

Best practices for sample prep and nuclei isolation for (sc)ATAC-seq

Karin Prummel

Postdoctoral fellow Zaugg and Saka groups

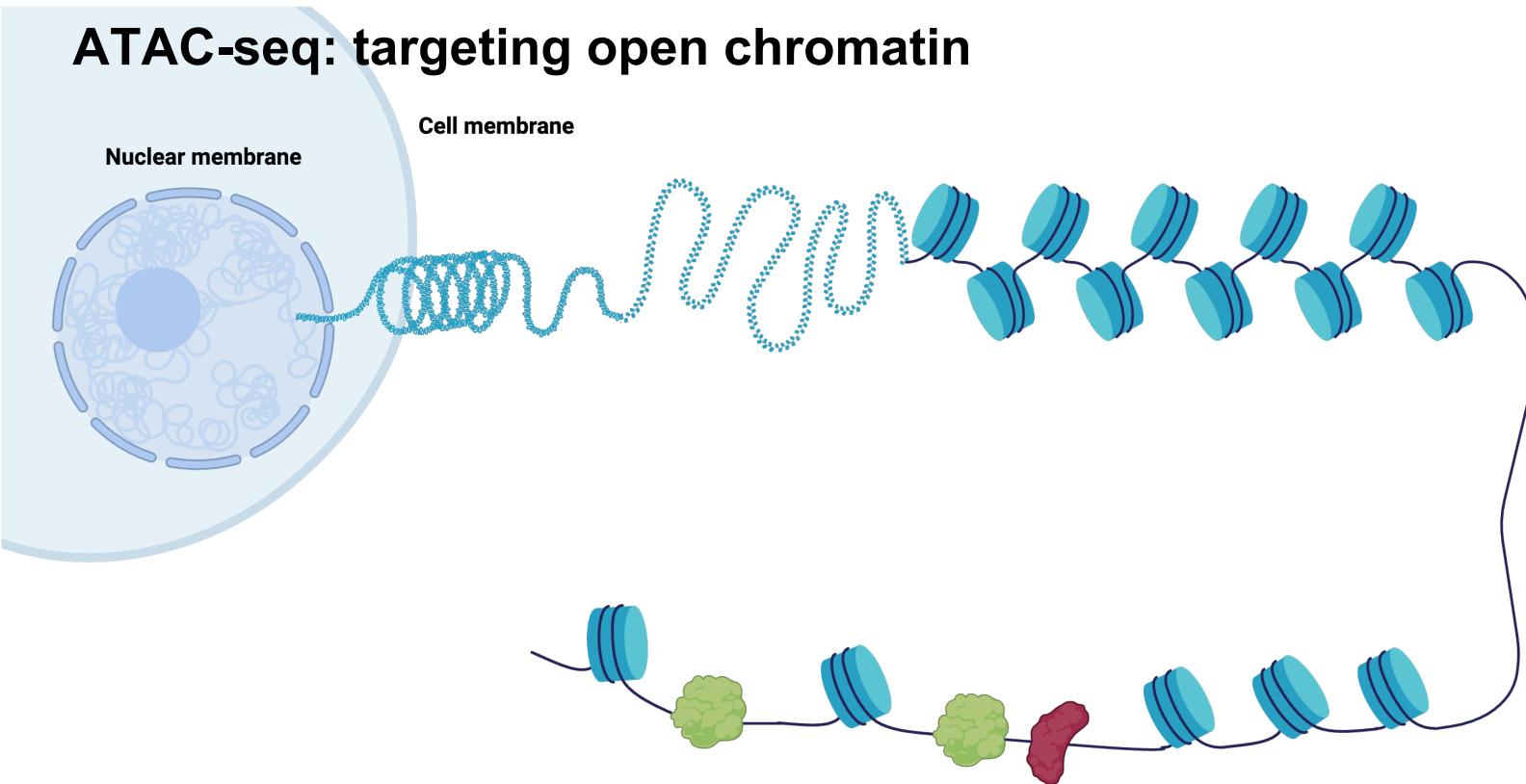
08.04.2024 EMBL scATAC-seq course



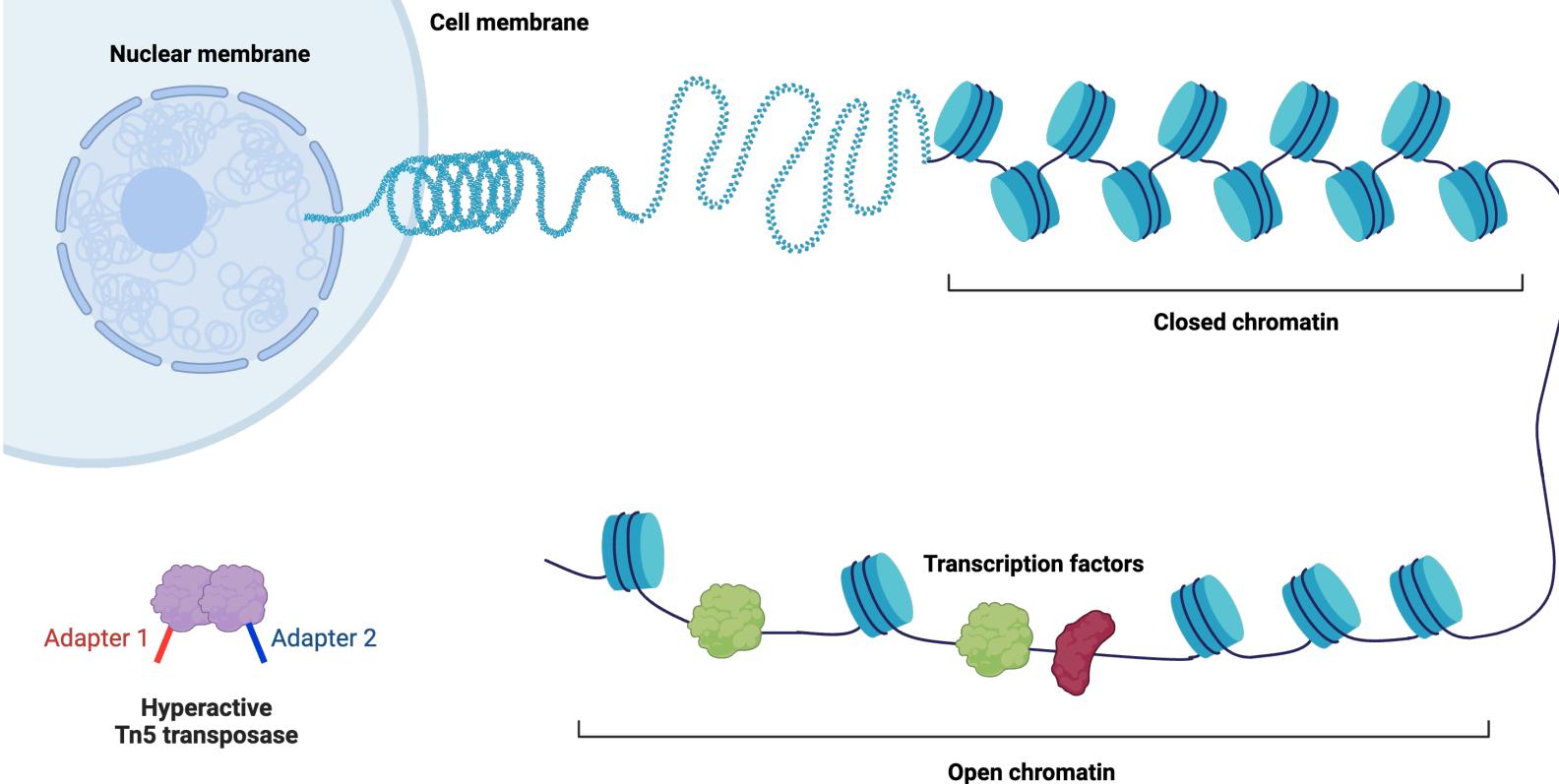
Outline

1. Nuclei isolation for ATAC sequencing
2. Methods and process of nuclei isolation / cellular permeabilization
3. Quality assessment
4. Challenges and considerations
5. Possibilities for fixation and freezing before ATAC-seq
6. Conclusions and Q&A

