

Today's schedule

Friday November 29

9.00 - 9.40	Single cell genomics Thijs Ettema B7:113a
9.40 - 10.00	Single cell genome assembly using SPAdes Jimmy Saw B7:113a
10.00 - 10.20	Coffee break
10.20 - 12.00	Tutorial Single cell genome assembly (Part 1-3) A6:003 Anders Andersson, Thijs Ettema, Ino de Bruijn, Jimmy Saw, Kasia Zaremba
12.00 - 12.45	Lunch
12.45 - 17.00	Tutorial Single cell genome assembly (Part 4-7) A6:003 Anders Andersson, Thijs Ettema, Ino de Bruijn, Jimmy Saw, Kasia Zaremba

Single cell genomics in a nutshell



Courtesy of Carrie Jackson

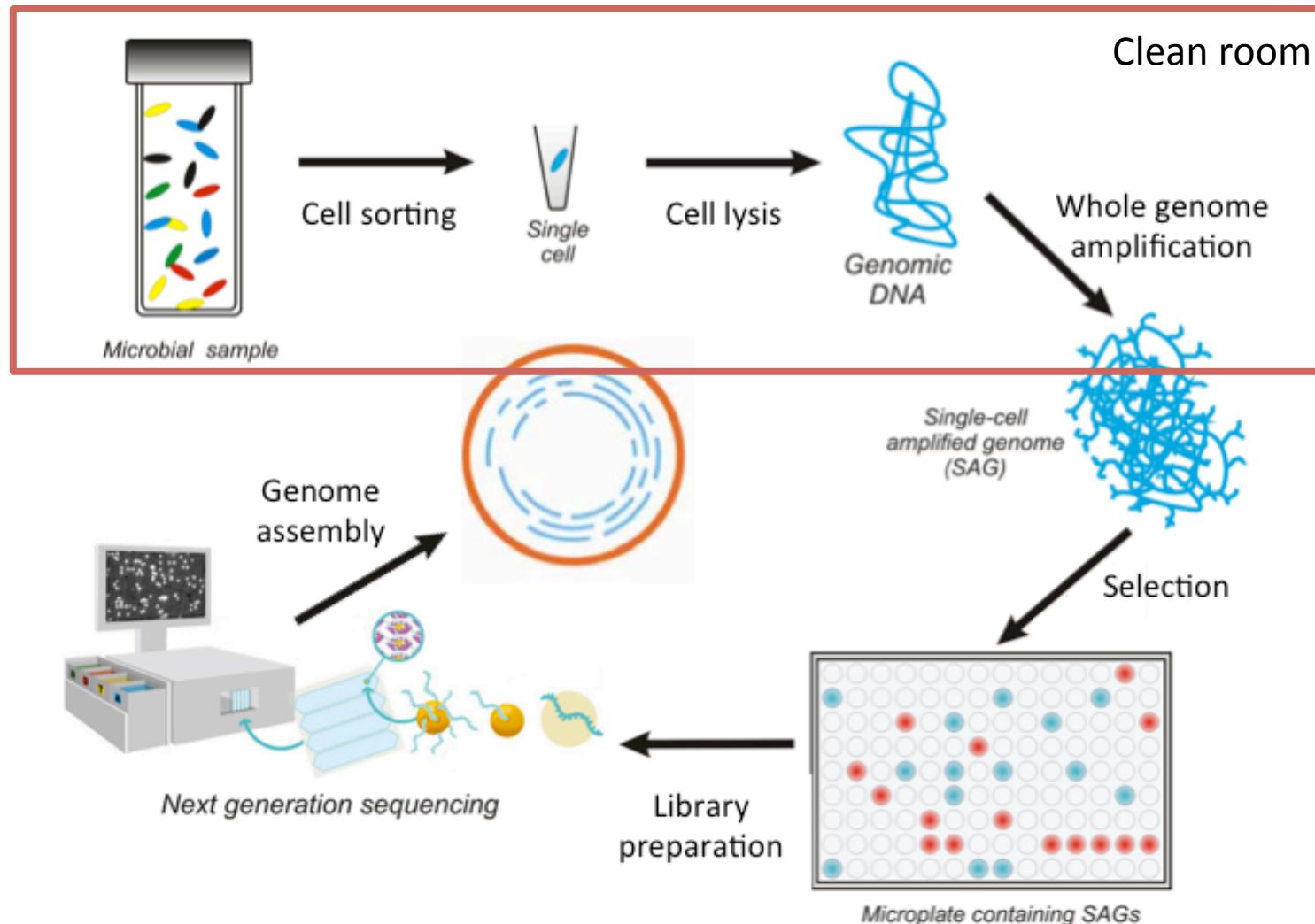
Thijs Ettema

Single cell genomics in a nutshell



- Single cell sorting
- Whole Genome Amplification (WGA)
- Library preparation and NGS
- Genome assembly (briefly – Jimmy Saw)
- SCG example study
- The Pros and Cons of SCG
- Future directions of SCG
- SiCell: the SciLife platform for Single Cell Genomics

Single cell genomics in a nutshell

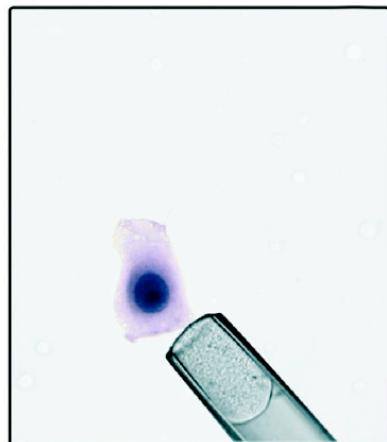


Single cell sorting

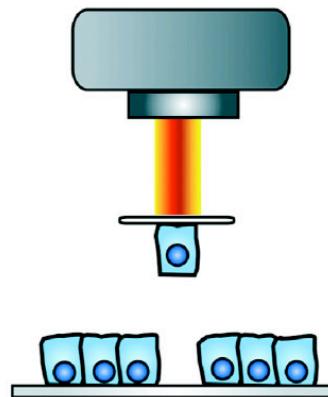
Cell material, some considerations

- Fresh material = better
- Intact cells (do not freeze without cryoprotectant)
- Cells should not be aggregated

