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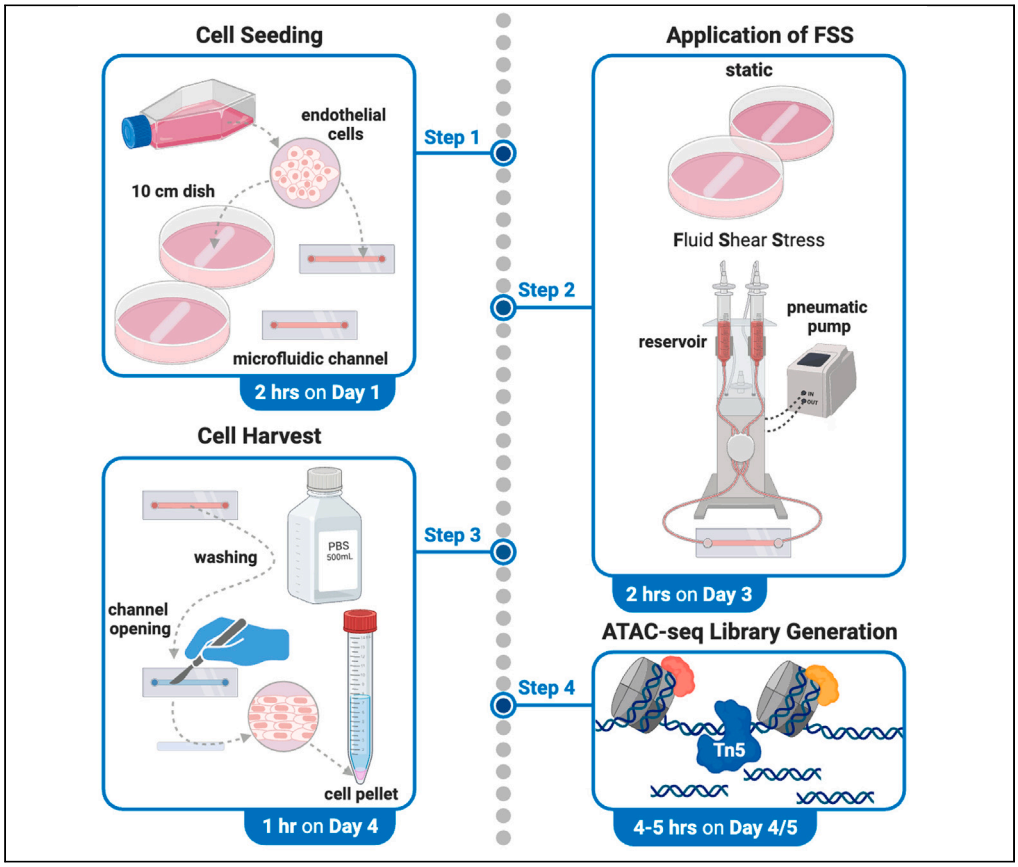
Protocol for chromatin accessibility profiling of human endothelial cells cultured under fluid shear stress using ATAC-seq



Chromatin accessibility influences gene regulation and can be quantified using assay for transposase-accessible chromatin with high-throughput sequencing (ATAC-seq). Recapitulating *in vivo* fluid shear stress (FSS) mechano-regimes *in vitro* allows the study of atheroprone and atheroprotective mechanisms. In this protocol, we show how to culture and harvest endothelial cells from microfluidic channels for the preparation of ATAC-seq, highlighting optional growth factor stimulation and different FSS rates. This extends the application of ATAC-seq to the analysis of *in vitro* mechanically stimulated cells.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Detailed ATAC-seq protocol for fluid shear stress (FSS)-stimulated endothelial cells

Cell harvest from microfluidic channels for the application of low-input seq assays

Growth factor stimulation and FSS rate modification in optional steps

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Protocol for chromatin accessibility profiling of human endothelial cells cultured under fluid shear stress using ATAC-seq

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SUMMARY

Chromatin accessibility influences gene regulation and can be quantified using assay for transposase-accessible chromatin with high-throughput sequencing (ATAC-seq). Recapitulating *in vivo* fluid shear stress (FSS) mechano-regimes *in vitro* allows the study of atheroprone and atheroprotective mechanisms. In this protocol, we show how to culture and harvest endothelial cells from microfluidic channels for the preparation of ATAC-seq, highlighting optional growth factor stimulation and different FSS rates. This extends the application of ATAC-seq to the analysis of *in vitro* mechanically stimulated cells.

For complete details on the use and execution of this protocol, please refer to Jatzlau et al.[1]

BEFORE YOU BEGIN

This protocol describes the specific steps for applying ATAC-seq[2–4] on human umbilical artery endothelial cells (HUAECs), cultivated under fluid shear stress (FSS) and additionally stimulated with BMP9. Furthermore, we applied the protocol to human aortic endothelial cells (HAoECs) stimulated under different FSS regimes.

Institutional permissions

For sequencing of genomic DNA from human samples permission must be obtained either directly from the individuals or informed consent must be guaranteed by the supplier of the samples.

Expand cells

Timing: 2–4 days

To confirm cell type specific morphology and growth, primary cells (passage 3 or 4) are expanded before starting the experiment. To ensure consistent experimental results, primary ECs at low passage number (passage 3 or 4) are freshly thawed and expanded before start of the experiment.

1. Freshly prepare full medium.
2. Pre-coat a T75 flask for 15 min with a dilution of 0.1% pork skin gelatin (Sigma-Aldrich) in DPBS.
3. Seed 5 × 105 primary Human Umbilical Artery Endothelial Cells (HUAECs; PromoCell GmbH, Germany) in 12 mL full medium to a T75 flask.