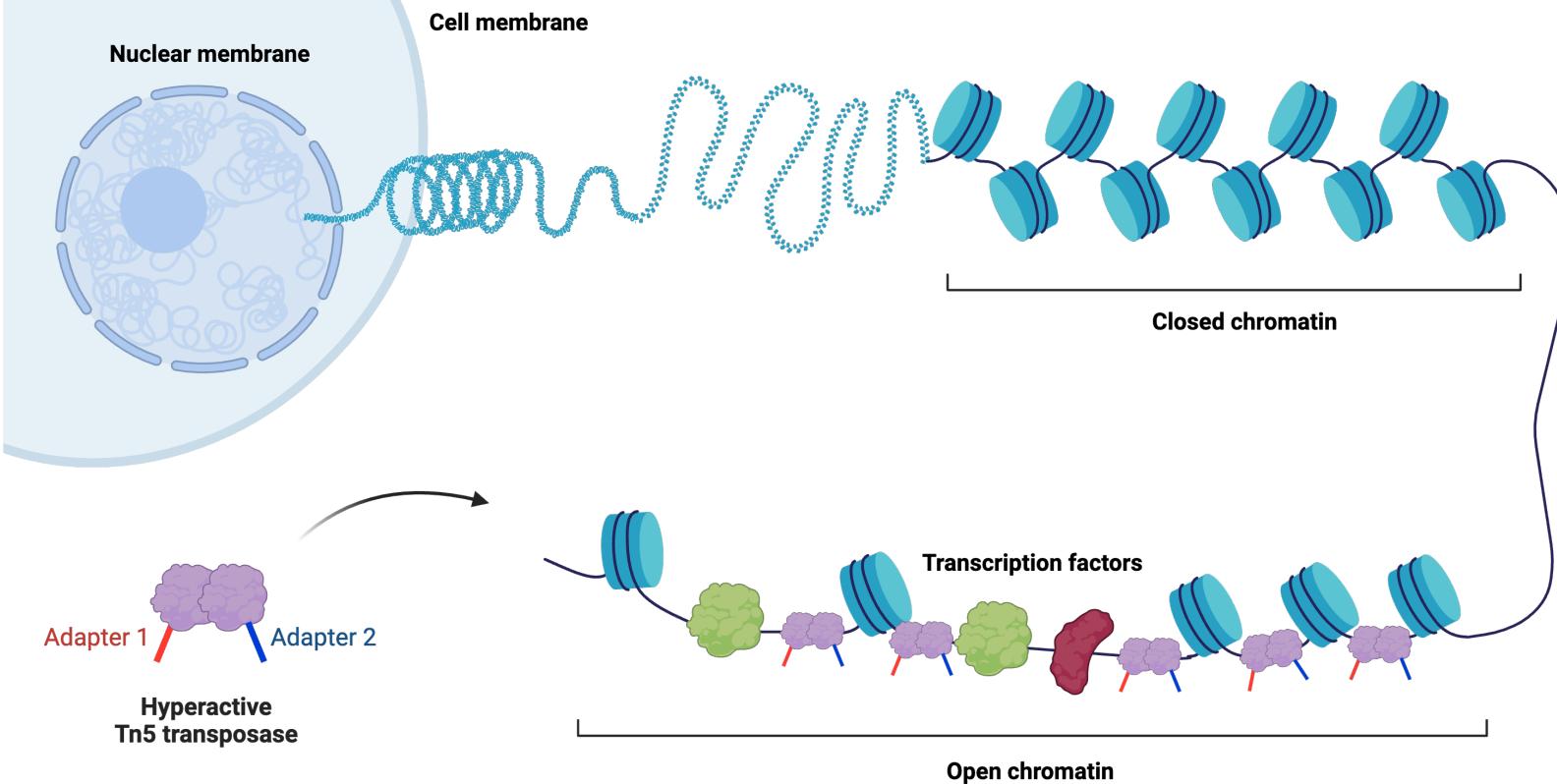
ATAC-seq: targeting open chromatin



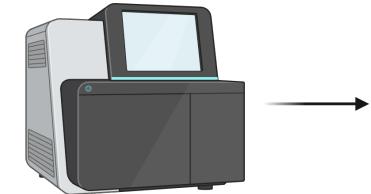
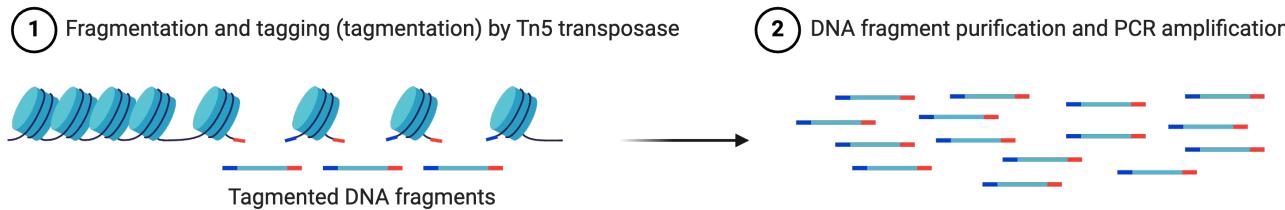
ATAC-seq: targeting open chromatin



ATAC-seq: targeting open chromatin



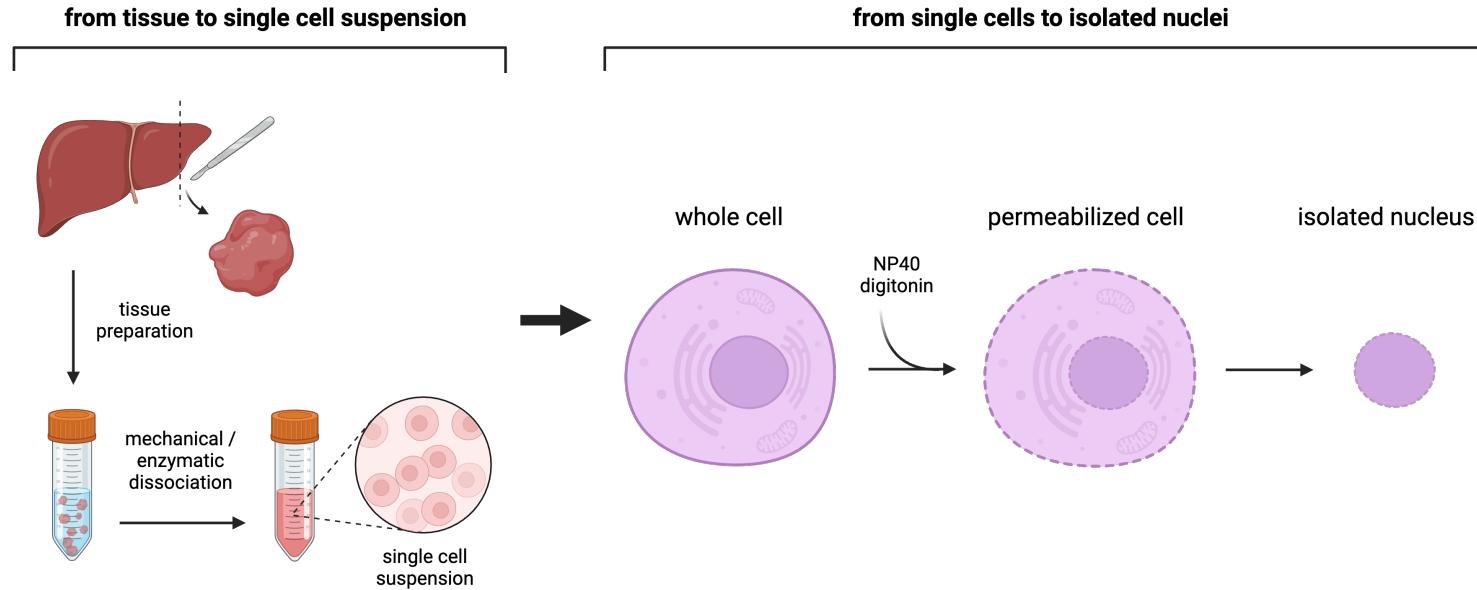
ATAC-sequencing



Important for a good quality ATAC-seq library:

- ❖ Chromatin intact
- ❖ Chromatin accessible for Tn5
- ❖ No cellular debris

Principle of nuclei isolation



Methods for nuclei isolation

Mechanical disruption: risk to damage cells / nuclei

- ❖ Dounce homogenization: soft tissue like brain
- ❖ Cryogenic



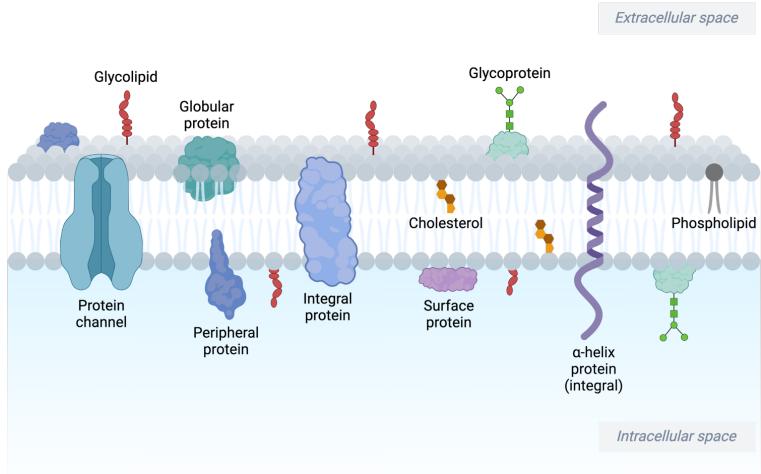
Enzymatic digestion: can lead to increased stress response in the cell

- ❖ Longer enzymatic digestion can result in nuclei isolation, by disrupting membrane structure
- ❖ Trypsin: epithelial tissues
- ❖ Collagenase: connective tissue
- ❖ Papain: soft tissue

Chemical lysis: cell membrane and nuclear membrane permeabilization

- ❖ Reagents that break down structures in the membranes, so enzymes can enter

Membrane permeabilization so enzymes like Tn5 can enter



Use of **mild detergents** to solubilize elements in the lipid bilayers

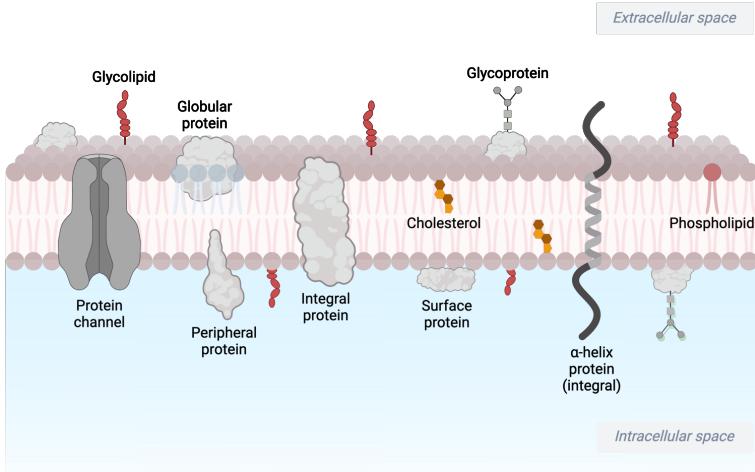
Detergents:

- ❖ **NP40 (Igepal)**: primary permeabilization reagent, allowing permeabilization of both plasma and nuclear membranes
- ❖ **Digitonin**: steroidal saponin that binds to cholesterol and related molecules
- ❖ **Tween-20**: weak permeabilization agent, serves primarily to prevent non-specific binding of mitochondria to the nuclear membrane
- ❖ **Triton X-100**: stronger permeabilization agent, not advised

Protective agents:

- ❖ **DTT**: reducing agent, e.g. preserves chromatin structure
- ❖ **RNase and/or protease inhibitors**: prevent RNA and protein degradation during isolation (i.e. for 10X Multiome)
- ❖ Spermidine and spermine: antioxidants and stabilizers

Membrane permeabilization so enzymes like Tn5 can enter



Use of **mild detergents** to solubilize elements in the lipid bilayers

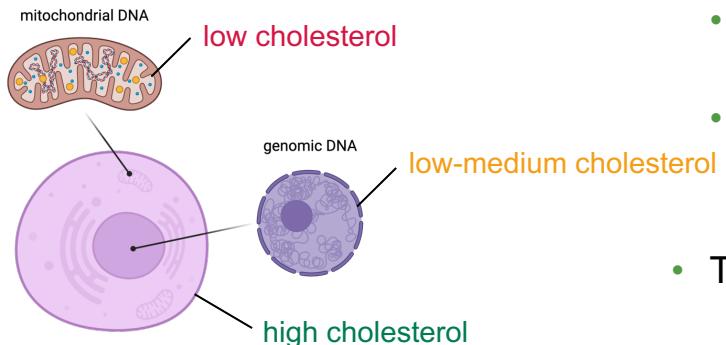
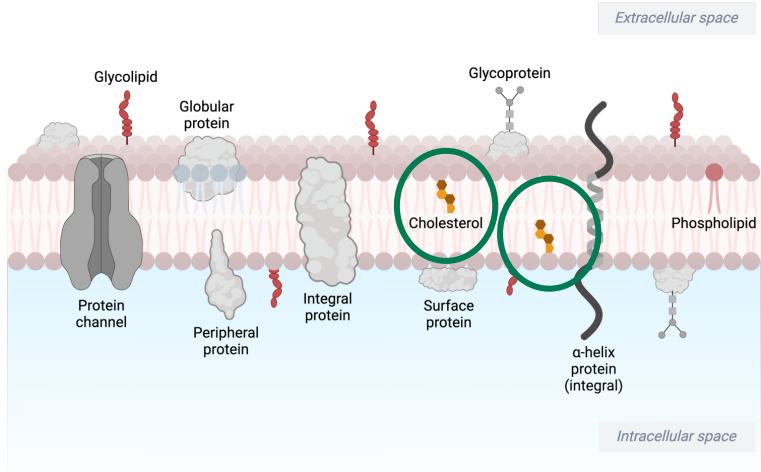
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Membrane permeabilization: think about their composition



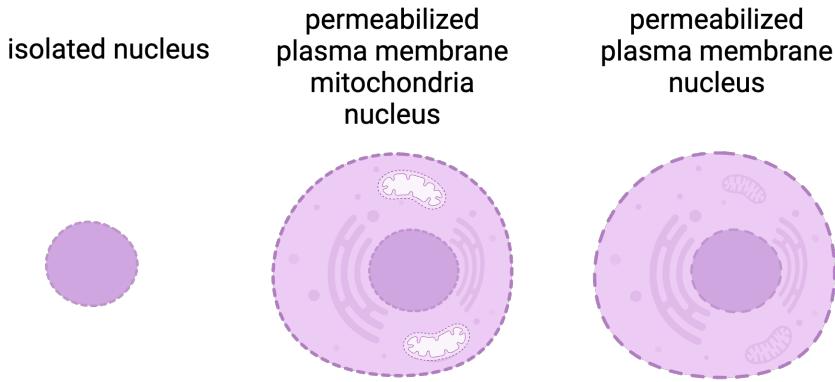
Mitochondria can be problematic

- Lysed mitochondria can lead to a high fraction of reads mapping to mito DNA
 - mito DNA = not chromatinized → Tn5-accessible
 - costly to capture enough reads of gDNA
- Keep **composition of membranes** in mind
 - Mitochondrial membrane: very low in cholesterol
 - Nuclear membrane: cholesterol present, but may vary in cell type and physiological condition
 - Plasma membrane: cholesterol major component of total lipid content
- Tween-20 and Digitonin important

Optimization of the lysis step

- Ensure that the majority of the cell membranes are lysed so Tn5 can access the nucleus
- **Prevent over-lysis** of the nuclear membrane, which can result in **leakage** of nuclear contents → increase in ambient material, so background signal
- Start with a sample with **high viability!** →
 - careful digestion
 - FACS enrichment
 - column-based clean up (StemCell, Miltenyi)
- Perform a **time course** for your sample / cell type:
 - ❖ Robust cell types: 5 – 20 min
 - ❖ Sensitive cell types: 2 – 5 min
- Play with the **buffer concentrations** for optimal dissociation (NP40 and Digitonin)
- Generally: work fast to prevent over-lysis!
 - ❖ Add wash buffers immediately
 - ❖ Centrifuge immediately

Different “severities” of lysis



omni-ATAC

10 mM Tris-HCl pH 7.4
10 mM NaCl
3 mM MgCl₂
0.1% NP40
0.1% Tween-20
0.01% digitonin
1% BSA

Low Loss Lysis (LLL)

10 mM Tris-HCl pH 7.4
10 mM NaCl
3 mM MgCl₂
0.1% NP40
1% BSA

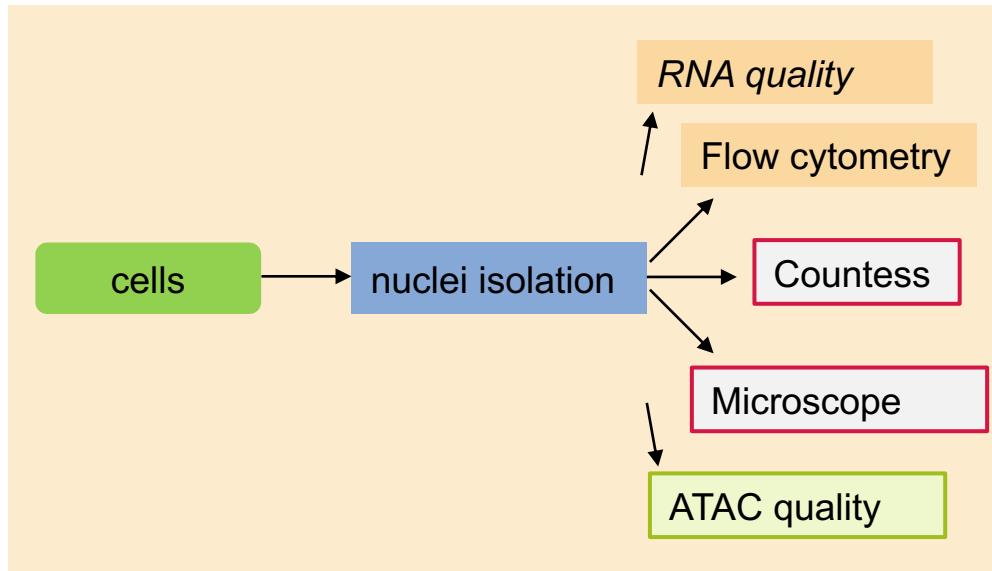
Digitonin permeabilization

20 mM Tris-HCl pH 7.4
150 mM NaCl
3 mM MgCl₂
0.01% - 0.20% digitonin

10X protocol

Omni-ATAC: Corces et al., 2017 Nat. Methods
LLL + digi: Mimitou et al., 2021, Nat. Biotech.

How to evaluate your nuclei quality?

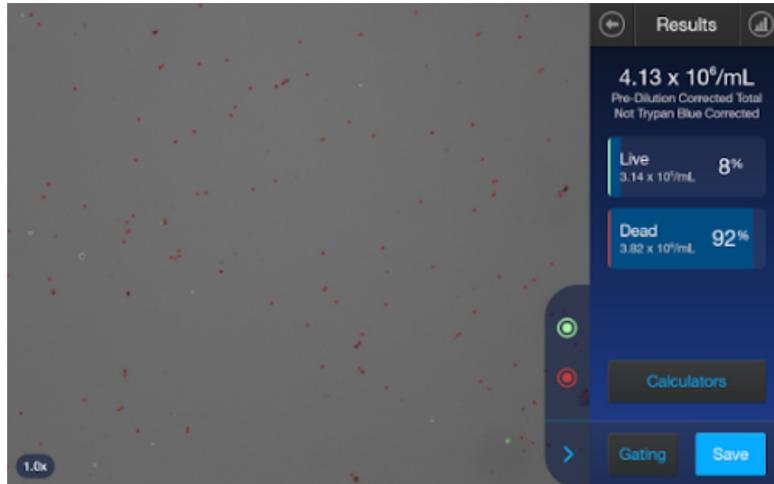


Cell counting and lysis assessment

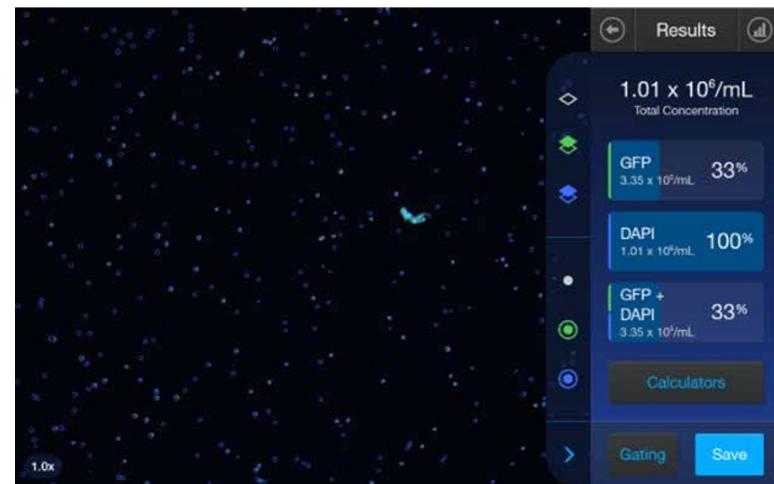
Countess

- Use DAPI (positive selection) or Tryphan blue (“negative” selection)
- Aim for > 90% lysis (DAPI+)

