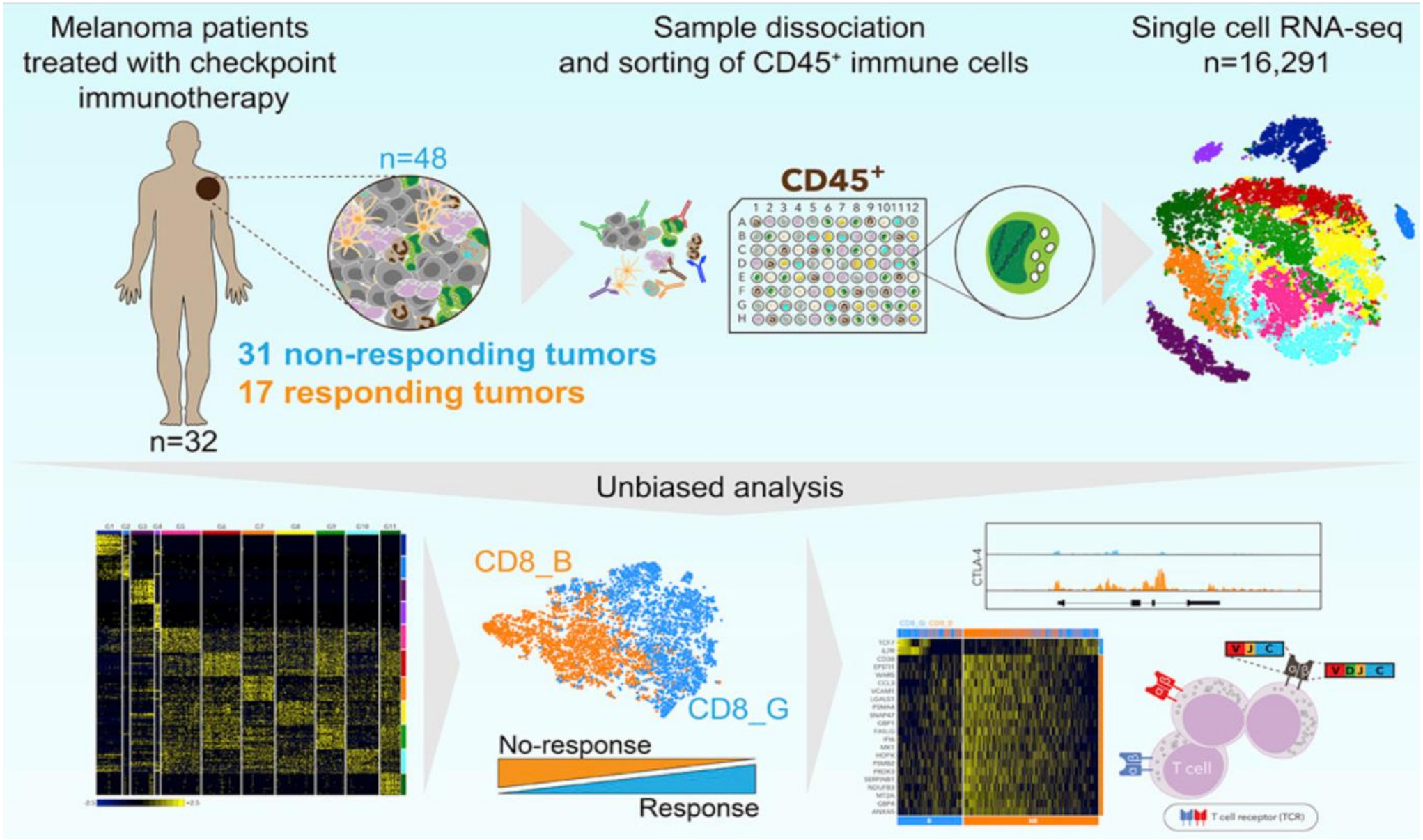
Molecular Insights on the Peripheral and Intratumoral Effects of Systemic High-Dose rIL-2 (Aldesleukin) Administration for the Treatment of Metastatic Melanoma

Geoffrey R. Weiss[1], William W. Grosh[1], Kimberly A. Chianese-Bullock[2], Yingdong Zhao[3], Hui Liu[4], Craig L. Slingluff Jr[2], Francesco M. Marincola[4], and Ena Wang[4]