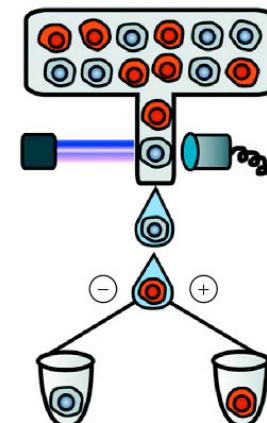
Different approaches



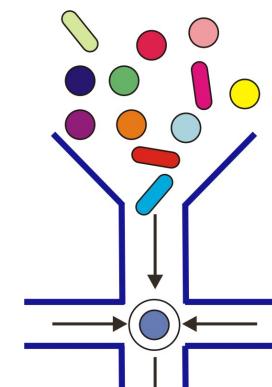
Micro-manipulation



Light-capturing
microscopy



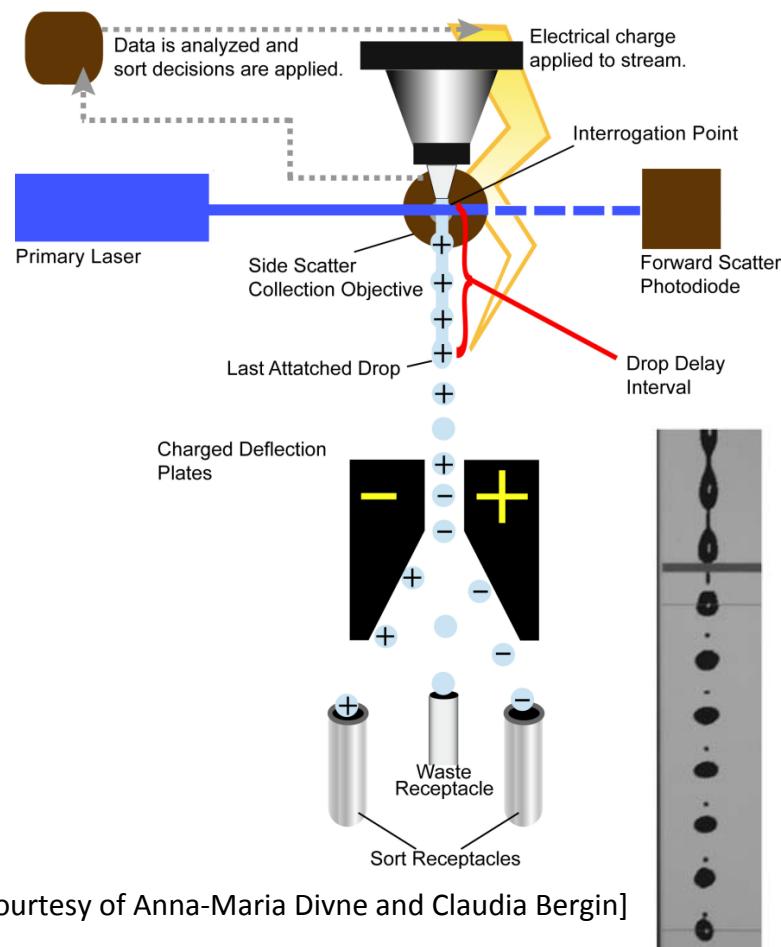
Fluorescence-activated
cell sorting (FACS)



Micro-droplet
based sorting

Single cell sorting

Fluorescence-assisted Cell sorting (FACS)

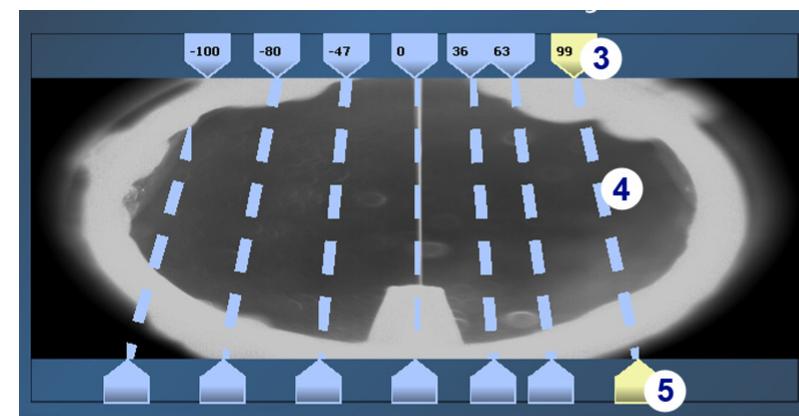
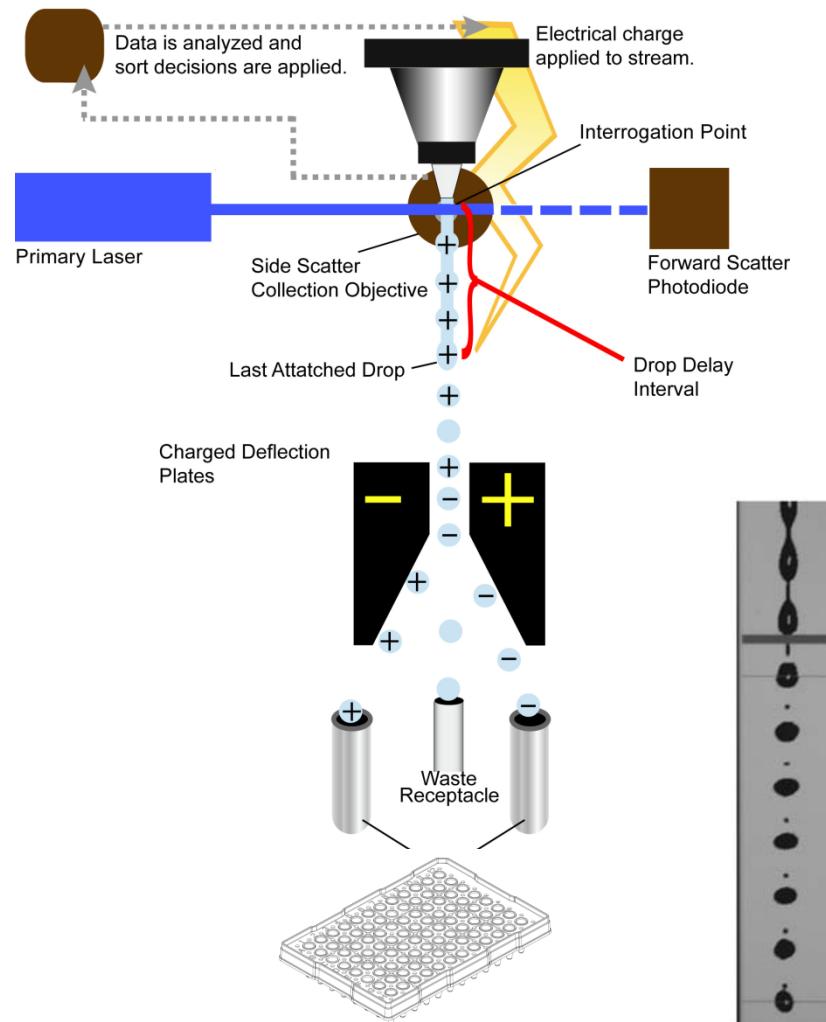


During a sort the following events occur:

1. Decision at the interrogation point: to sort or discard a cell.
2. If a cell is to be sorted, the system waits until the cell reaches the last attached drop.
3. The last attached drop breaks off carrying a charge that can be *positive*, *negative*, or *neutral* depending on the direction it will be sorted.
4. As the charged droplet falls through the electric field created by the Sort Deflection Plates, it is deflected into the proper sorting receptacle.

Single cell sorting

Fluorescence-assisted Cell sorting (FACS)

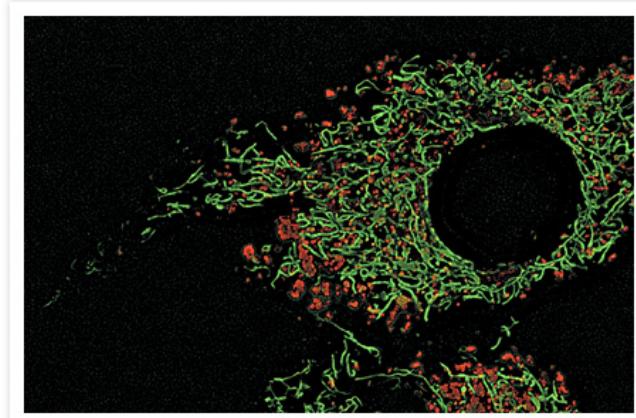
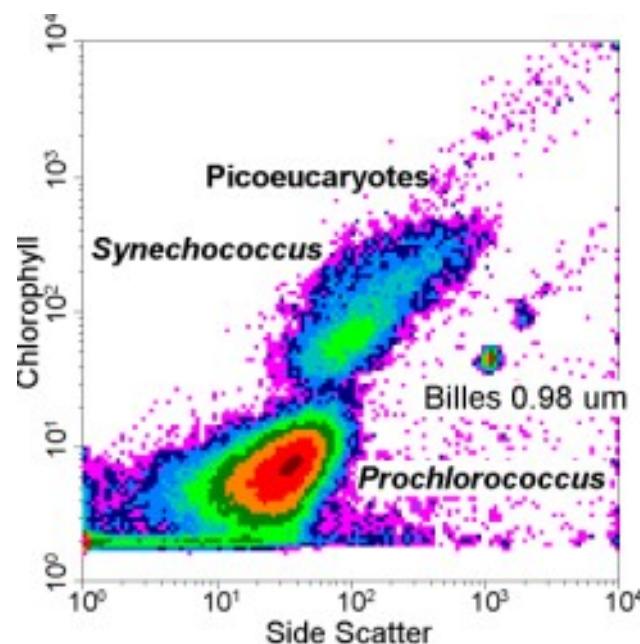