Tryphan blue

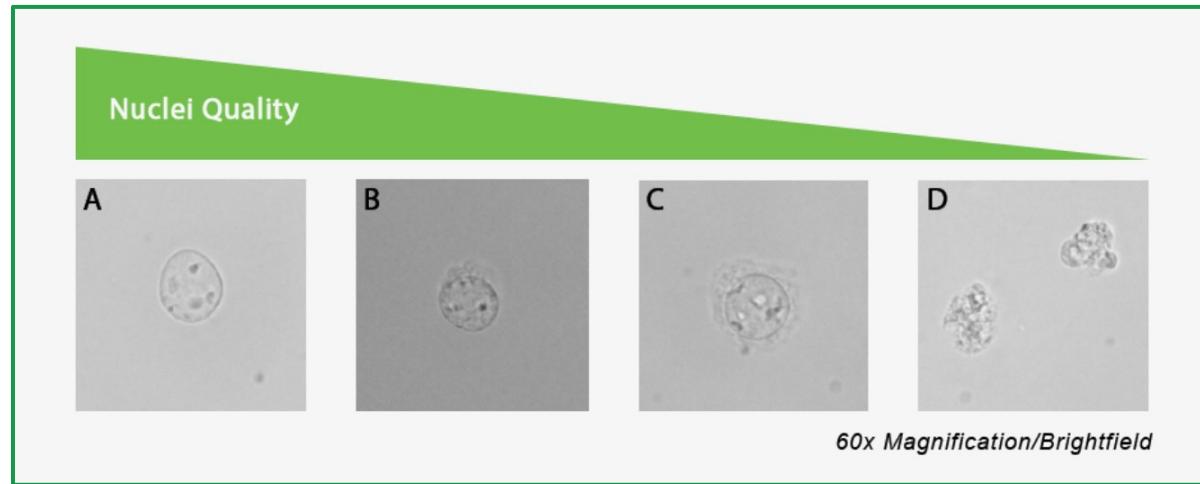


DAPI



Microscopy for morphology

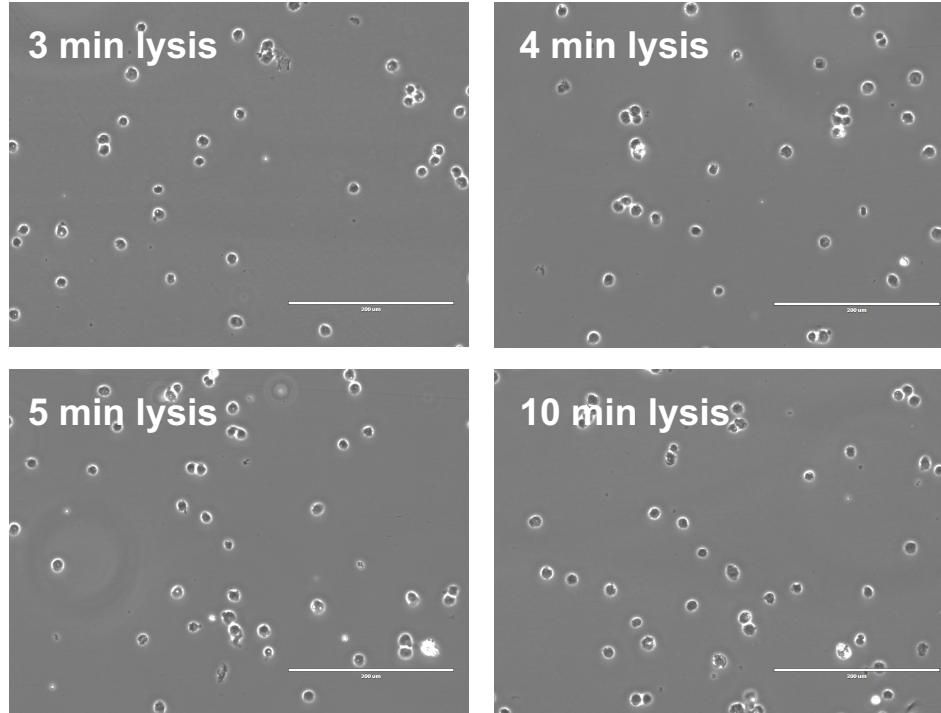
Microscope



Microscopy for morphology

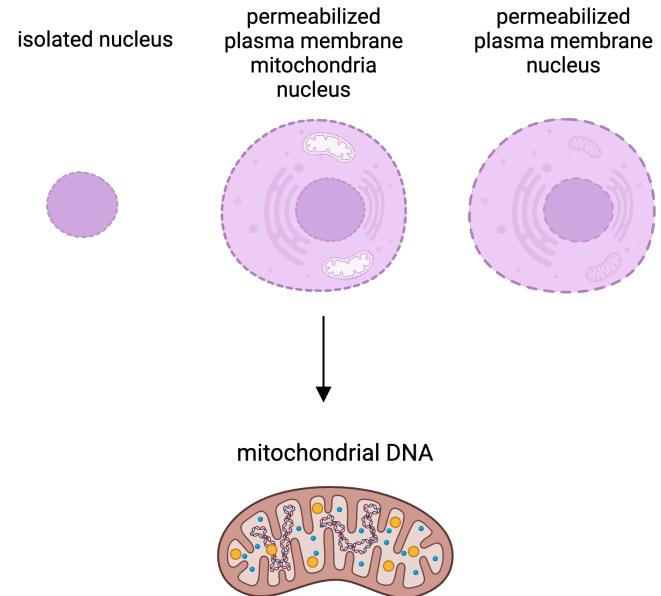
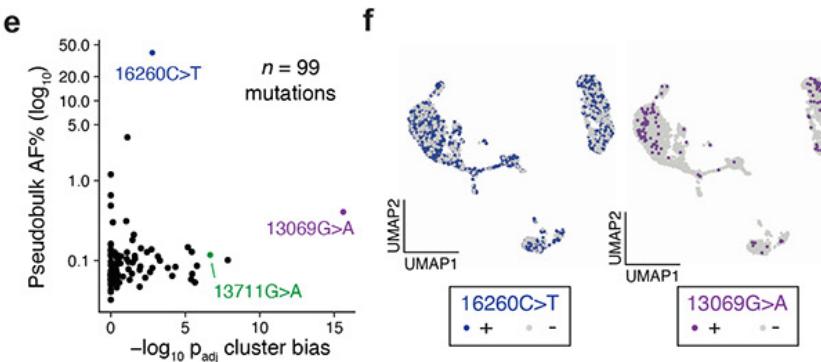
Microscope

Our mouse ESCs 😊



When would you like to keep mito DNA?

- Profiling mito DNA mutations in ATAC-seq data
- Can be used for **clonal tracking**

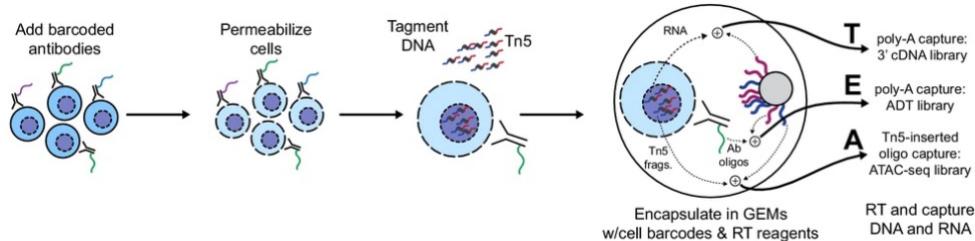


- A bit of mtDNA in your library *could* be cool 😊

How much permeabilization needed depends on your experiment!

Evaluating RNA + ATAC + surface proteome from the same cells

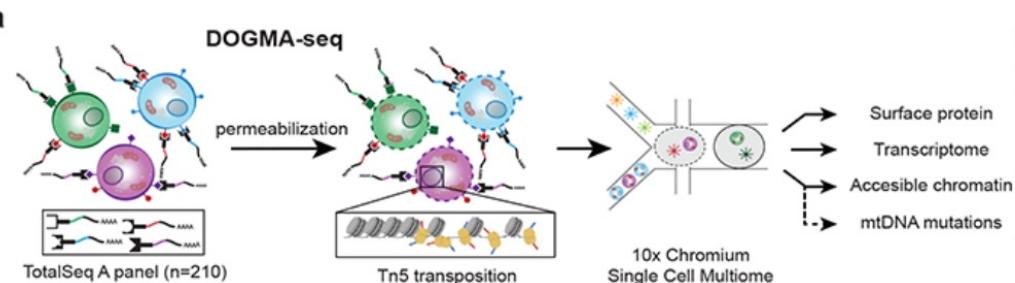
TEA-seq (Swanson et al., 2021, *Elife*)



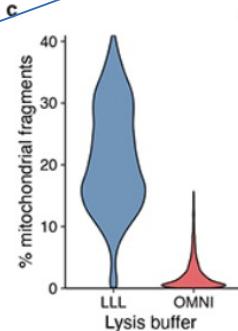
Digitonin perm.
20 mM Tris-HCl pH 7.4
150 mM NaCl
3 mM MgCl₂
0.01% - 0.20% digitonin

Low loss lysis (LLL)
10 mM Tris-HCl pH 7.4
10 mM NaCl
3 mM MgCl₂
0.1% NP40
1% BSA

DOGMA-seq (Mimitou et al., 2021, *Nat. Biotech.*)



Multiome	DOGMA
Standard	Digitinin ✓ LLL ✓
✓	✓ ✓ ✓
✓	✓ ✓ ✓
✗	✗ ✓ ✓



Optimize whole nuclei isolation workflow for your sample!