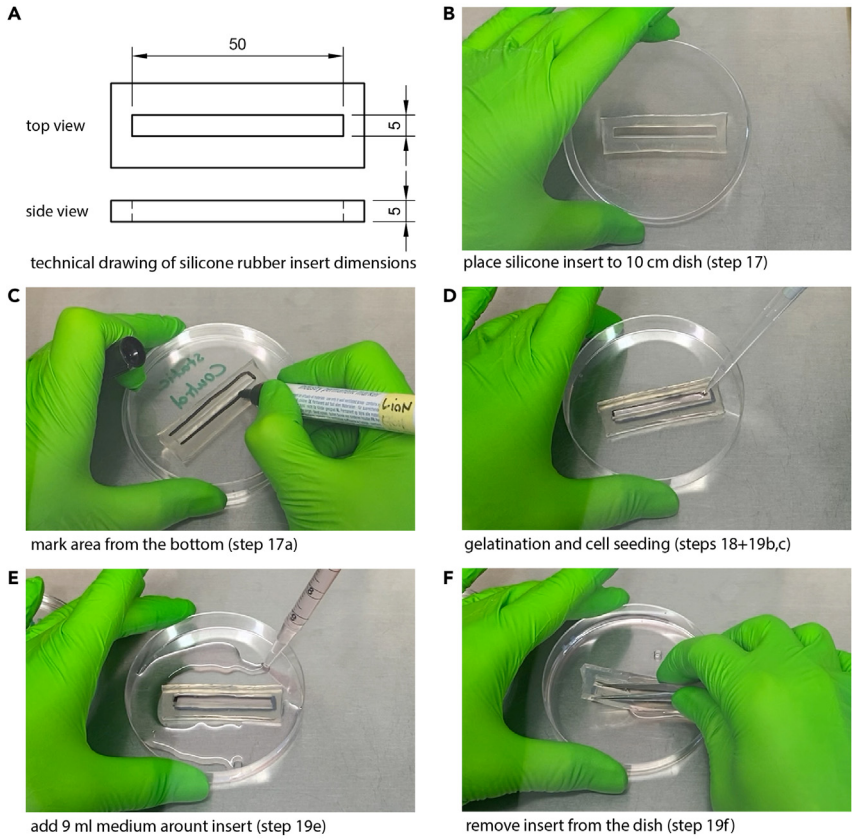

Figure 1. Custom silicone rubber inserts for gelatin coating and cell seeding of static control samples

(A) Technical drawing of the inserts with relevant measures in mm.

(B) Silicone insert placed in a 10 cm Petri dish for one sample.

(C) Petri dish with insert, turned upside down to mark the area for coating and cell seeding.

(D) Insert restricts area and volume for gelatination and cell seeding.

(E) After successful cell attachment, the dish is filled with the remaining amount of medium, before (F) removing the insert.

4. Replace medium with fresh 12 mL full medium every 2 days until cells reach 100% confluency.

Manufacture silicone rubber insert for static controls

Timing: 1 day

ECs cultured in flow channels are prone to starvation in the absence of constant medium exchange due to a small channel volume. To ensure nutrient availability to static control samples, while maintaining a similar medium/cell ratio, we used custom silicone rubber inserts to restrict gelatin coating and cell seeding (Figure 1A) to a smaller area in a 10 cm Petri dish. Manufacturing the reusable inserts can be performed at a regular laboratory bench at RT.

5. Mix ELASTOSIL RT 625 components A and B in a 9:1 (w/w) ratio.

Note: The silicone rubber starts vulcanizing at room temperature once both components are mixed. Hence, further handling needs to be finished in due time.

6. Degas mixture using a desiccator until bubbles are fully removed.
7. Pour mixture into a petri dish or a similar mold to a height of 5–10 mm.

8. Wait for 2 h until elastomer hardens.
9. Use scalpel to cut out inserts.
   a. Outer dimensions: 20 mm × 50 mm.
   b. Inner dimensions: 5 mm × 20 mm.
10. Bake inserts at 130°C for 4 h for final vulcanization.
11. Cook inserts in ddH_2O for 1 h to remove remaining monomers.
12. Steam autoclave inserts without heat drying before usage in cell culture.

⚠ CRITICAL: Silicone rubber inserts should be tested in cell culture for biocompatibility before starting experiments, using cell viability assays (problem 1). To do so, seed and culture cells as described (steps 17 to 20). Viability should not differ significantly from cells cultured without using the inserts.

Note: Inserts can be reused many times. To do so, wash inserts with soap and water after each experiment and steam autoclave without heat drying. Dry at room temperature.

Seeding to ibidi µ-Slide $I^{0.4}$ channels

Timing: 1 h, day 1

Before FSS can be applied to the cells, they need to be seeded into channel slides which can then be connected to the perfusion set of an ibidi pump system. For the formation of a uniform monolayer of ECs, seed 3×10^5 cells per µ-Slide $I^{0.4}$ channel 2 days prior to applying the flow, followed by daily medium exchanges.

⚠ CRITICAL: All following steps are conducted under a biosafety cabinet (BSC).

⚠ CRITICAL: To transfer slides between BSC and incubator use the enclosed lids to cover the reservoirs. For ease of handling, slides can be kept in the plastic bottom of the fabrication enclosure or in a larger petri dish. No additional cover is needed.

13. Pre-coat each channel for 15 min with 0.1% pork skin gelatin (Sigma-Aldrich) in DPBS.
    a. Add 100 µL gelatin solution to each channel.
    b. Incubate for 15 min (37°C, 95% relative humidity (RH), 5% CO2).
    c. Aspirate solution with vacuum pump or pipette.
14. Harvest cells from expansion.
    a. Aspirate medium from the flask.
    b. Wash cells: add and remove 10 mL DPBS two times.
    c. Add 1 mL Trypsin for 5 min.
    d. Stop reaction with 4 mL of full medium and collect solution in 15 mL falcon tube.
    e. Count cells using a hemocytometer or another suited method.
    f. While counting, spin down cells (200 *g*, 5 min, low brakes) and discard supernatant.
    g. Resuspend cell pellet in full medium as to obtain 3×10^6/mL cells.
15. Seed 3×10^5 cells per ibidi µ-Slide $I^{0.4}$ channel (ibidi application note 03: 2.1).[5]
    a. Add 100 µL cell suspension (3×10^6/mL cells) per channel.
    b. Transfer µ-Slide to incubator to allow cell attachment for 30 min - 1 h.
    c. Add 60 µL full medium per reservoir.
    d. Culture in incubator (37°C, 95% RH, 5% CO2) for 24–48 h until cells form uniform monolayer before applying FSS.
16. Exchange medium every 24 h (ibidi Application Note 03: 2.2)[5] until a uniform cell monolayer is formed.
    a. Gently remove medium from both reservoirs, so that the cells are still covered with medium.

b. Slowly add 150 μL pre-warmed full medium to the left reservoir while removing flow-through from the right reservoir.
c. Repeat a. and b. once to achieve a full medium exchange.
d. Add 60 μL full medium to both reservoirs.
e. Place channel back into the incubator.