- Advanced Cell sorter can do 6-way sorting, i.e. sorting in different quadrants.
- Cells 0.2 – 100 um in diameter
- Typically in multiwell plates, e.g. 96, 384 or 1536-well plates

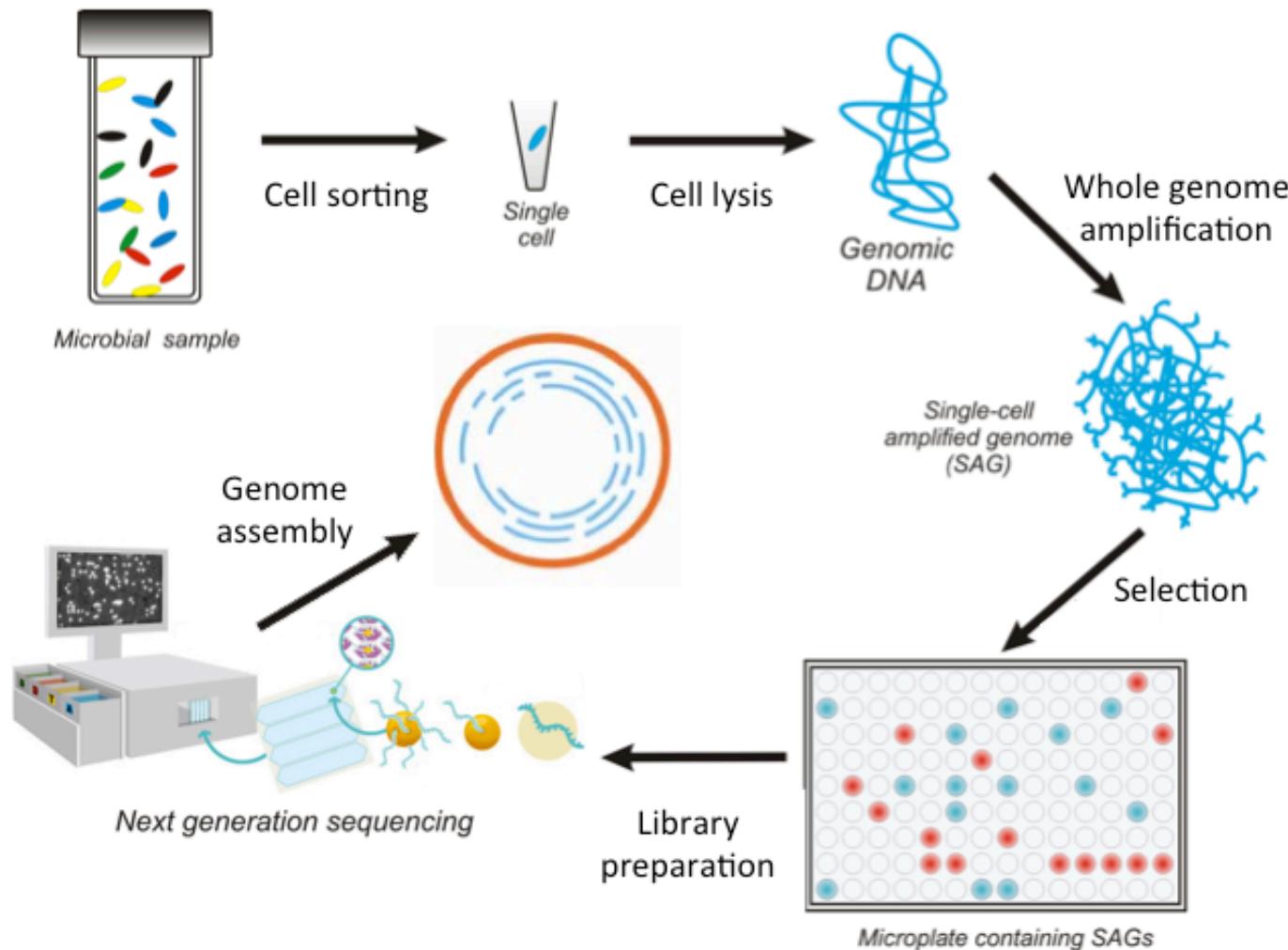
Single cell sorting

- Labelling possible
 - 'Endogenic' markers (fluorophores)
 - Cell size (Side scatter)
 - Specific probes

1. Total, High and Low Nucleic Acids (staining with SYTO-9)
2. Prokaryotes with active electron transfer system (probing with CTC)
3. Prokaryotes with intact cell membranes (staining with SYTO-9 and propidium iodide)
4. Prokaryotes with esterase activity (probing with CFDA)
5. Mitotracker and Lysotracker dyes for staining of mitochondria resp. lysosomes in eukaryotic cells

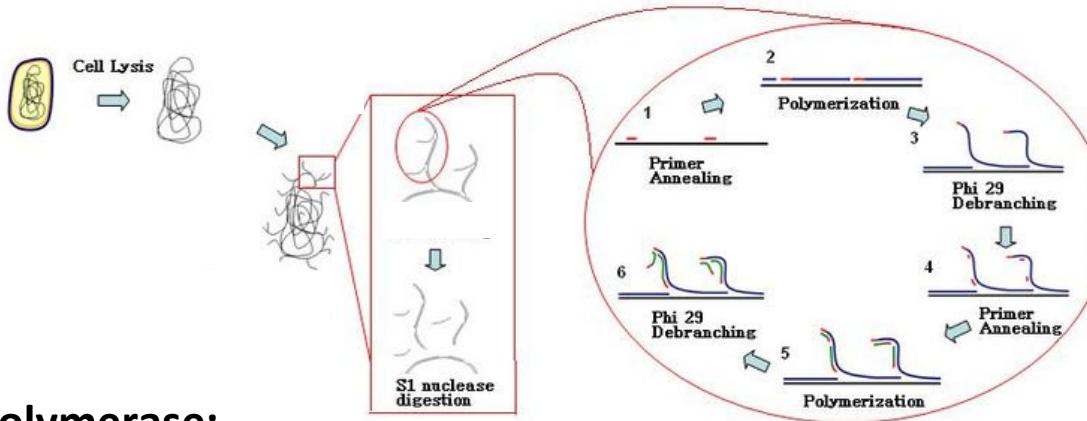


Single cell genomics in a nutshell



Whole Genome Amplification

Multiple-strand Displacement Amplification (MDA)