- The **ATAC-seq assay itself** requires little optimization of the reaction parameters for different cell types
 - Tn5 is not as sensitive as DNase and MNase to over- or underdigestion
 - Tn5 is a non-catalytic enzymatic reaction: 1 Tn5 molecule can only perform 1 single transposition reaction
- **Nuclei isolation** requires sample and assay specific optimization!
 1. Method of tissue dissociation (if needed): mechanical or enzymatic
 2. Chemical isolation for permeabilization: **concentration** of detergents in buffers and **lysis times**, composition wash buffers
 3. Nuclei clean up

Cleaning up your isolated nuclei suspension

As little as debris as possible

- prevent clumping / clogging 10X chip
- will reduce the background in the seq data

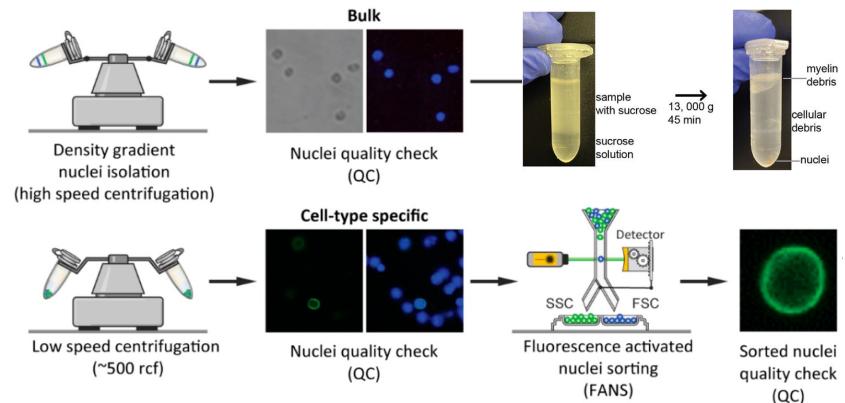
❖ Filtering: 40 µm → removes clumps



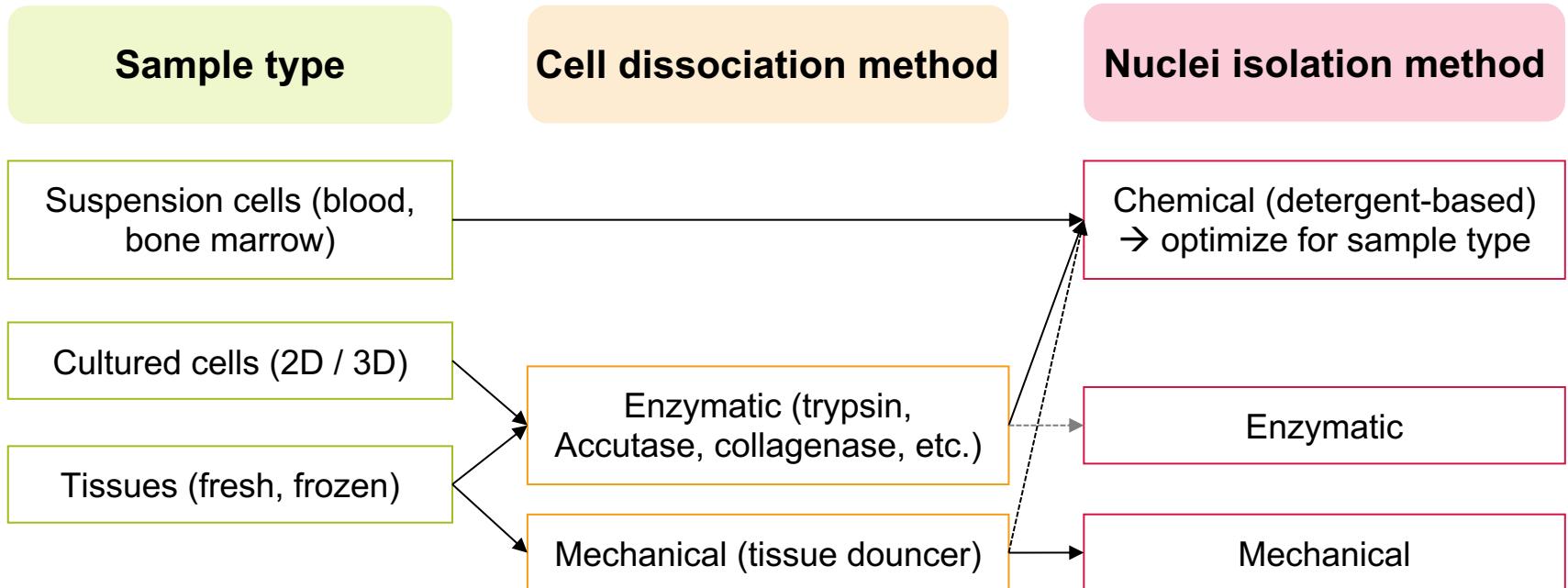
Dirty sample?

1. Sucrose gradient (density gradient)
2. FACS / FANS
3. Glycerol pelleting

- lots of cell-loss with clean up
- start with high cell number

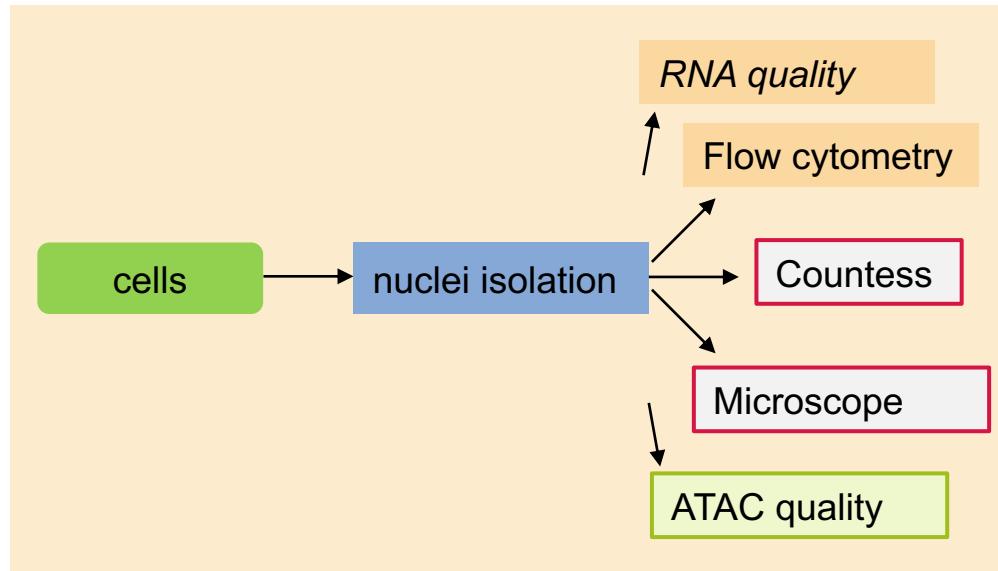


Nuclei isolation guideline depending on your sample type



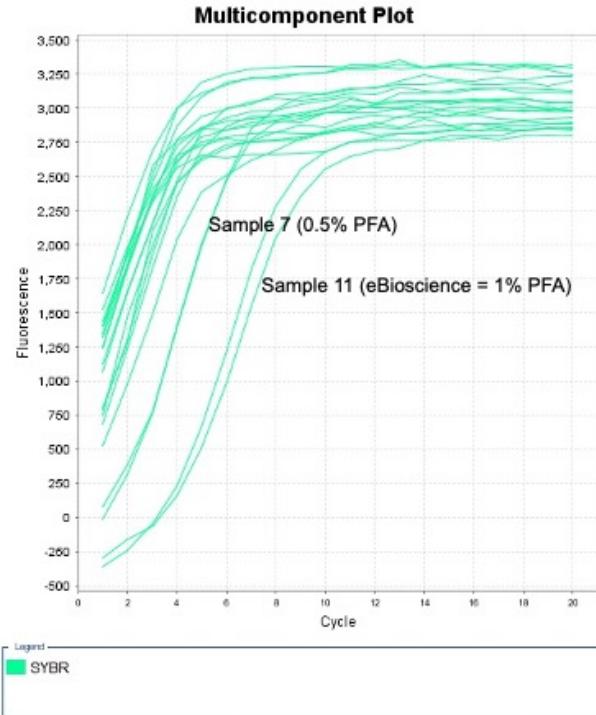
mechanical: cell dissociation and nuclei isolation often coupled

How to evaluate your nuclei quality?



How to evaluate a successful ATAC library?