Seeding to 10 cm dishes (static control)

Timing: 1 h, day 1

In order to compare FSS stimulation with the absence of flow, a control sample is needed. This section describes how to prepare the cells alongside the FSS stimulation experiment. Key to the control sample condition is a nutrient, O_2 and CO_2 availability comparable to the condition under FSS. Therefore, seeding of 3×10^5 cells in an area restricted by a silicone rubber insert, inside a 10 cm Petri dish, is necessary. ECs are exposed to 10 mL medium in the 10 cm Petri dish after removing the silicone rubber insert, which is the same amount of medium as in one flow unit.

Note: Start together with seeding to ibidi μ-Slide $I^{0.4}$ channels.

Note: Steps 17 to 19 are explained in Methods video S1 and Figures 1B–1F.

17. For as many control samples as needed, stick one custom silicone rubber insert to the bottom of one 10 cm petri dish to restrict gelatin coating and cell seeding to the same area as in one channel.
    a. Mark area of cell seeding with ethanol resistant marker from the bottom of the dish.
18. Pre-coat restricted area for 15 min with 0.1% pork skin gelatin (Sigma-Aldrich) in DPBS.
    a. Add 500 μL gelatin solution to each insert.
    b. Incubate for 15 min (37°C, 95% RH, 5% CO2).
    c. Completely aspirate solution with vacuum pump.
19. Seed 3×10^5 cells per dish.
    a. Prepare cell suspension with 3×10^6 cells/mL in full medium.
    b. Add 1 mL full medium to the insert.
    c. Add 100 μL cell suspension drop wise to the medium in the insert.
    d. Transfer to incubator to let cells attach for 30 min - 1 h.
    e. Add 9 mL full medium per dish around the insert.
    f. Remove insert with tweezers and store for cleaning and re-use.
    g. Culture in incubator (37°C, 95% RH, 5% CO2) (problem 1).

Optional: Cell seeding can also be done by preparing a 3×10^5 cells/mL cell suspension and by adding 1 mL of this solution directly to the insert.

20. Exchange medium every 48 h (do not wait until pH indicator turns yellow!).
    a. Gently remove medium from the side of the dish.
    b. Freshly add 10 mL pre-warmed full medium to the side of the dish.
    c. Place dish back into the incubator.

Note: In case the experiment starts within 48 hours, no additional medium exchange is necessary.

⚠ CRITICAL: Regularly inspect cells between steps under a phase contrast microscope to confirm de-/attachment, viability and cell type-typical morphology (Figure 2).

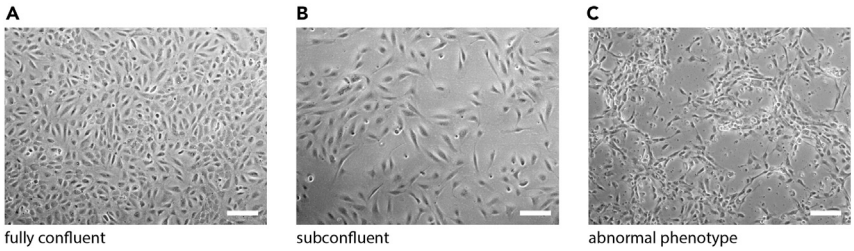

Figure 2. Endothelial cell morphology

(A–C) Endothelial cells under a phase contrast microscope. Scale bars: 100 μm (A) Before applying FSS, the cell monolayer should be grown to full confluency. (B) Since growth rates of different primary ECs varies, cells can be subconfluent on the day of the intended experiment start. In that case it is advised to delay the experiment one or two days until confluency is reached. (C) If ECs exhibit an abnormal phenotype, i.e., no (A) cobble-stone like EC-monolayer, on the day of the experiment, it is advised to restart the experiment.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
DPBS, w/o: Ca and Mg w/o: phenol red	Capricorn	Cat#PBS-1A
Endothelial Cell Growth Medium 2 Kit (including all growth factors for medium preparation)	PromoCell	Cat#C-22111
Fetal calf serum	PromoCell	Cat#C-22111
EGF (epidermal growth factor [recombinant human])	PromoCell	Cat#C-22111
FGF (basic fibroblast growth factor [recombinant human])	PromoCell	Cat#C-22111
IGF (insulin-like growth factor [long R3 IGF, recombinant human])	PromoCell	Cat#C-22111
VEGF (vascular endothelial growth factor 165 [recombinant human])	PromoCell	Cat#C-22111
Ascorbic acid	PromoCell	Cat#C-22111
Heparin	PromoCell	Cat#C-22111
Hydrocortisone	PromoCell	Cat#C-22111
Penicillin 10.000 U/mL / Streptomycin 10 mg/mL	PAN-Biotech	Cat#P06-07100
AMPure XP Beads	Beckmann Coulter	Cat#A63881
NEBNext high-fidelity 2X PCR master mix	New England Biolabs	Cat#M0541L
SYBR Green I	Thermo Fisher Scientific	Cat#7563
rhBMP9/GDF2	PeproTech	Cat#120-07
NP40/IGEPAL CA-630	Sigma-Aldrich	Cat#I8896
Tween 20	Serva	Cat#37470
Digitonin	Millipore	Cat#300410
ELASTOSIL RT 625	Wacker	Cat#RT 625 A/B
Critical commercial assays		
MinElute Reaction Cleanup Kit	QIAGEN	Cat#28206
Tagment DNA Enzyme and Buffer Large Kit	Illumina	Cat#20034198
Deposited data		
ATAC-seq data of BMP9, FSS, and BMP9/FSS stimulated human umbilical artery endothelial cells (HUAECs)	Jatzlau et al.[1]	https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE227588