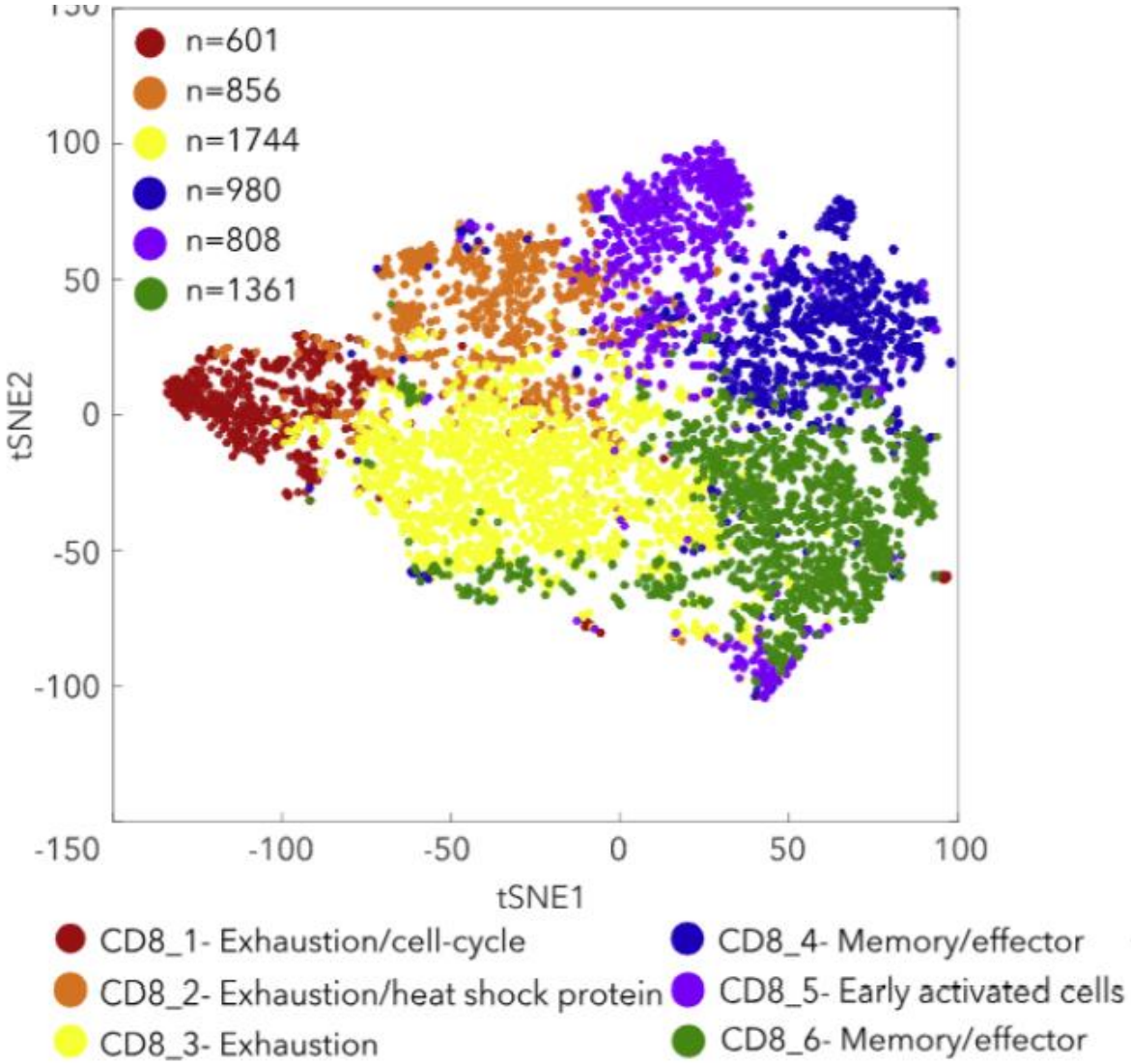


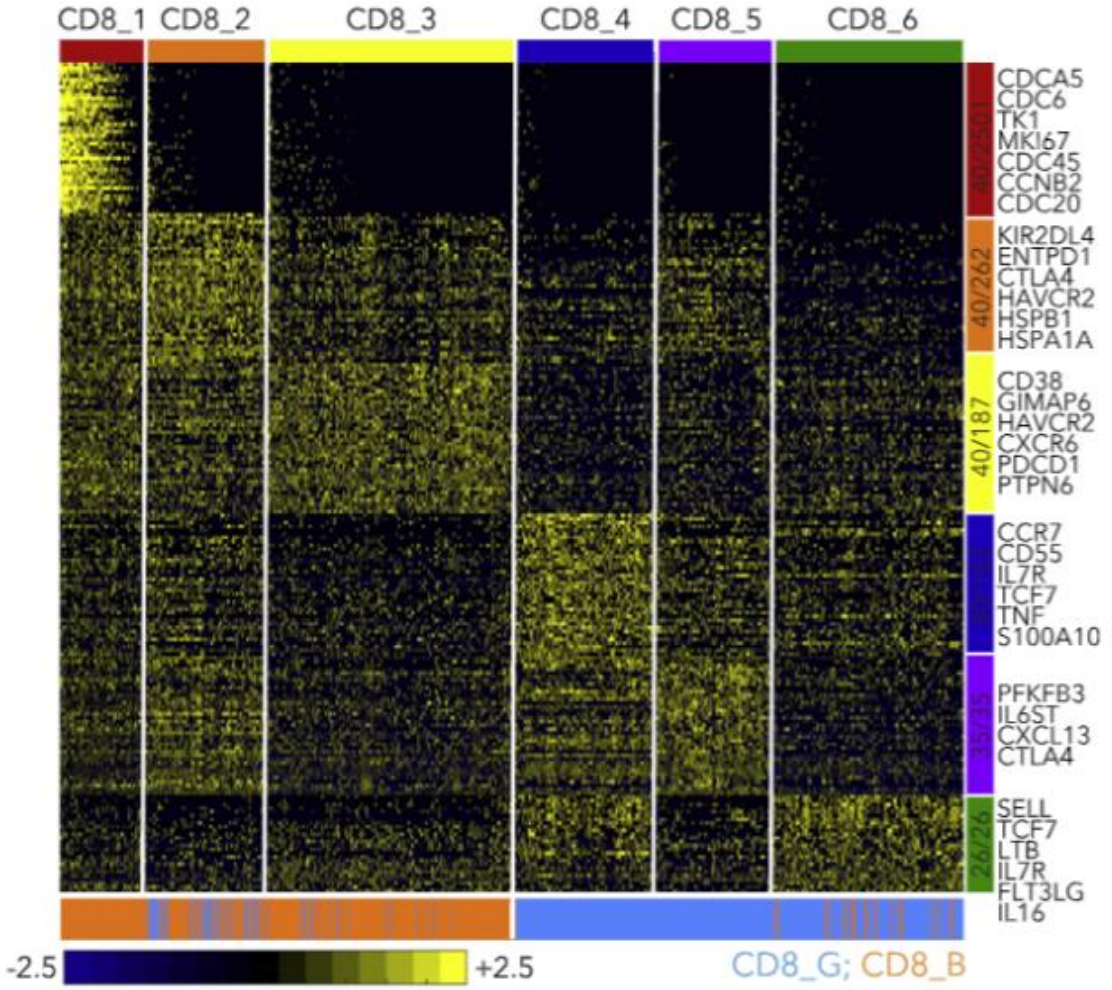
Defining T Cell States Associated with Response to Checkpoint Immunotherapy



Sade-Feldmam, M et al. Cell. 2018 Nov 1;175(4):998-1013.