ATAC quality



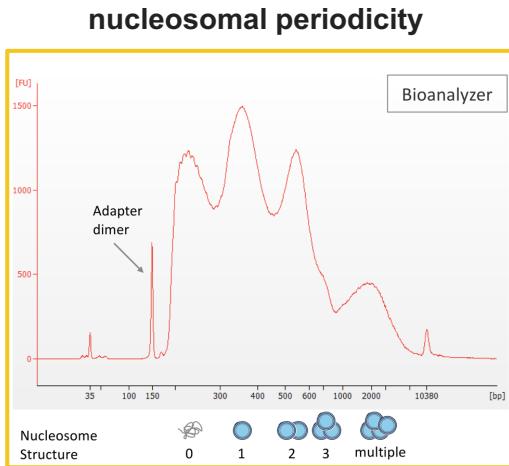
When optimizing your samples:
generate a bulk ATAC library for new sample types
before using 10X protocols

- qPCR to determine library size / complexity
- Determine how many cycles of library amplification is necessary for a sufficient library size
- Lots of extra cycles:
 - Low quality library
 - Low cell input
 - Very transcriptionally inactive cells

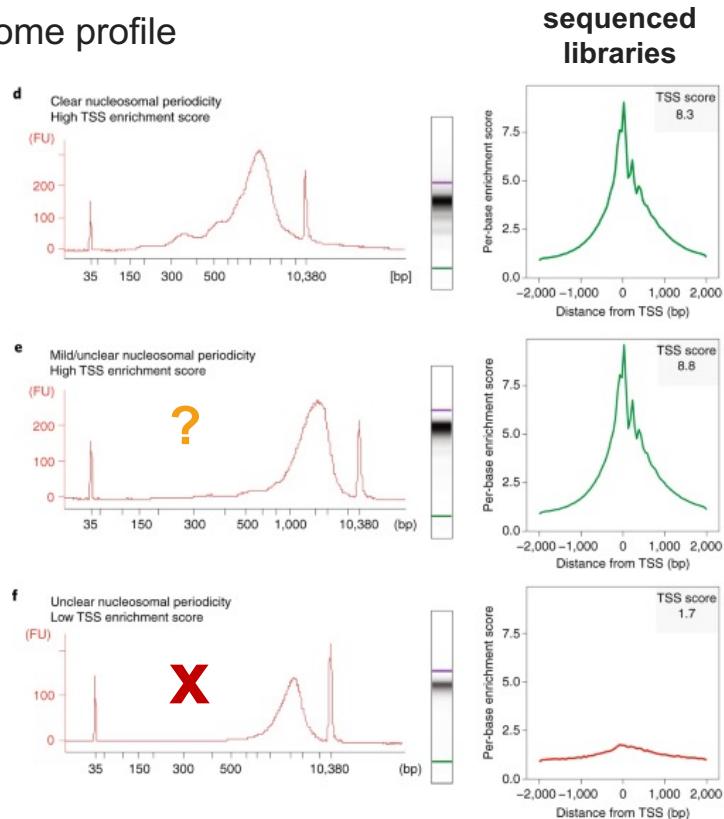
How to evaluate a successful ATAC library?

ATAC quality

- Bioanalyzer electropherogram for nucleosome profile



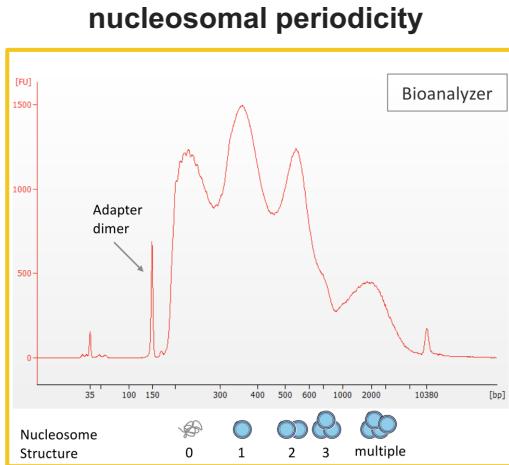
TSS = transcription start site
Open chromatin around promoter
QC for library quality



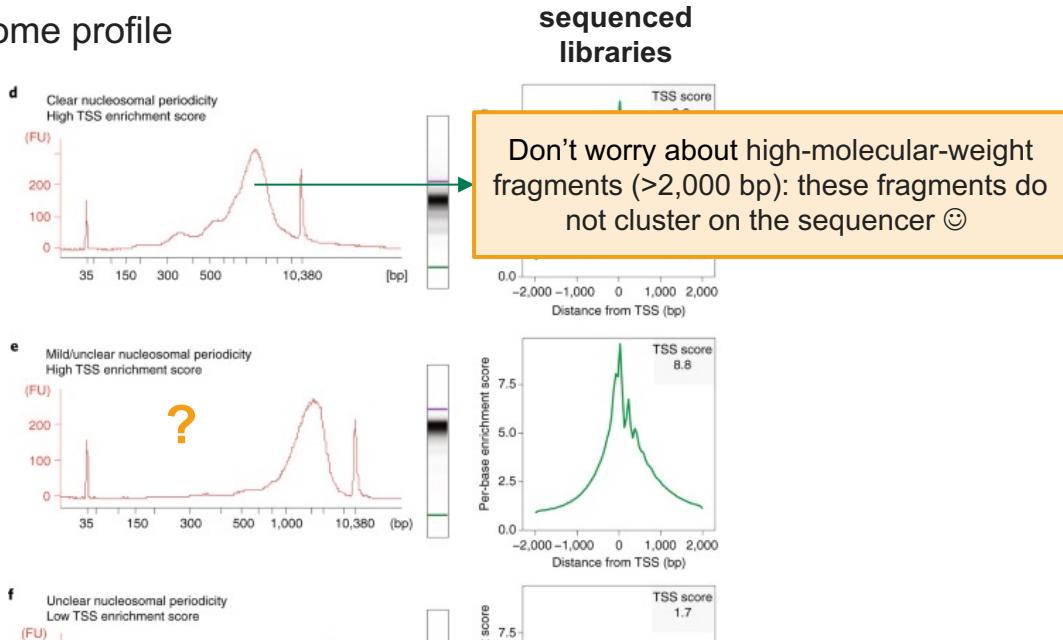
How to evaluate a successful ATAC library?

ATAC quality

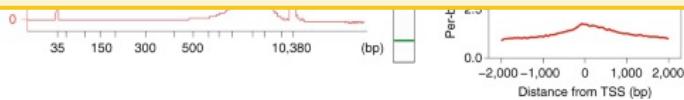
- Bioanalyzer electropherogram for nucleosome profile



TSS = transcription start site
Open chromatin around promoter
QC for library quality



Tissues / cell types have different transcriptional activity!



How to evaluate a successful ATAC library?

- Option: low-depth sequencing → 50,000– 100,000 reads / sample of shallow sequencing
- Tn5-accessible chromatin can be found at **promoters**, located proximal to the transcription start site (TSS), and at intergenic regions corresponding to **enhancers**, insulators or silencers

- (i) enrichment of transposase insertions in regions of known chromatin accessibility (signal-to-background ratio)
- (ii) total number of unique fragments (library complexity)
- (iii) ratio of sequencing reads mapping to the nuclear genome (desired) versus those mapping to the mitochondrial genome (undesired)
- (iv) the fragment size distribution

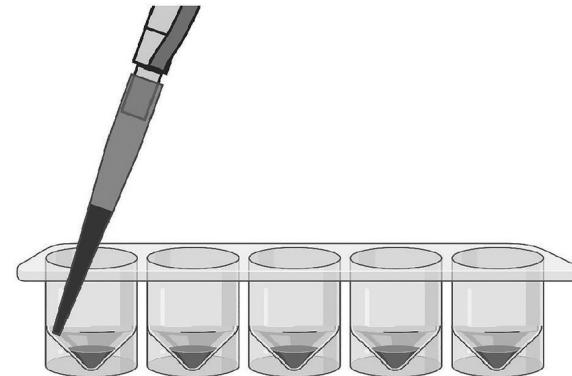
Handling low cell numbers

- As low as 10K cells possible, but > 50K advised prior starting nuclei isolation

Aim for 10X scATAC v2: loading 16,000 cells to recover 10,000 cells

1. Use **low-attachment** plastics and/or **coat** plastic material with 5% BSA/PBS
2. Use conical V-bottom 96 plates
3. Pipet carefully and reduce to a minimum
4. Bit of Tween-20 to improve pelleting
5. Improve cell recovery: centrifugation at appropriate speed (certain cell types / small cells need slightly *faster* or *longer*)
6. Consider **fixation**

Round conical V-bottom 96 well plates



High-throughput single cell 'omics studies: cells often have to go a long way...

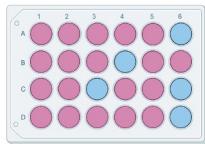
animal cohorts



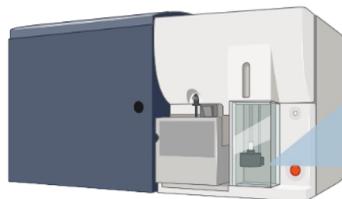
patient samples



time courses



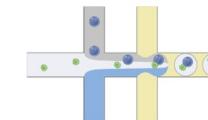
tissue dissociation



long sorting times

(nuclei isolation)

droplet-based "multi omics" methods



scRNA-seq



scATAC-seq

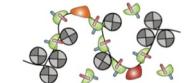
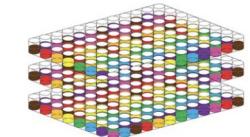


plate-based (high-throughput)
single cell methods

SMART-seq



combinatorial barcoding
(sci-RNA/ATAC,
SUM-seq, etc.)