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Experimental models: Cell lines		
Human umbilical artery endothelial cells (HUAECs), single donor, female	PromoCell	Cat#C-12200
Oligonucleotides		
ATAC-seq barcode adapters, Table S1	Buenrostro et al.[4] ordered at idtdna.com	N/A
Software and algorithms		
UCSC browser	Nassar et al.[6]	https://genome.ucsc.edu
2100 Expert Software (vB.02.11.SI824 [SR1])	Agilent Bioanalyzer Software	https://www.agilent.com/en/product/automated-electrophoresis/Bioanalyzer-systems/Bioanalyzer-software/2100-expert-software-228259
Other		
µ-Slide I Luer 0.4 mm	ibidi	Cat#80176
ibidi pump system	ibidi	Cat#10902
HS DNA Bioanalyzer chip	Agilent	Cat#5067-4626
Perfusion Set YELLOW and GREEN	ibidi	Cat#10964
Gelatin from porcine skin, type A	Sigma-Aldrich	Cat#G2500
Scalpel blades for handle no. 3, 12	Carl Roth	Cat#1PY4.1

MATERIALS AND EQUIPMENT

Full medium - EGM2 10% FCS (Endothelial Cell Growth Medium 2, PromoCell GmbH, C-22111)		
Reagent	Final concentration	Amount
Fetal Calf Serum	10%	5 mL
EGF (Epidermal Growth Factor (recombinant human))	5 ng/mL	50 µL
FGF (Basic Fibroblast Growth Factor (recombinant human))	10 ng/mL	50 µL
IGF (Insulin-like Growth Factor (Long R3 IGF, recombinant human))	20 ng/mL	50 µL
VEGF (Vascular Endothelial Growth Factor 165 (recombinant human))	0.5 ng/mL	50 µL
Ascorbic Acid	1 µg/mL	50 µL
Heparin	22.5 µg/mL	50 µL
Hydrocortisone	0.2 µg/mL	50 µL
Penicillin-Streptomycin (10000 U/mL // 10 mg/mL)	100 U/mL // 100 µg/mL	500 µL
EBM2 (Endothelial Cell Basal Medium 2)	N/A	44 mL
Total	**N/A**	**50 mL**
Storage conditions: 4°C in the dark, maximum storage time: 6 weeks		

Starvation medium - EGM2 2% FCS (Endothelial Cell Growth Medium 2, PromoCell GmbH, C-22111)		
Reagent	Final concentration	Amount
Fetal Calf Serum	2%	1 mL
EGF (Epidermal Growth Factor (recombinant human))	5 ng/mL	50 µL
FGF (Basic Fibroblast Growth Factor (recombinant human))	10 ng/mL	50 µL
IGF (Insulin-like Growth Factor (Long R3 IGF, recombinant human))	20 ng/mL	50 µL
VEGF (Vascular Endothelial Growth Factor 165 (recombinant human))	0.5 ng/mL	50 µL
Ascorbic Acid	1 µg/mL	50 µL
Heparin	22.5 µg/mL	50 µL
Hydrocortisone	0.2 µg/mL	50 µL
Penicillin-Streptomycin solution (10000 U/mL and 10 mg/mL)	100 U/mL and 100 µg/mL	500 µL
EBM2 (Endothelial Cell Basal Medium 2)	N/A	48 mL
Total	**N/A**	**50 mL**
Storage conditions: 4°C in the dark, maximum storage time: 6 weeks		

Resuspension buffer		
Reagent	Final concentration	Amount
1 M Tris pH 7.5	10 mM	100 μL
5 M NaCl	10 mM	20 μL
300 mM MgCl2	3 mM	100 μL
H_2O (DEPC treated)	N/A	9.780 mL
Total	**N/A**	**10 mL**
Storage conditions: store at RT, maximum storage time: 12 months		

Lysis buffer (50 μL per sample, prepare together for all samples, add spare volume)		
Reagent	Final concentration	Amount
Resuspension buffer	N/A	44 μL
10% NP40 / IGEPAL CA-630	0.1%	0.5 μL
10% Tween 20	0.1%	0.5 μL
0.1% Digitonin	0.01%	5 μL
Total	**N/A**	**50 μL**
Storage conditions: prepare freshly, keep on ice		

⚠ CRITICAL: NP40 / IGEPAL CA-630 is harmful if swallowed, causes skin irritation, causes serious eye damage, and is very toxic to aquatic life with long lasting effects. When handling, wear protective gloves, eye protection and face protection.

⚠ CRITICAL: Digitonin is toxic if swallowed and may cause damage to organs through prolonged or repeated exposure. When handling, wear protective gloves, eye protection and face protection.

⚠ CRITICAL: For deposition of hazardous waste please refer to your locally applying regulations.

Tagmentation buffer (50 μL per sample, prepare together for all samples, add spare volume)		
Reagent	Final concentration	Amount
Illumina Tagment DNA Buffer	N/A	25 μL
Tween 20 (10%)	0.1%	0.5 μL
Digitonin (0.1%)	0.01%	5 μL
Tn5 (Illumina Tagment DNA TDE1 Enzyme)	N/A	2.5 μL
H_2O (DEPC treated)	N/A	17 μL
Total	**N/A**	**50 μL**
Storage conditions: prepare freshly *without enzyme*, keep on ice, add enzyme right before usage		

- 3 nM BMP9 stimulation solution: aseptically, add 0.3 μL 1000 nM BMP9 (PeproTech) in DPBS solution to 999.7 μL DPBS.

Storage conditions: prepare freshly at RT.

- 10% FCS in PBS: aseptically, add 5 mL FCS to 45 mL DPBS.

Storage conditions: store at 4°C for one week.

- Wash buffer (50 μL per sample, prepare together, add spare volume): aseptically, add 0.5 μL 10% Tween 20–49.5 μL resuspension buffer.

Storage conditions: store at 4°C for one week.

- 80% EtOH in H_2O: add 4 mL EtOH (absolute for analysis) to 1 mL H_2O (DEPC).

Storage conditions: prepare freshly at RT.

STEP-BY-STEP METHOD DETAILS

Application of fluid shear stress

Timing: 1–2 h, day 3 (setup); 6–24 h (stimulation)

The application of FSS to EC monolayers *in vitro* allows recapitulation of physiological and patho-physiological mechano-regimes. This section describes how to apply FSS to HUAECs, optionally in combination with growth factor stimulation, for downstream analysis with ATAC-seq. The following steps describe how to set up the FSS stimulation. After setting up and starting the flow, cells are exposed to FSS for 6–12 h.