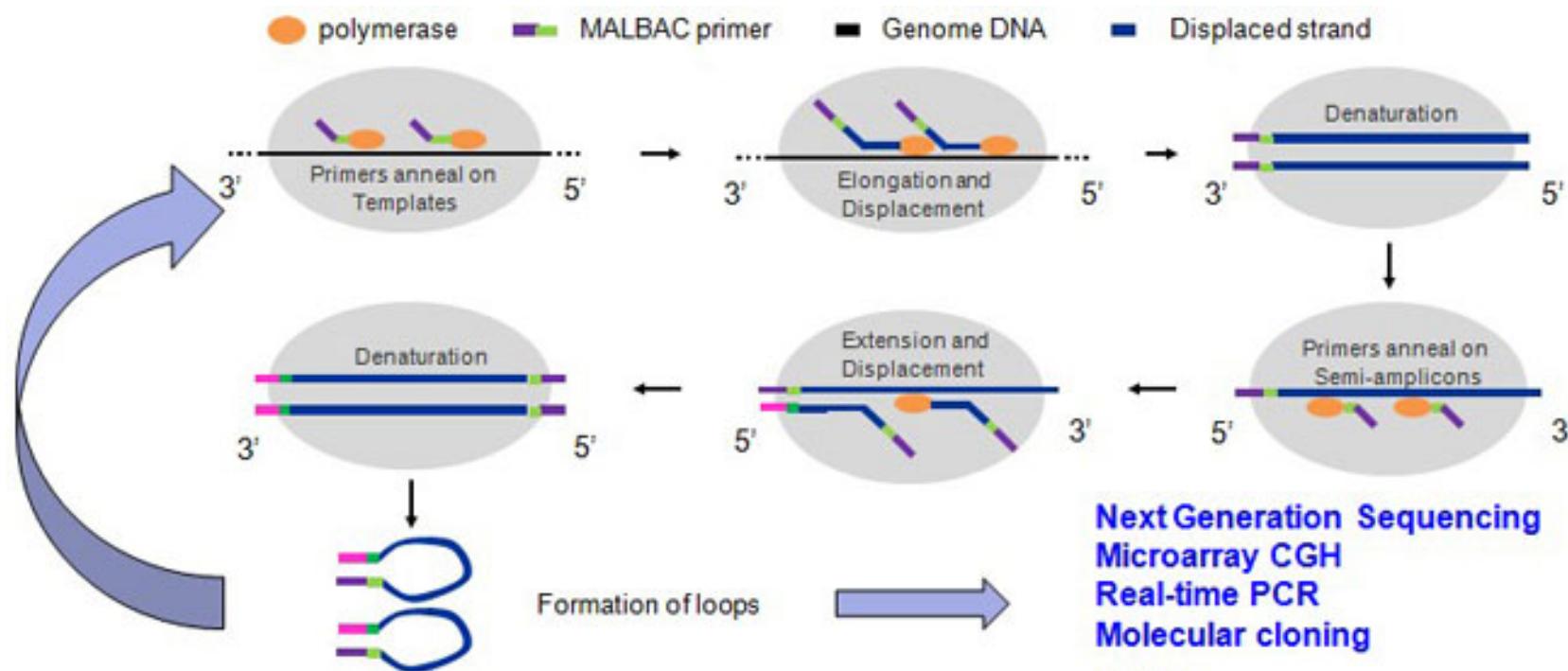


Phi29 DNA polymerase:

- Highly processive (>70 kb DNA fragments)
- Strand-displacement activity allows for efficient isothermal (30 C) DNA amplification
- 3'→5' exonuclease (proofreading) activity:
 - 3'-modified primers recommended
 - High accuracy (~1 error in 10⁶–10⁷ bases)
- Displacement activity causes chimeric DNA fragments (1 chimeric junction per ~5 kb)
 - Large insert libraries not recommended
- Amplification is not uniform: severe amplification bias
- Amplification is not complete (typically 30 – 90%)

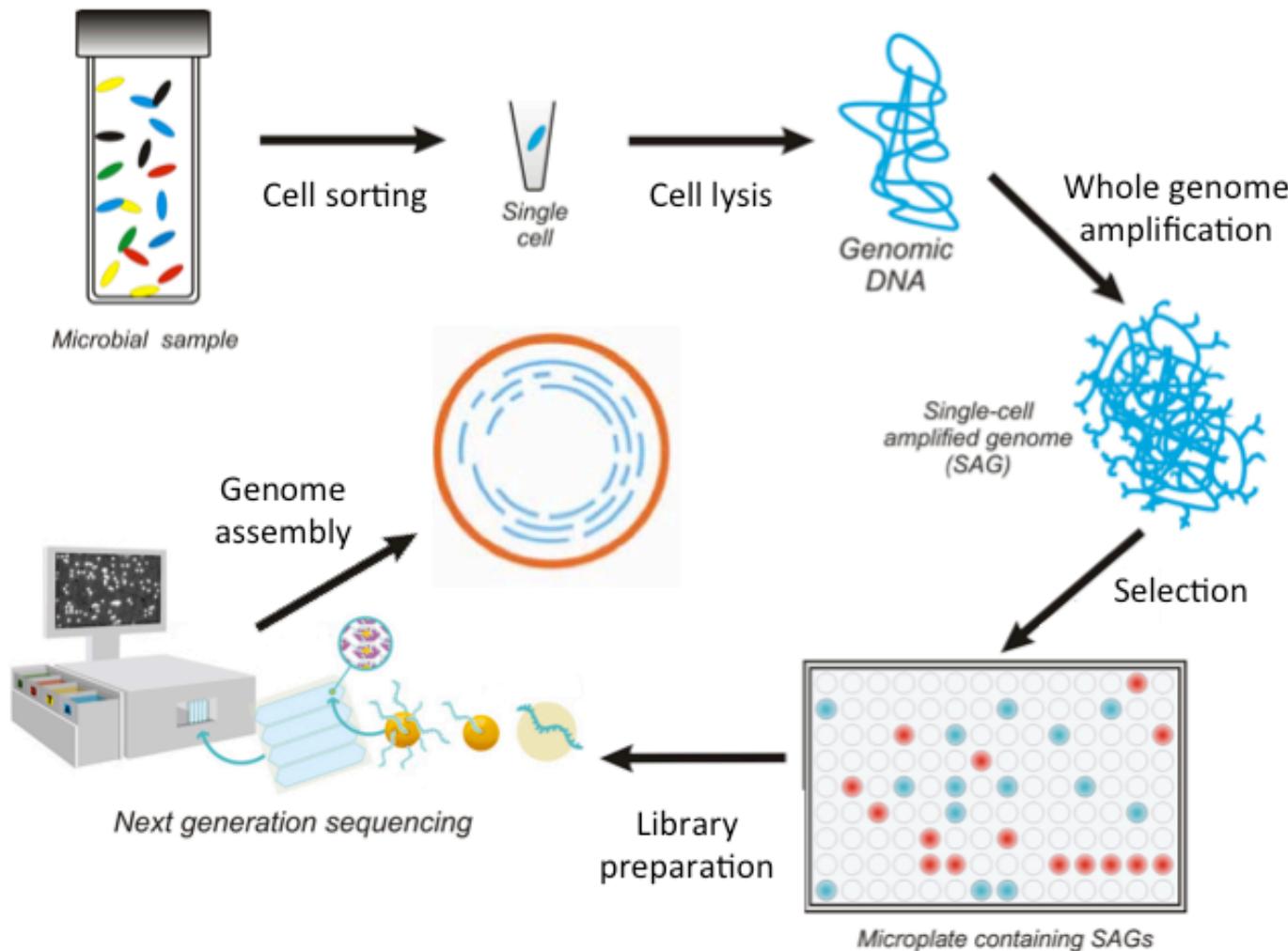
Whole Genome Amplification

Multiple Annealing and Looping Based Amplification Cycles (MALBAC)



- Similar to MDA, except that:
 - Special “MALBAC primers” are used that ‘loop out’ after one amplification cycle
 - Prevents extreme amplification bias and results in more even and more complete genome amplification