Fixation and freezing of cells prior of single cell 'omics

Advantages:

- ★ fixation "locks" cells in current **cellular state**
- ★ cells / nuclei **more robust** for downstream handling (sorting, pipetting, low cell #, etc.)
- ★ **molecular processes blocked**: stress signatures can't further increase
- ★ fixation + freezing allows **pooling** of samples across **time** and **space**
- ★ **reduces time pressure** in long experimental protocols (= happier scientists :))

Important notes:

- ❖ not every fixative is compatible with downstream protocols
- ❖ same epitopes are affected by certain fixatives → maybe influence sorting strategy
- ❖ not all freezing methods are compatible with certain single cell read outs
 - **careful with snap freezing!** histones do not like it
 - use a cryoprotectant (DMSO, glycerol)

Several fixatives + sequencing published / preprinted

FixNCut: Single-cell genomics through reversible tissue fixation and dissociation

Laura Jiménez-Gracia, Domenica Marchese, Juan C. Nieto, Ginevra Caratù, Elisa M. Vicente, Joana Petral, Jasmin Article | Open Access | Published: 24 September 2021
doi: [https://doi.org/10.1038/s41564-021-01102-w](#)

High-throughput RNA sequencing of paraformaldehyde-fixed single cells

Hoang Van Phan, Michiel van Gent, Nir Drayman, Anindita Bhattacharya, Daniel J. S. H. Lee, Michael A. P. Stadhoudor, Daniel J. L. Veldkamp, Jeroen J. M. van der Velde, Marcel A. Lauterbach, Stephan E. Lehnhart, Tobias Moseley, Tiago F. Outeiro, and Mark J. van der Wijngaart. [Nature Communications](#) 12, Article number: 5636 (2021). doi: [https://doi.org/10.1038/s41564-021-01102-w](#)



Resource

Glyoxal as an alternative fixative to formaldehyde in immunostaining and super-resolution microscopy

Katharina N Richter^{1,2,*}, Natalia H Revelo^{1,1,*}, Katharina J Seitz^{1,3}, Martin S Helm^{1,3}, Deblina Sarkar⁴, Rebecca S Saleem⁵, Elisa D'Este⁶, Jessica Eberle⁷, Eva Wagner^{8,9}, Christian Vogl^{10,12}, Diana F Lazaro^{12,13}, Frank Richter^{3,11}, Javier Coy-Vergara¹⁰, Giovanna Coceano¹⁰, Edward S Boyden¹⁷, Rory R Duncan¹⁸, Stefan W Hell¹⁹, Marcel A Lauterbach²⁰, Stephan E Lehnhart^{8,5}, Tobias Moseley^{10,11}, Tiago F Outeiro^{12,13}, and Mark J van der Wijngaart^{1,2}. [Methodology article](#) | Open Access | Published: 19 May 2017

Cell fixation and preservation for droplet-based single-cell transcriptomics

Jonathan Alles, Nikos Karaikos, Samantha D. Praktiknjo, Stefanie Grosswendt, Philipp Wahle, Pierre-Louis Ruffault, Salah Ayoub, Luisa Schreyer, Anastasia Boltengagen, Carmen Birchmeier, Robert Zinzen, Christine Kocks & Nikolaus Rajewsky. [BMC Biology](#) 15, Article number: 44 (2017) | [Cite this article](#)

Methodology | Open Access | Published: 17 July 2018

PBMC fixation and processing for Chromium single-cell RNA sequencing

Cheung, Rongye Shi, Huizhi Zhou, Wenrui Lu & CHI Consortium [al Medicine](#) 16, Article number: 198 (2018) | [Cite this article](#)
Citations | 12 Altmetric | Metrics

Method | Open Access | Published: 08 April 2021

dissociation: a versatile cell fixation-dissociation method for single-cell transcriptomics

J-Castro, Nathan J. Kenny, Marta Iglesias, Patricia Alvarez-Campos, Vincent Mason, K. Anna Schönauer, Victoria A. Sleight, Jakke Neiro, Aziz Aboobaker, Jon Permanyer, Arnau Sebé-Pedrós & Jordi Solana. [Biology](#) 22, Article number: 89 (2021) | [Cite this article](#)

Research article | Open Access | Published: 05 June 2021

effect of methanol fixation on single-cell RNA sequencing data

Yang, Lei Yu & Angela Ruohao Wu. [Genomics](#) 22, Article number: 420 (2021) | [Cite this article](#)

reprint and has not been certified by peer review [what does this mean?]. [Glyoxal fixation facilitates transcriptome analysis after antigen staining and cell sorting by flow cytometry as alternative fixative for single cell RNA sequencing](#)

1371/journal.pone.0240769

Article | Open Access | Published: 23 July 2019

DMSO cryopreservation is the method of choice to preserve cells for droplet-based single-cell RNA sequencing

Wohnhaas, Germán G. Leparc, Francesc Fernandez-Albert, David Kind, Florian Gantner, et, Tobias Hildebrandt & Patrick Baum. [Sports](#) 9, Article number: 10699 (2019) | [Cite this article](#)

article | Open Access | Published: 15 May 2023

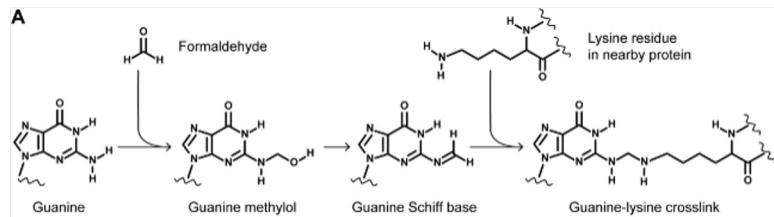
Methanol fixation is the method of choice for droplet-based single-cell transcriptomics of neural cells

Ina Gutiérrez-Franco, Franz Ake, Mohamed N. Hassan, Natalie Chaves Cayuela, Loris Mularoni & Mireya Lllass. [Communications Biology](#) 6, Article number: 522 (2023) | [Cite this article](#)

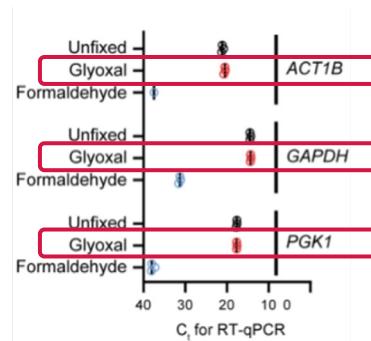


Paraformaldehyde (PFA) not ideal for most downstream ‘omics...

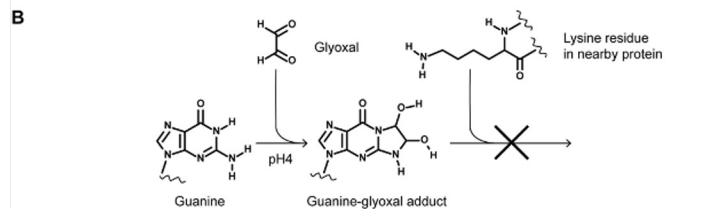
PFA induces crosslinks between nucleic acids and proteins



PFA poor recovery with qPCR

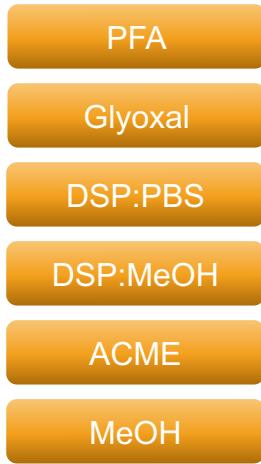


Glyoxal interesting alternative

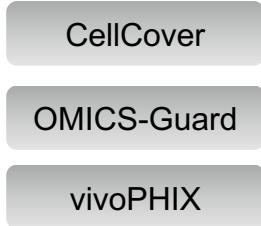


Systematic evaluation of various fixation methods for multi omics

fixatives



perservatives



Project entails:

1. general cellular morphology
2. transcriptomic readouts (scRNA-seq)
3. chromatin accessibility ((sc)ATAC-seq)
4. antibody-based readouts (flow cytometry)

Zaugg group

Gwen Bauersachs
Sara Lobato
Karin Prummel

GeneCore

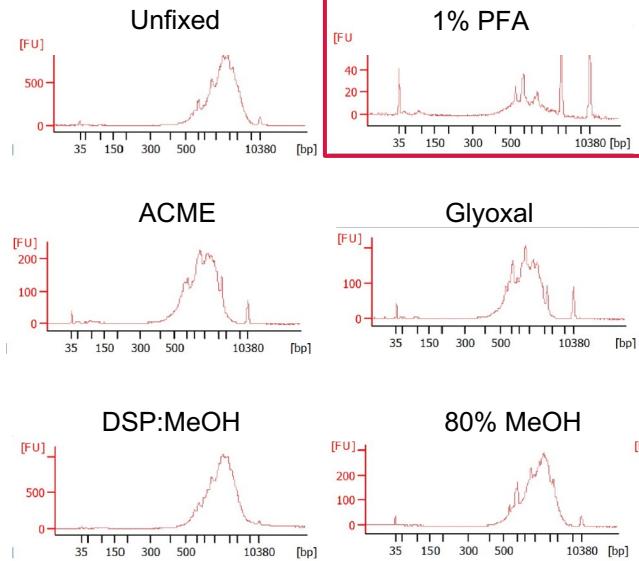
Laura Villacorta
Jonathan Landry
Vladimir Benes