Note: The ibidi Pump System was used combining manufacturer instructions[7] and application notes.[5,8]

1. Confirm uniform cell monolayer under a microscope (Figure 2).
   a. In case of a subconfluent cell layer, continue medium exchange until a uniform monolayer is formed (before you begin, step 16).
2. Per condition, mount one perfusion set YELLOW-GREEN to a fluidic unit.

Optional: To apply oscillatory shear stress (OSS) or pulsatile shear stress (PSS) refer to the manufacturer instruction manual[7] chapter 7.3 and 7.4, accordingly, to set up the experiment correctly. In our experiments, OSS and PSS experiments did not include a ramp phase and shear stress exposition was prolonged (see options after step 6).

3. Fill the reservoirs of each perfusion set with 10 mL pre-warmed starvation medium.
   a. For static controls, exchange full medium in 10 cm dishes with 10 mL pre-warmed starvation medium.
4. Run program "Remove Air Bubbles" (problem 2).
5. Per condition, connect 2 channels sequentially to one ibidi perfusion set (Ibidi Application Note 25: Serial Connection of Luer-Slides for Flow Experiments).[8]
6. Using the software PumpControl (v1.4.2) set up a laminar flow experiment with the following cycles.

Flow experiment cycles in PumpControl software

Cycle #	Duration [min]	Shear stress [dyn]	Switching times [s]	Phase
1	60	2.5	5	Ramp
2	30	4.5	20	
3	30	5.5	20	
4	30	7.5	10	
5	30	10	10	
6	30	12.5	6.84	
7	30	15	6	
8	60	20	5	
9	infinite	30	4	FSS stimulation

Optional: Dependent on good adhesion of the selected cell type (e.g., HUAECs and HAoECs) ramp phase (cycle # 1–8) can be omitted for maximum shear stress levels of up to 20 dyn or higher.

Optional: Application of FSS can be extended up to 24 hours without medium exchange to observe long term adaptation of cells to the stimulus.

7. Start the flow.
8. After 6 h (5 h of ramp phase (cycle # 1–8), and 1 h 30 dyn (cycle # 9), step 6) of FSS stimulation, proceed with BMP9 stimulation (see next section: ligand stimulation).

Ligand stimulation

Timing: 2.5 h

Co-stimulation of growth factors to FSS-exposed ECs reveals mechano-dependent modulation of signal transduction. Here is shown how to stimulate with BMP9 at physiological serum concentrations to elucidate, if BMP9-dependent chromatin modulation changes in the absence or presence of FSS.

Note: If no ligand stimulation is desired, continue with FSS until cell harvest (step 13).

9. Prepare 1 mL 3 nM BMP9 stimulation solution per perfusion set and 10 cm dish.
10. Pause the flow.
11. Remove and discard 1 mL medium from each perfusion set or static sample.
    a. Transfer each fluidic unit, including the perfusion set and the connected channels, to a biosafety hood.
    b. Remove the filters from the reservoirs.
    c. Remove 500 µL of medium from each reservoir.
12. Add 1 mL stimulation solution (1 mL DPBS for controls) per perfusion set or 10 cm dish to reach a final concentration of 0.3 nM BMP9.
    a. Add 500 µL stimulation solution to each reservoir.
    b. Close the reservoirs with the filters.
    c. Transfer the setups back to the incubator and re-connect cables and tubing.
13. Resume flow for 2 more hours.

Cell harvest

Timing: 30 min to 1 h

Harvesting the cells for ATAC-seq from the channel slides is the most critical step to ensure a good readout quality. Cell numbers and viability are key for successful chromatin accessibility readout. The following steps describe how to extract the adherent cells from the channels. Firstly, channel slides are disconnected from the perfusion set. Secondly, the bottom of the slide is cut out with the attached cell layer and finally, cells are detached from the cut out-strips by trypsin treatment for further processing. Corresponding steps for harvesting the static control samples are explained briefly.

Note: Steps 16 to 26 are shown in Methods video S2 and Figure 3.