Single cell genomics in a nutshell



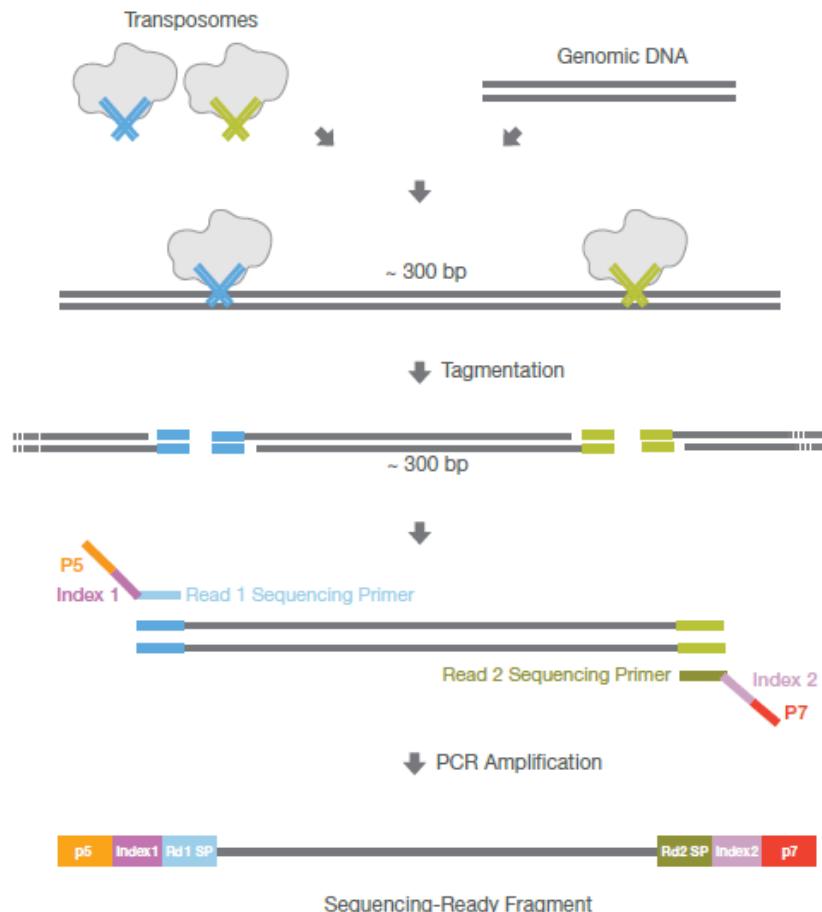
NGS-based SAG sequencing

- Select SAGs or random
- Library creation:
 - Re-MDA (expect more redundancy!)
 - Alt. Use low-input library kits
- Chimera formation
 - Constraints regarding creating LIPE libraries
- Illumina HiSeq2000: sequence 96 SAGs per lane (200-300X per SAG)



Low-input library kits

Illumina NextEra library kit



- Transposon-based DNA “tagmentation”
- NextEra: input = 50 ng (\$80/library)
- NextEra XT: input = 1 ng (\$40/library)
- Insert size ~300 bp
- Multiplexing: Currently 96 barcode combinations available (8X12)

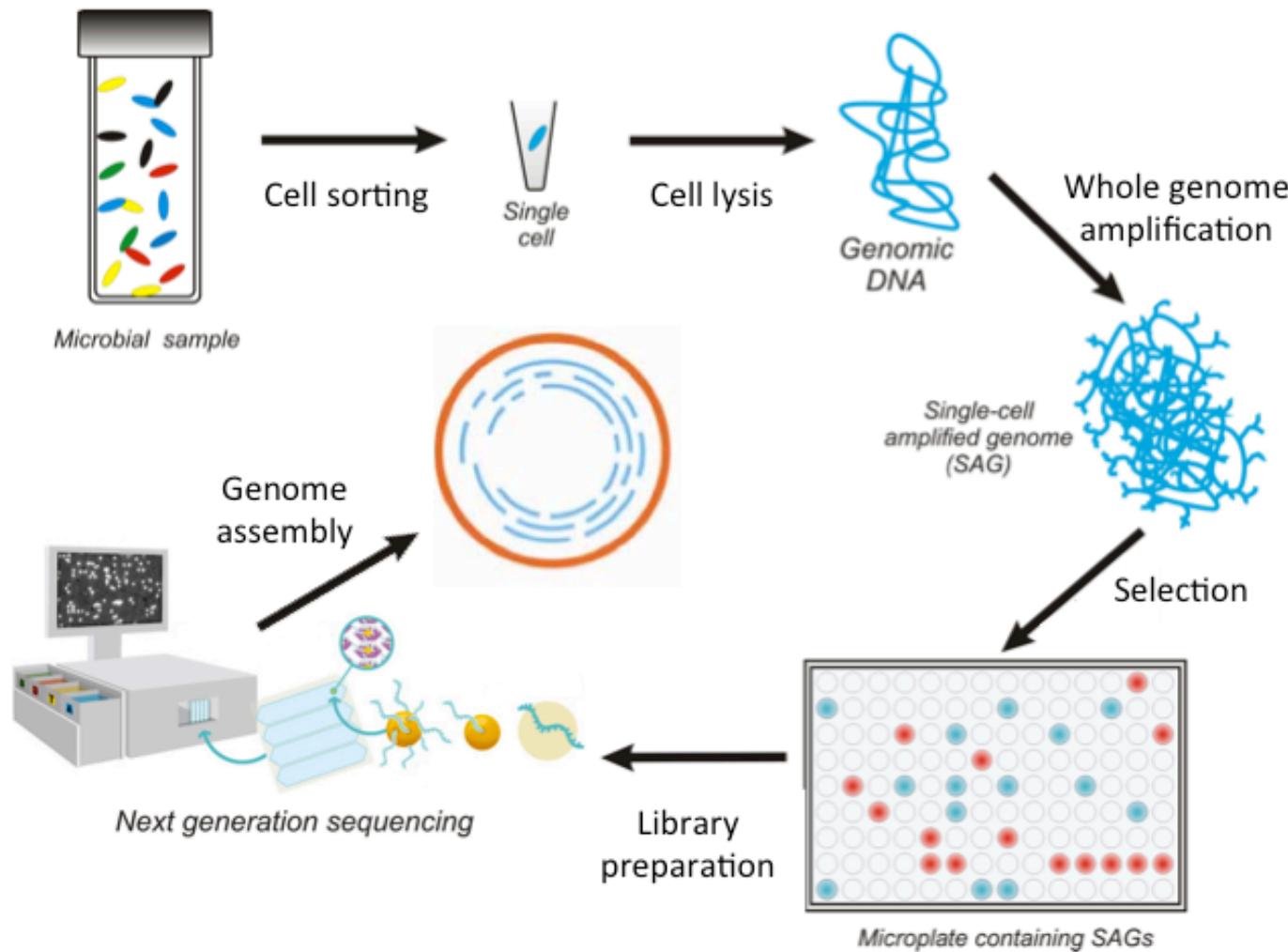
Alternative low-input libraries available from New England Biolabs (NEBNext-Ultra) and several others