CD8 subsets identified





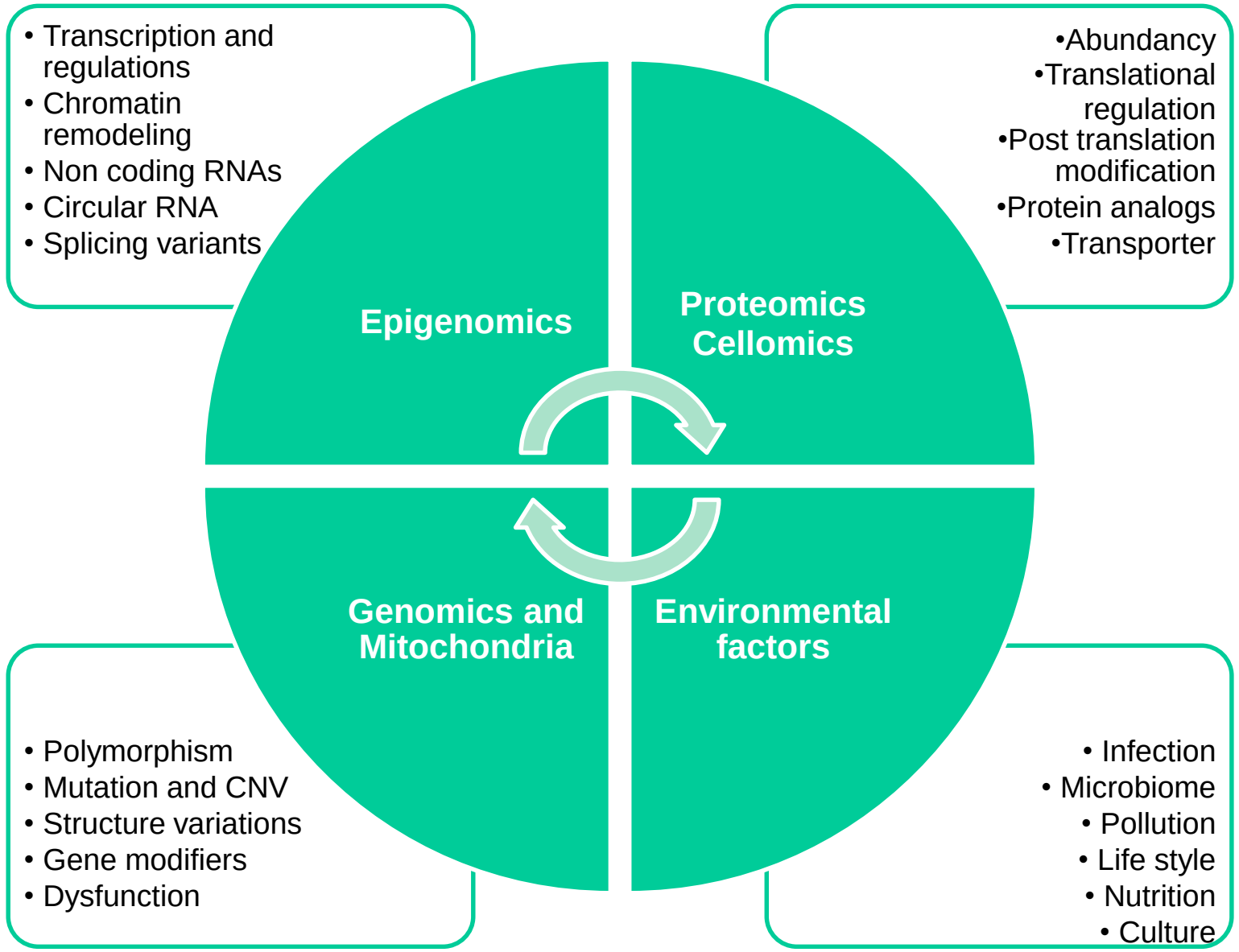
Sade-Feldmam, M et al. Cell. 2018 Nov 1;175(4):998-1013

The heterogeneity of tumor microenvironment

1. Broad spectrum of immune infiltration from immune deserted, excluded to inflamed
2. Complex components coexisting at the tumor microenvironment
3. Diverse architecture and vasculature of tumor
4. Balance between immune competent vs immune compromised status
5. Insufficient knowledge about immune cell subset functional and activation threshold.
6. Potential ways to tip the balance between innate and adaptive immune stimulatory and immune regulatory mechanisms

The complexity of tumor rejection - multi factory phenomena

Classes of factors influencing immune responsiveness



US NGS commercial providers

Provider Name-Commercial	Provider Name-Commercial	Provider Name-Commercial	Provider Name-Commercial
Accelrys	David H Murdock Research Institute	Integrated Genomics	HERA Biosciences
ACGT, Inc	DNAStar	Kashi Clinical Laboratories	PrimBio Research Institute
Alpha BioLab	EA Quintiles	Laragen, Inc.	Otogenetics
AltheaDx	Elim Biopharmaceuticals	LC Sciences	Fulcrum Genomics
Ambry Genetics	EnGencore	Lofstrand Labs	Epic Sciences
Amplicon Express	EpigenDx	Longhorn Vaccines & Diagnostics	Macrogen Clinical Laboratory
Ana-Gen Technologies	Eton Bioscience	MCLAB	Gene Peak
Asuragen	Eureka Genomics	PerkinElmer	Sequencing.com
BGI Americas	Expression Analysis	Polymorphic DNA Technologies	Omega Biosciences
Bio Applied Technologies Joint	Floragenex	Research and Testing Laboratory	Eurofins Genomics
BioReliance	Functional Biosciences	Selah Genomics	MOgene
Centrillion Biosciences	Gene by Gene	Sequetech	Lucigen
Childrens Mercy Hospitals	Gene Codes	SeqWright	SeqMatic
City of Hope Integrative Genomics Core	Gene Link	SeqXcel	Medgenome, Inc.
Claritas Genomics	GeneDx	Sorenson Genomics	NGX Bio
Cleveland Genomics	GENEWIZ, Inc	Taueret Laboratories	Ashion
Cofactor Genomics	Genome Explorations	Wyzer Biosciences	Admera Health
Complete Genomics	Genome International	Zymo Research	CD Genomics
Covance Genomics Laboratory	Genoptix Medical Laboratory	Human Longevity	SeqLL, LLC
Creative Proteomics Lilly Green	Golden Helix	Fulgent Genetics	