14. Before starting prepare the following.
    a. Warm Trypsin (37°C).
    b. Warm DPBS (37°C).

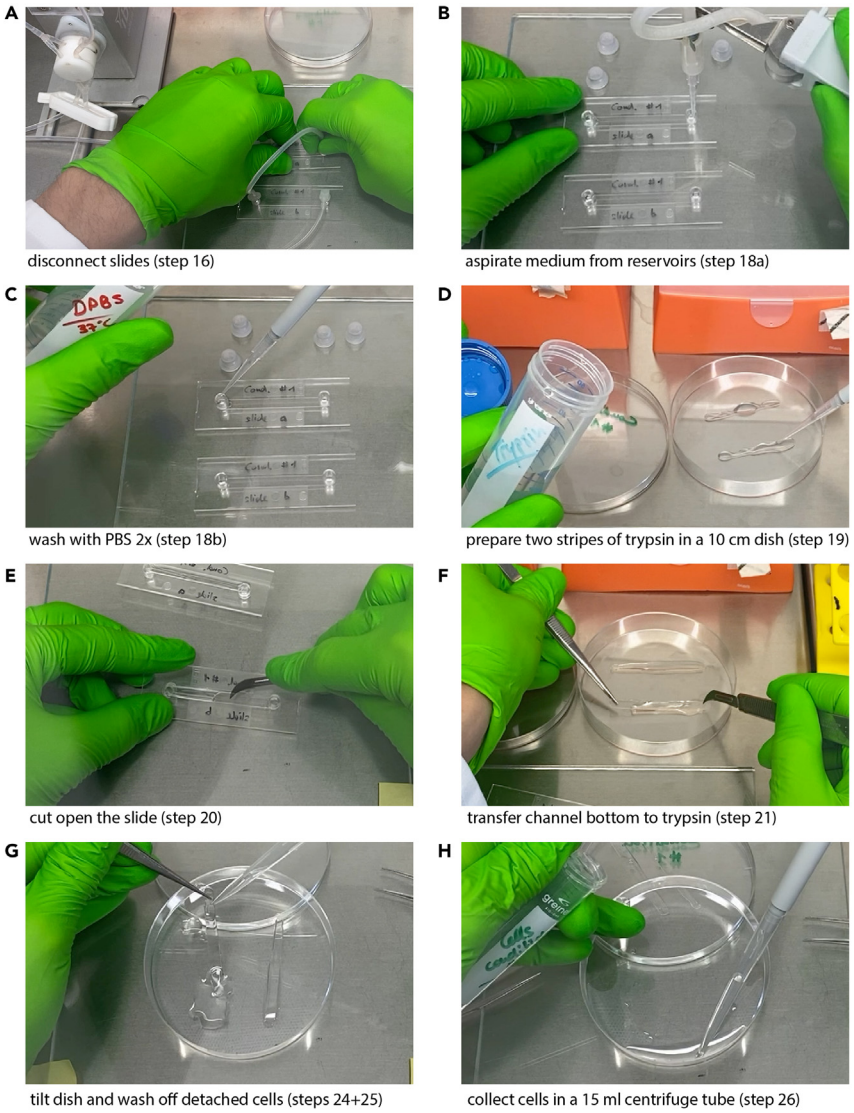

Figure 3. Key steps of cell harvest from channel slides

Video stills of Methods video S2 show how to (A) disconnect the tubing (step 16), (B and C) replace medium with PBS (step 18) before (D) preparing stripes of trypsin in a dish (step 19), followed by (E) cutting out the channel bottom (step 20), (F) transferring the plastic strip to the trypsin lines (step 21), (G) washing off the cells from the cut-outs (step 25) and (H) collecting the cells (step 26) for counting and centrifugation.

   c. Cold DPBS with 10% FCS (4°C).
   d. Cold tabletop centrifuge for 1.5 mL tubes (4°C).
   e. 1 labeled 1.5 mL DNA low bind tube per sample.
15. Stop flow.
16. Disconnect slides from perfusion set.
17. Confirm intact cell monolayer under a microscope (problem 2).
18. Aseptically, replace medium by pre-warmed PBS (37°C).
   a. Aspirate medium from reservoirs.
   b. 2x flush with 150 µL PBS (as in Application Note 03).[5]
   c. Aspirate PBS from reservoirs.
19. Per FSS condition, prepare the following.
   a. 10 cm dish.

b. Pipette two stripes of 200 μL warm trypsin into the dish.

20. Cut open the slide with a scalpel (blade #12).
    a. Turn the channel slide upside-down.
    b. Carefully introduce the scalpel tip at a reservoir end of the channel.
    c. Slice out the channel bottom along the channel edges until the strip containing the cell monolayer is completely cut loose.
21. Use sterile tweezers to transfer the plastic strip containing the cell layer to the trypsin, so the cells face the liquid.
22. Incubate for 5 min (37°C, 95% RH, 5% CO2).
23. Confirm detachment of cells under a microscope.
24. Place one edge of the dish on top of the lid so the bottom is tilted and liquid flows to one side.
25. Lift the plastic strip with tweezers and wash off the cells using 800 μL cold PBS with 10% FCS.
    a. Repeat the flushing with the same liquid several times.
    b. Avoid bubbles.
26. Transfer cell suspension to a 15 mL falcon tube, keep on ice from now on.
27. For static conditions.
    a. Wash dishes twice with 1 mL pre-warmed PBS.
    b. Add 200 μL Trypsin drop wise to cell area.
    c. Incubate for 5 min (37°C, 95% RH, 5% CO2).
    d. Wash off the cells using 800 μL cold PBS w/ 10% FCS.
28. Count cells (problem 3).
29. Transfer 5×10^4 cells to a new 1.5 mL DNA low bind tube.
30. Spin down cells (200 *g*, 5 min, low brakes) and discard supernatant.

Tagmentation

Timing: 1.5–2 h

In this section we describe how to perform the tagmentation of the ATAC-seq protocol,[9,10] optimized for HUAECs cultured under FSS.

31. Before starting the tagmentation, prepare the following.
    a. Cold lysis buffer (4°C).
    b. Cold wash buffer (4°C).
    c. Cold tagmentation buffer **without Tn5** (4°C).
    d. MinElute Reaction Cleanup Kit (QIAGEN).
    e. 1 labeled 1.5 mL DNA low bind tube per sample.
    f. 1 labeled MinElute Spin Colum per sample (4°C).

Optional: Lysis/permeabilization can be performed jointly with tagmentation, since the tagmentation buffer already contains Tween 20 and Digitonin. To do so, skip steps 32 to 36 and directly proceed with step 37. Successful tagmentation has to be evaluated based on the size distribution (step 75), which can vary in between different cell types.

32. Resuspend cell pellet in 50 μL lysis buffer.
33. Incubate for 3 min on ice.
34. Add another 50 μL wash buffer.
35. Spin down 500 *g* for 10 min at 4°C.
36. Discard supernatant.
37. Add Tn5 to Tagmentation buffer.
38. By gently pipetting up and down, resuspend pellet in 50 μL Tagmentation buffer.
39. Incubate at 37°C, 800–1000 rpm shaking for 30 min.
40. Get Qubit dye to warm up to RT (min. 30 min before measurement).

41. Clean-up with MinElute Reaction Cleanup Kit.
    a. Elute in 11–12 μL (instead of 10 μL, to get out 10 μL in the end).
    b. Elute for 3–5 min minimum (instead of 1 min, for higher yield).
42. Measure DNA concentration w/ Qubit (problem 4).

Optional: To measure DNA concentrations later, freeze 2 μL sample in a separate PCR tube.

Pause point: Purified and tagmented DNA fragments can be stored at -20°C for at least one week.

Library generation

Timing: 2.5–3 h

The transposase ligates two adapters to the DNA fragments while cutting the double strands. To obtain libraries with fragments ready for high-throughput sequencing, unique barcoded primers with common sequencing ends have to be ligated to each samples' DNA fragments. The necessary steps to perform library generation are described in this section.

43. Per sample, choose a suitable indexing primer combination (i5/i7 primer pair)[4] to allow for multiplexing in a sequencing flow cell.
44. To ligate the indexing primers to the fragments and amplify the library, combine the following reagents in a PCR tube and run on a PCR cycler with the following program.