NGS-based SAG sequencing

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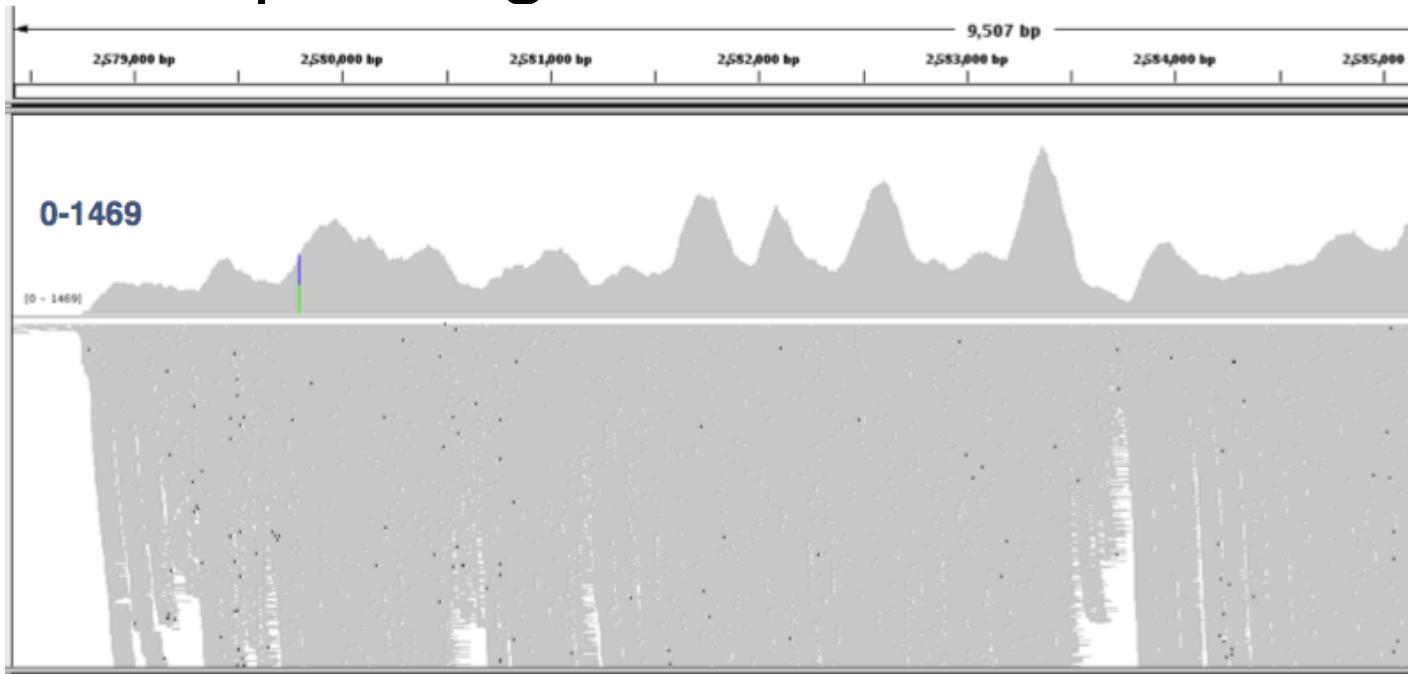


Single cell genomics in a nutshell



Assembly

- Uneven coverage is bottleneck in assembly of SC sequencing data



Available SC-assemblers:

- Velvet-SC (Chitsaz *et al.* (2011) Nat. Biotech. 29, 915-21)
- SPADes (Bankevich *et al.* (2012) J Comput Biol. 19, 455-77)
- IDBA-UD (Peng *et al.* (2012) Bioinformatics 28, 1420-8)

Assembly

- Dedicated SC assemblers outperform ‘normal’ assemblers
 - Velvet-SC: iterative removal of low-depth erroneous contigs
 - IDBA-UD: iterate k -values from small to large to build a more efficient de Bruijn graph
 - SPAdes: Error-correction, Paired de Bruijn graphs (PDBGs), k -bimer adjustment (see lecture Jimmy)

Assembly	NG50	# contigs	Largest	Total length	MA	MM	IND	GF (%)	# genes
Single-cell <i>E. coli</i>									
A5	14399	745	101584	4441145	8	12.01	0.17	89.880	3444
ABYSS	68534	179	178720	4345617	6	3.32	1.68	88.268	3704
CLC	32506	503	113285	4656964	2	5.53	1.42	92.291	3768
EULER-SR	26662	429	140518	4248713	17	10.87	35.67	84.898	3416
Ray	45448	361	210820	4379139	17	6.29	2.83	88.372	3636
SOAPdenovo	1540	1166	51517	2958144	1	1.87	0.11	57.672	1766
Velvet	22648	261	132865	3501984	2	2.19	1.23	73.765	3080
E+V-SC	32051	344	132865	4540286	2	2.35	0.73	91.744	3771
IDBA-UD contigs	98306	244	284464	4814043	8	5.09	0.27	95.210	4045
IDBA-UD scaffolds	109057	229	284464	4813609	8	5.14	0.77	95.199	4052
SPAdes2.5 contigs	109825	250	268493	4799522	1	2.97	0.50	94.917	4032
SPAdes2.5 scaffolds	112342	240	268493	4801710	1	3.54	1.11	94.939	4039

The pros and cons of SCG (cf. metagenomics)

- Pros
 - Obtaining genomic information of individual cell (“Who is doing what?”, within population variation)
 - Assessment of rare cell types via targeted sorting
 - Low sequencing costs, low computational demands
- Cons
 - Current throughput is limited
 - High contamination risk
 - Formation of chimeric artefacts
 - Incomplete genomes