Flow Cytometry Facility

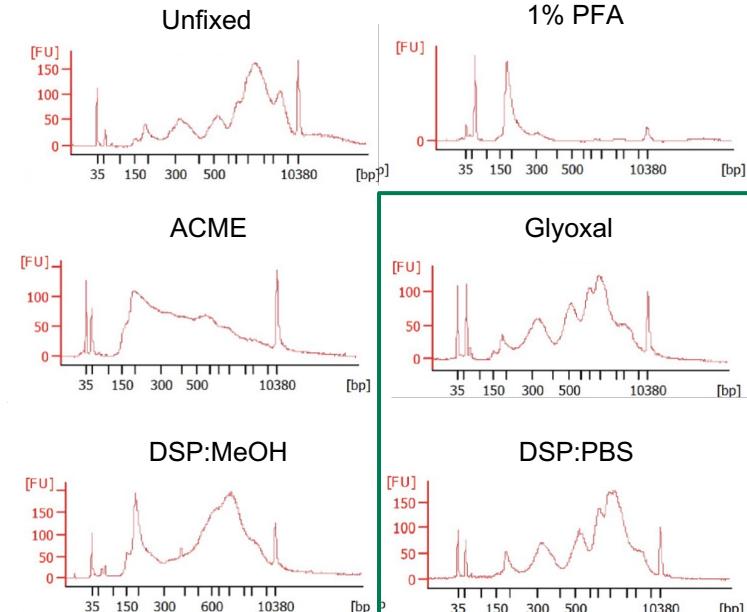
Diana Ordonez

Effect of fixation on RNA yield/quality and ATAC quality

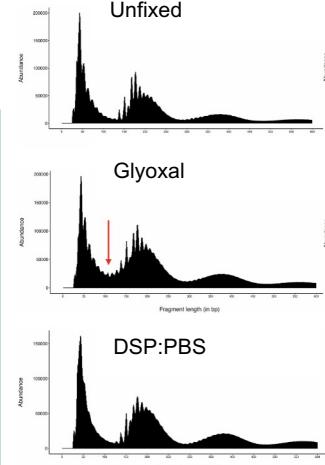
single cell SMART-seq



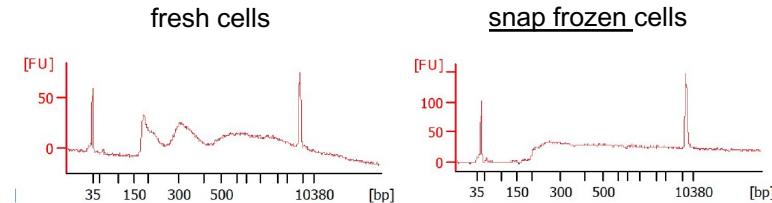
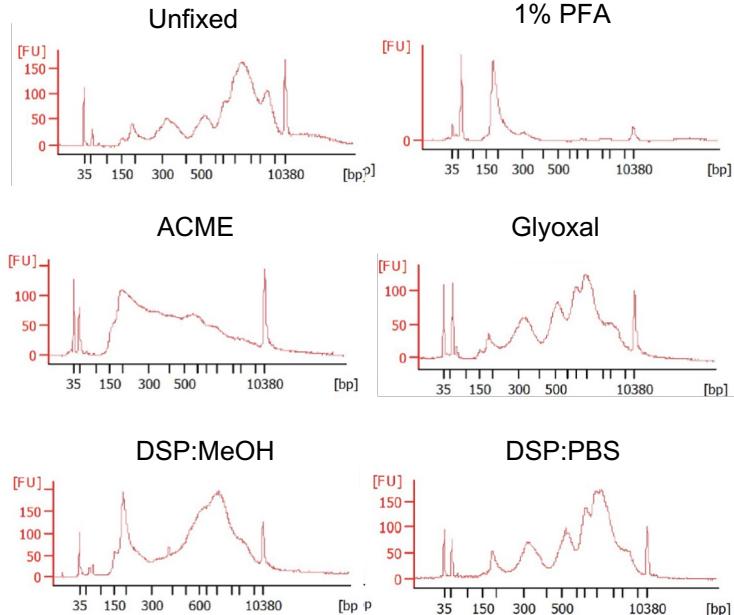
bulk ATAC-seq



nucleosome profile



Fixation and freezing



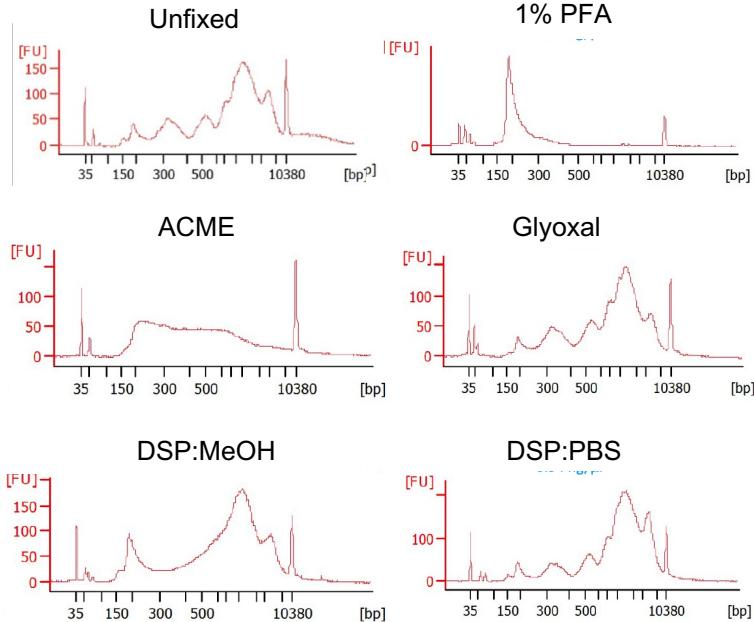
DO NOT

- ❖ snap freeze cells / nuclei for ATAC
- ❖ use MeOH / EtOH-based fixatives

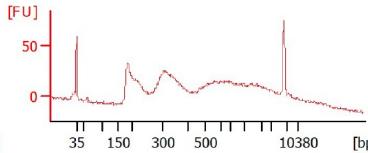
DO ☺

- ❖ use glycerol or DMSO-based buffers
- ❖ use glyoxal or DSP:PBS as fixative

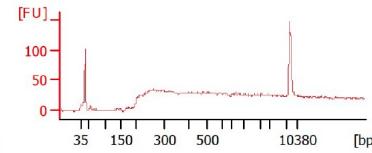
Fixation and freezing



fresh cells



snap frozen cells



DO NOT

- ❖ snap freeze cells / nuclei for ATAC
- ❖ use MeOH / EtOH-based fixatives

DO ☺

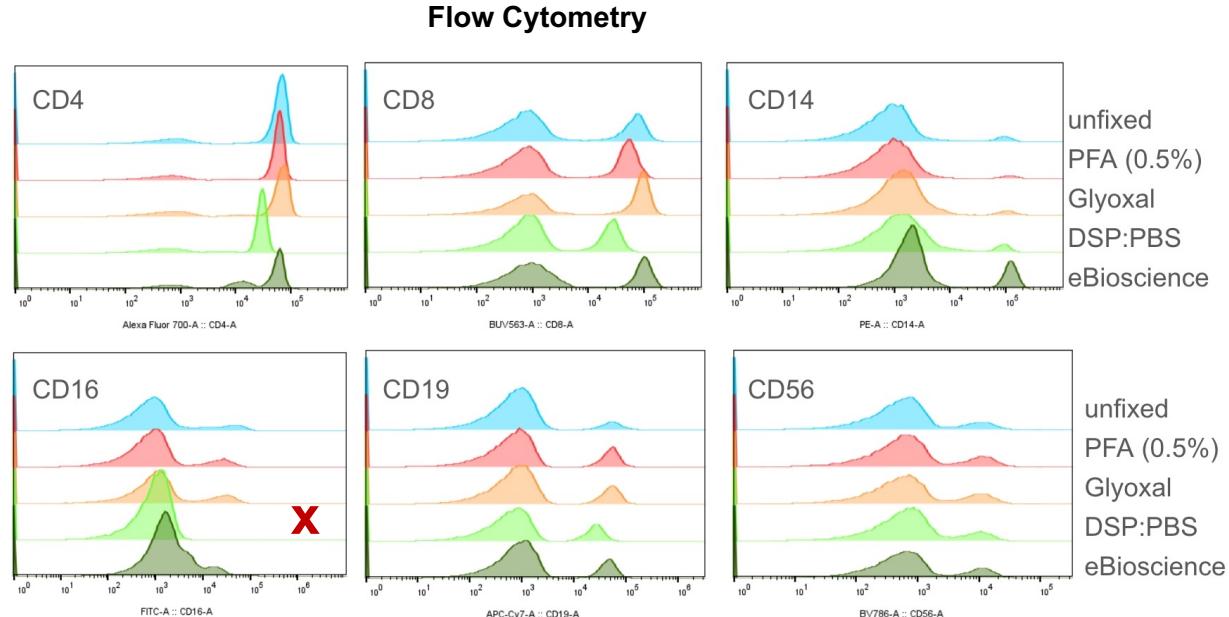
- ❖ use glycerol or DMSO-based buffers
- ❖ use glyoxal or DSP:PBS as fixative

Fixation and antibodies against surface markers: FACS-compatibility

PBMCs fixed and then stained with panel of surface markers

- PFA
- Glyoxal
- DSP:PBS

➤ Testing for your markers needed!



Summary

- Optimize the sample prep and nuclei isolation for your tissue / cell type
 1. Cell isolation / enrichment optimization
 2. Check how many viable cells you can recover
 3. Freezing and / or fixation necessary for your project?
 4. Nuclei isolation + permeabilization optimization
 5. Check morphology by microscope and cell counting
 6. Check library structure / complexity by qPCR (bulk ATAC) and Bioanalyzer
- Optimizations possible for scMultiome / scCITE-seq (DOGMA- / TEA-seq)
- Fixation compatible with ATAC-seq and FACS for cell population enrichment

Acknowledgements

Approach us for your experimental design and sample prep questions!



Karin



Gwen



Sara

PBMC
Bone marrow
Cell lines
HSC cultures
Zebrafish

PBMC
Bone marrow
Cell lines
Brain organoids

Cell lines
iPSCs
Neuronal cultures



GeneCore

Vladimir Benes
Laura Villacorta
Jonathan Landry

FCCF

Diana Ordonez

Future reading

- Chromatin accessibility profiling by ATAC-seq (<https://doi.org/10.1038/s41596-022-00692-9>)
- Nuclei on the Rise: When Nuclei-Based Methods Meet Next-Generation Sequencing (<https://doi.org/10.3390/cells12071051>)
- Omni-ATAC: Corces et al., 2017 Nat. Methods
- TEA-seq: Swanson et al., 2021, *Elife*
- DOGMA-seq: Mimitou et al., 2021, Nat. Biotech.