PCR reaction mix	
Reagent	Amount
purified transposed DNA	10 μL
H_2O (DEPC treated)	10 μL
Ad1.x i5 indexing primer (25 μM)	2.5 μL
Ad2.x i7 indexing primer (25 μM)	2.5 μL
NEBNext High-Fidelity 2X PCR Master Mix	25 μL

PCR cycling conditions: Library generation			
Steps	Temperature	Time	Cycles
Initial extension	72°C	5 min	1
Initial denaturation	98°C	30 s	
Denaturation	98°C	10 s	5
Annealing	63°C	30 s	
Extension	72°C	1 min	
Hold	4°C	forever	

45. To estimate the required number N of additional PCR cycles for library amplification without creating a PCR bias due to saturation, combine the following and run on a RT-qPCR setup:

RT-qPCR reaction mix	
Reagent	Amount
partially amplified library	5 μL
H_2O (DEPC treated)	2.5 μL
Ad1.x i5 indexing primer (25 μM)	0.5 μL

(*Continued on next page*)

Continued	
Reagent	Amount
Ad2.x i7 indexing primer (25 μM)	0.5 μL
10X SYBR Green I	1.5 μL
NEBNext High-Fidelity 2X PCR Master Mix	5 μL

RT-qPCR cycling conditions			
Steps	Temperature	Time	Cycles
Initial denaturation	98°C	30 s	1
Denaturation	98°C	10 s	20
Annealing	63°C	30 s	
Extension	72°C	1 min	

46. Estimate number N of additional PCR cycles *per sample* by plotting the relative fluorescence vs. cycle no. and use the rounded C_T value (Figure 4) as N for further amplification.

Note: Do not exceed 6 additional PCR cycles to minimize above mentioned PCR bias.

47. Continue the initial PCR without addition of any reagents (45 μL remaining) with the following program.

PCR cycling conditions: additional library amplification			
Steps	Temperature	Time	Cycles
Initial denaturation	98°C	30 s	1
Denaturation	98°C	10 s	N (see above)
Annealing	63°C	30 s	
Extension	72°C	1 min	
Hold	4°C	forever	

Library size selection

Timing: 1.5 h

The obtained fragments usually contain primer dimers (≈120 bp) from the adapter ligation step. Additionally, an undesired fraction of large fragments (>1000 bp) is also often visible (Figure 5D). To enrich for the inter-, mono-, di-, and tri-nucleosomal fragments (150 bp – 1000 bp, Figure 5A), a bead based, double-sided size selection approach with AMPure XP beads is used. In a first round of selection, large fragments are removed with a lower bead/sample ratio. The second selection aims at binding the desired fragments (>150) to the beads with a higher bead/sample ratio. The resulting purified library is eluted from the beads in the last step.

The size selection can be done in standard 1.5 mL or 0.5 mL PCR reaction tubes, depending on the available magnetic rack. Usage of a magnetic rack for small tubes is preferred since no transfer is necessary.

48. Before starting the size selection, vortex and equilibrate AMPure XP beads to RT for 30 min on a rotator.
49. (Optional) Transfer each sample to a new 1.5 mL reaction tube.
50. Refill each sample to 50 μL (add 5 μL water).

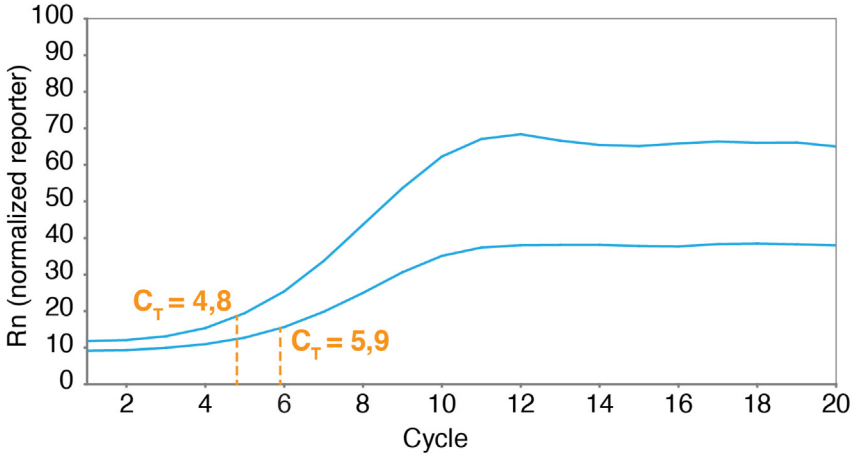

Figure 4. qPCR amplification plot

qPCR is performed (step 45) with 5 µL of the generated library from step 44. To estimate number of additional PCR cycles (step 46) for sufficient library amplification without introducing PCR bias, round C_T value to closest cycle number (here 5 and 6) or calculate cycle number for 1/3 of maximum reporter intensity to use for further amplification (step 47).

51. Add 0.55× of sample volume (27.5 µL) AMPure XP beads.
52. Mix thoroughly by pipetting 10×.
53. Incubate at RT for 10 min.
54. Place reaction tube into magnetic rack.
55. Wait until liquid becomes clear.
56. Transfer supernatant to new tube.
57. Add 0.9× of original sample volume (45 µL) AMPure XP beads to the transferred sample.
58. Incubate at RT for 10 min.
59. Place reaction tube into magnetic rack.
60. Wait until liquid becomes clear.
61. Discard supernatant.
62. Wash beads with 200 µL 80% EtOH.
    a. Gently add and remove EtOH to the bottom of the tube 2 times.
63. Remove EtOH completely without disturbing the pellet.
64. Make sure all drops of EtOH on the tube walls are removed.
65. Let residual EtOH evaporate while on magnetic rack.
66. Dry pellets until appearance changes from glossy to silk-like.

⚠ CRITICAL: Avoid any EtOH residuals.

⚠ CRITICAL: Do not over-dry the pellets and avoid cracks. Otherwise only a reduced fraction of the DNA fragments will elute from the beads.

67. Remove the tube from the magnetic rack.
68. Resuspend the beads in 21 µL water (DEPC) by pipetting 10×.
69. Place reaction tube into magnetic rack.
70. Wait until liquid becomes clear.
71. Transfer supernatant to final 1.5 mL tube.