SCG example study

ARTICLE

OPEN

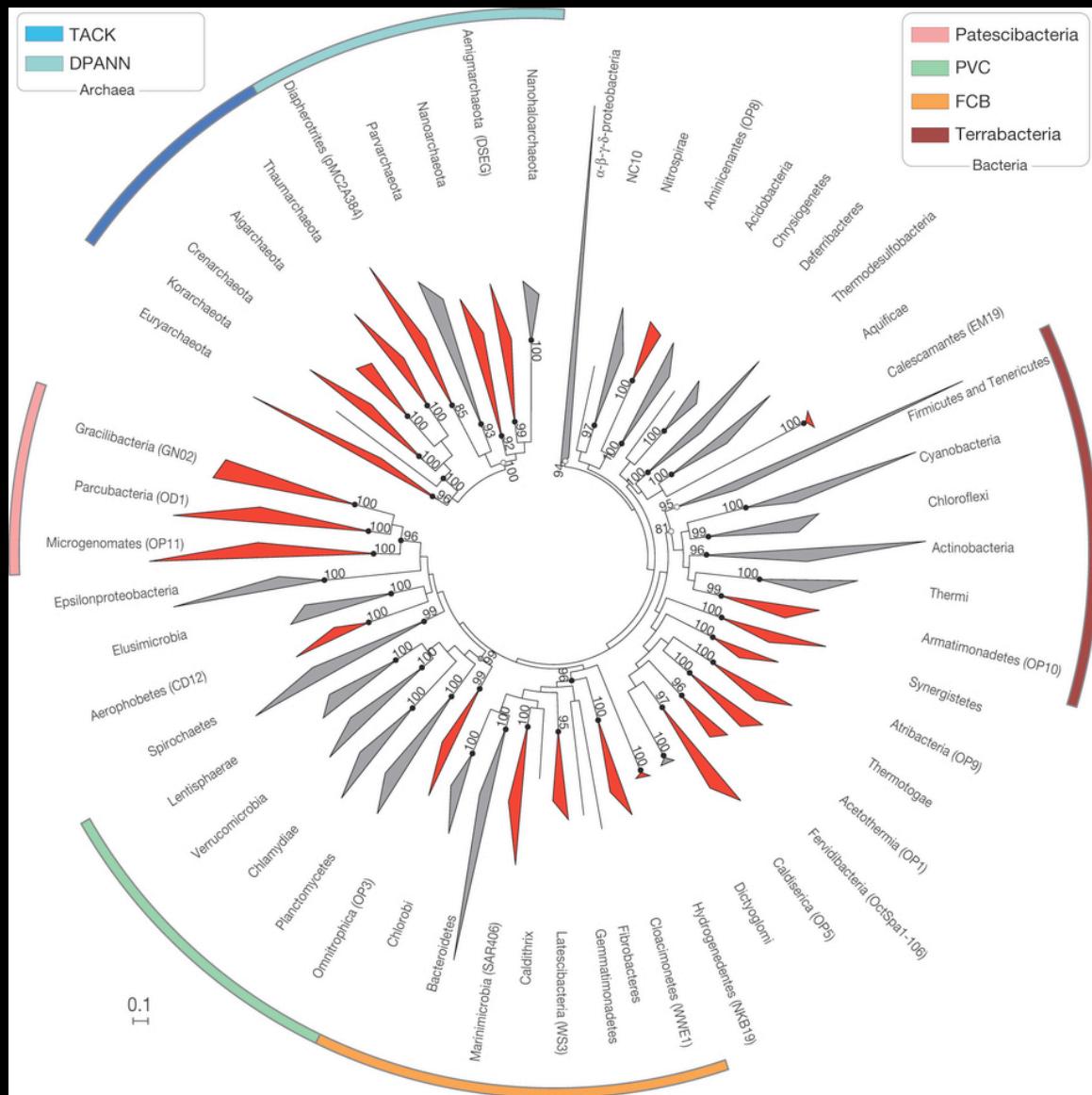
doi:10.1038/nature12352

Insights into the phylogeny and coding potential of microbial dark matter

Christian Rinke¹, Patrick Schwientek¹, Alexander Sczyrba^{1,2}, Natalia N. Ivanova¹, Iain J. Anderson^{1†}, Jan-Fang Cheng¹, Aaron Darling^{3,4}, Stephanie Malfatti¹, Brandon K. Swan⁵, Esther A. Gies⁶, Jeremy A. Dodsworth⁷, Brian P. Hedlund⁷, George Tsiamis⁸, Stefan M. Sievert⁹, Wen-Tso Liu¹⁰, Jonathan A. Eisen³, Steven J. Hallam⁶, Nikos C. Kyrpides¹, Ramunas Stepanauskas⁵, Edward M. Rubin¹, Philip Hugenholtz¹¹ & Tanja Woyke¹

Genome sequencing enhances our understanding of the biological world by providing blueprints for the evolutionary and functional diversity that shapes the biosphere. However, microbial genomes that are currently available are of limited phylogenetic breadth, owing to our historical inability to cultivate most microorganisms in the laboratory. We apply single-cell genomics to target and sequence 201 uncultivated archaeal and bacterial cells from nine diverse habitats belonging to 29 major mostly uncharted branches of the tree of life, so-called 'microbial dark matter'. With this additional genomic information, we are able to resolve many intra- and inter-phylum-level relationships and to propose two new superphyla. We uncover unexpected metabolic features that extend our understanding of biology and challenge established boundaries between the three domains of life. These include a novel amino acid use for the opal stop codon, an archaeal-type purine synthesis in Bacteria and complete sigma factors in Archaea similar to those in Bacteria. The single-cell genomes also served to phylogenetically anchor up to 20% of metagenomic reads in some habitats, facilitating organism-level interpretation of ecosystem function. This study greatly expands the genomic representation of the tree of life and provides a systematic step towards a better understanding of biological evolution on our planet.

SCG example study

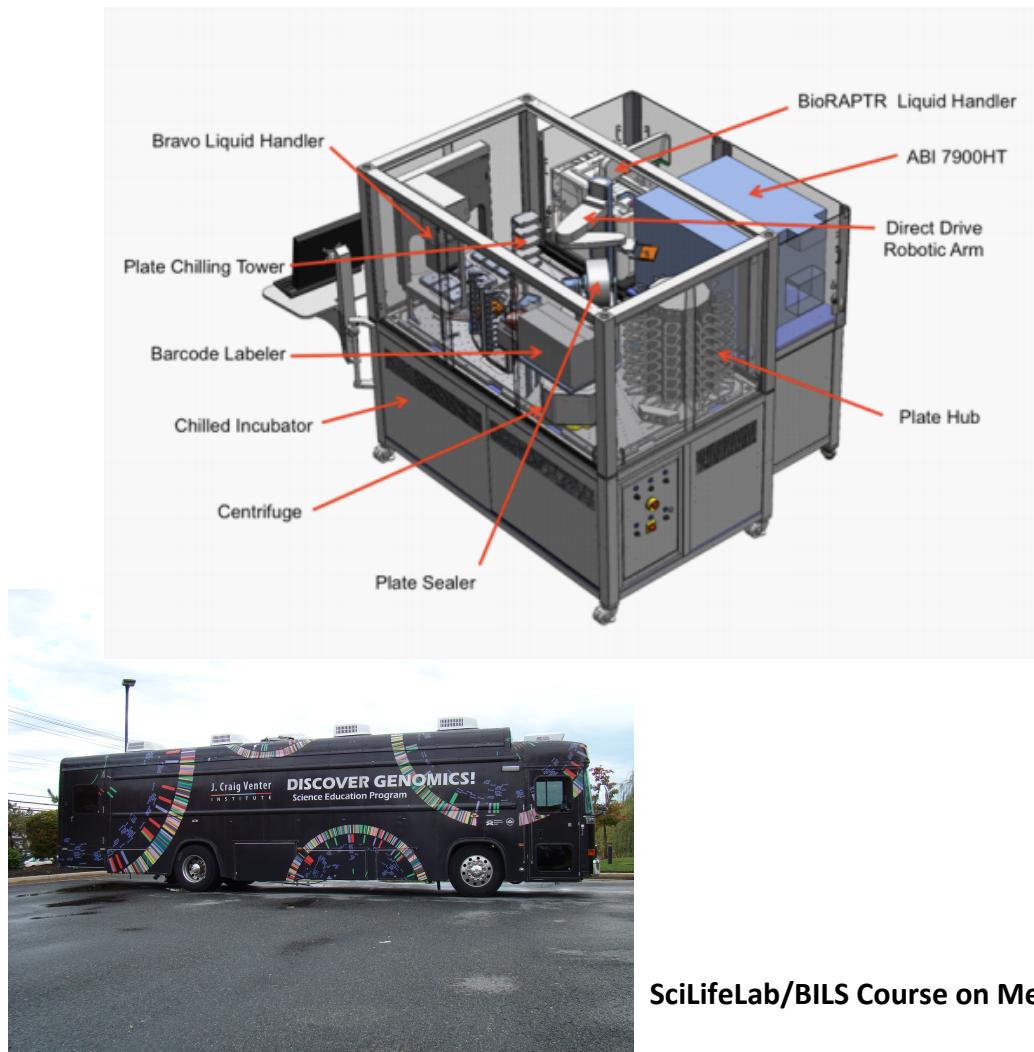


Highlights

- Sequencing of 201 SAGs across the Tree of Life
- Several representatives of proposed novel bacterial and archaeal phyla
- Several newly proposed superphyla:
 - Terrabacteria (B)
 - Patescibacteria (B)
 - DPANN (A)
- Several cases of HGT across the Domains of life
- Bacteria with alternative genetic code (opal stop codon)

Future directions of SCG

- Fully automated-based approaches

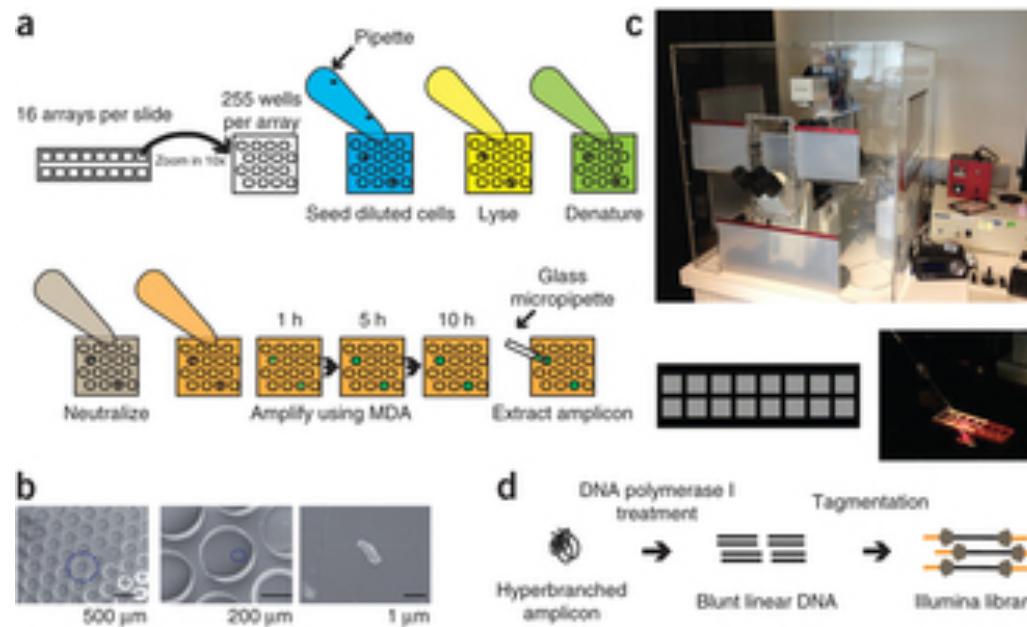


JCVI

- Custom-made SCG robotics setup
- “Normal” FACS-based single cell sorting
- All steps fully automated, except FACS and qPCR screen
- Throughput 5000 SAGs/week

Future directions of SCG

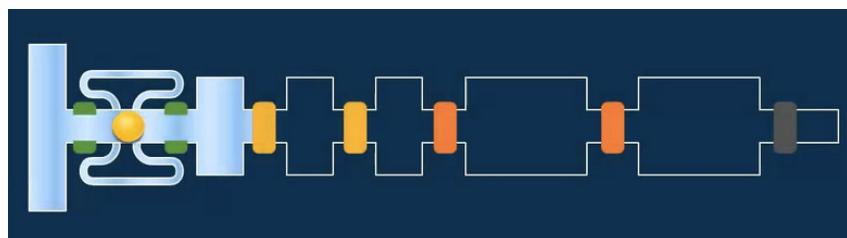
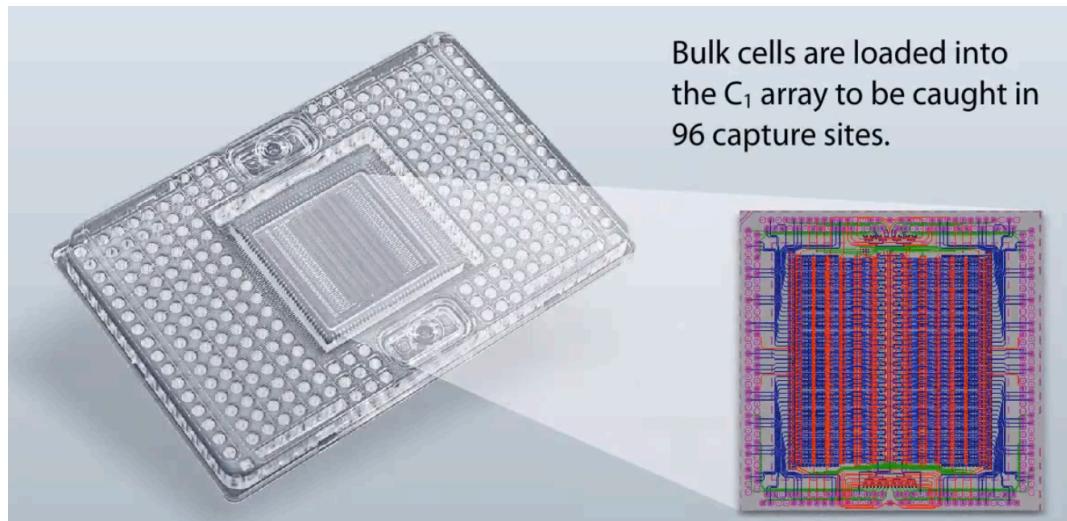
- Microwell-based approaches: MIDAS



- Microwell displacement amplification system (MIDAS)
- Random deposition of cells in microgrid
- Limited amplification (MDA), limits amplification bias
- Limited amplification bias: **increased genome completeness** (avg. 80-90%)
- High throughput: 4080 wells per chip

Future directions of SCG

- Microfluidics-based approaches

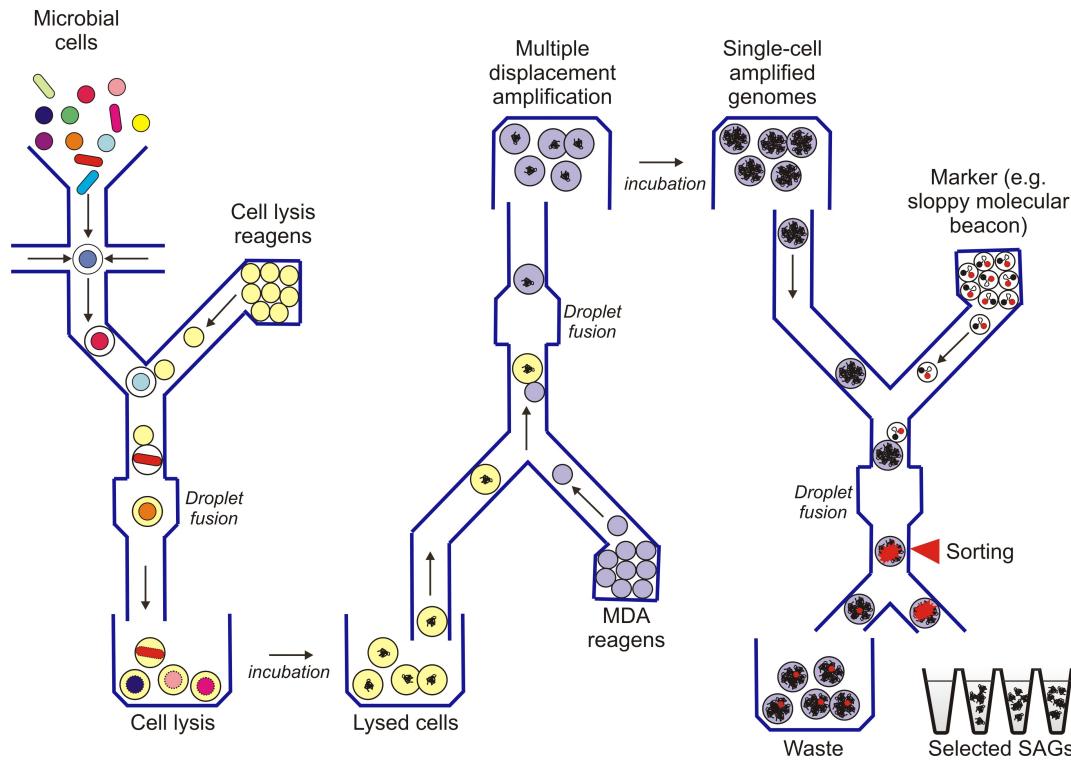


- Fluidigm C1: Integrated solution for single cell WGS and **RNA-seq**
- On-chip cell capture, lysis, (cDNA synthesis) and amplification.
- Limited cell sizes (diameter ~5-25 um)
- Up to 96 wells per chip



Future directions of SCG

- Picoliter droplet-based approaches



Maria Hammond,
Postdoc at KTH

Picoliter volume droplets

Droplet based cell sorting, lysis, amplification and screening

Throughput: Up to 20,000,000 droplets per experiment

SciLifeLab SiCell

Platform for Single Cell Genomics @ Uppsala University



Stefan Bertilsson
Director



Thijs Ettema
Platform manager



Claudia Bergin
Scientist



Anna-Maria Divne
Scientist

- Currently running test projects
- Open as a service-based platform late 2013/early 2014

SciLifeLab SiCell



MoFlo AstriosEQ FACS

- 5 lasers (355, 405, 488, 532 & 642 nm)
- 6-way sorting
- IntelliSort - beadless drop-delay determination
- Biosafety hood



Biomek NxP liquid handler

- 384-well pipetting head
- Automated serial plate dilution
- Setting up cell lysis incubation and qPCR reactions



BMG Fluostar Omega Platereader + Stacker

- Filter-based fluorescence, absorbance and luminescence
- Top and bottom reading
- Temp control 5° C – 42° C
- Stacker has capacity of 50 plates