Library quality assessment

Timing: 30 min

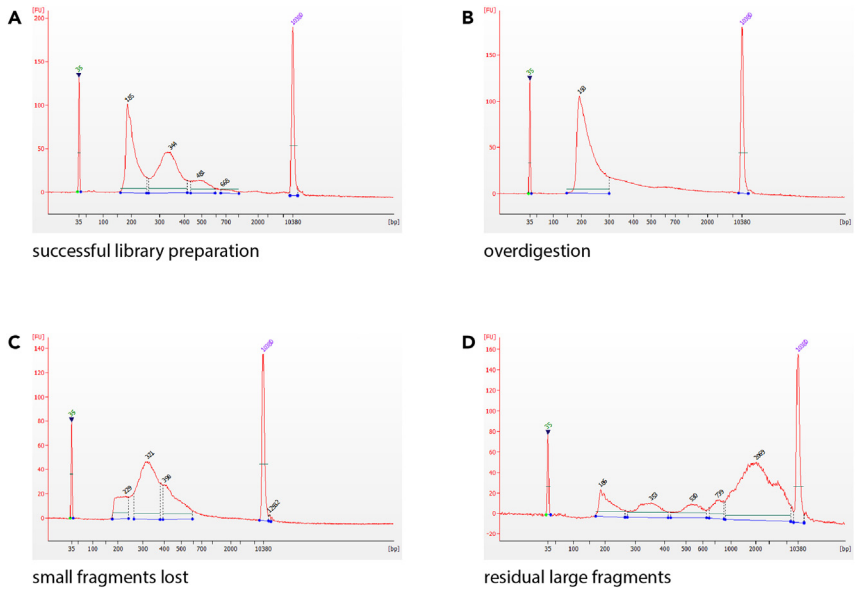

Figure 5. Quality control of ATAC sequencing libraries

Fragment size quality control of sequencing libraries with automated electrophoresis can reveal (A) successful library preparation, (B) overdigestion (problem 6), (C) removal of small fragments during size selection (problem 7) or (D) residual large fragments (problem 8). Blue lines below peaks resemble baseline, numbers above peaks show fragment size of highest value.

Quality control of the purified libraries is essential to ensure expected fragment distribution and avoid contamination by primer-dimers. To test DNA concentrations and gain insights on the size distribution of the purified libraries, fluorometer and electrophoresis measurements should be conducted as follows.

72. In a separate PCR tube, dilute 1 µL sample in 3 µL H_2O.
73. Measure DNA concentration using 1 µL of the 1:4 dilution with a Qubit Fluorometer.
74. Further dilute the 1:4 dilution to obtain a 1 ng/µL DNA dilution.

Note: When using the above volumes and the concentration of the diluted sample C_{dil}, the calculation for the additional volume of water V_{add} is:

$$V_{add} = C_{dil} \times 3\mu L \times 1\frac{\mu L}{ng} - 3\mu L$$

75. Run 1 µL of the diluted library on a Bioanalyzer HS DNA chip or a TapeStation D5000 HS ScreenTape to validate the fragment distribution (Figure 5, problems 5, 6, 7, 8).

Pause point: Store ready-to-run libraries at −20°C until sequencing.

Sequencing

Timing: depending on sequencing service availability

The above protocol showed how to prepare ATAC libraries ready for sequencing. The next steps explain how we recommend to sequence the samples to obtain fastq files containing the sequences

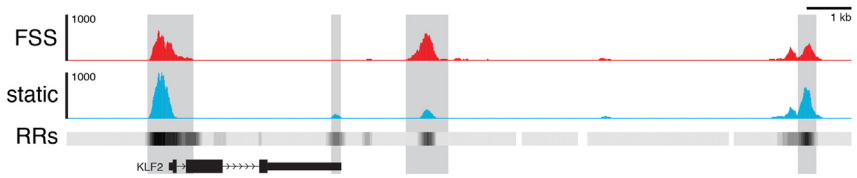

Figure 6. Sequencing output of HUAECs stimulated with FSS

Read coverage of regions with altered chromatin accessibility from static vs. FSS stimulated HUAECs is highlighted for the *KLF2* gene locus in this adjusted UCSC[6] screenshot (genome.ucsc.edu). FSS-sensitive chromatin accessibility is observed in known EC regulatory regions (RRs,[11] UCSC track with filtering for the term '*EC').

of the enriched fragments. Each fragment's end resembles the cut site of a Tn5 enzyme, which is why we highly recommend paired-end sequencing.

76. High-quality libraries are sequenced using the 2 × 100 bp PE (paired-end) Illumina NovaSeq 6000 setup to a minimum depth of 50×10^6 reads or other comparable settings.
    a. In case of technical replicates, the *combined* read depth should be 50×10^6 reads.
77. For transcription factor (TF) footprinting analysis, sequencing depths of minimum 200×10^6 reads are recommended.

EXPECTED OUTCOMES

This protocol shows how to prepare and sequence libraries for ATAC-seq on HUAECs stimulated with FSS and BMP9. The expected fragment distribution shows no fragments <150 bp (Figure 5A). This minimal length represents the shortest possible fragments, ligated to the two barcoded sequencing adapters (i5 and i7). The subsequent peaks resemble DNA strands wrapped around histone complexes 0, 1, 2, etc. times (n-nucleosomal fragments). The shortest, inter-nucleosomal fragments are the most interesting as they represent the open fraction of DNA in the nucleus. Fragments shorter than 150 bp, usually around 125 bp, are primer-dimers which should be avoided to reduce sequencing noise.

After successful library preparation, sequencing and consecutive mapping, ATAC-seq tracks can be compared between different experimental conditions, such as FSS regimes or growth factor treatments. Differential accessible regions can further be compared with known TF-binding sites or cis regulatory regions (Figure 6).

Quantification of cell numbers and DNA concentrations

When harvesting cells, measuring tagmented DNA fragments as well as quantifying library concentrations, values can vary depending on various parameters. Table 1 provides real values from different samples processed with the above protocol.

LIMITATIONS

In this protocol we demonstrate the preparation of ATAC-seq libraries from ECs stimulated with FSS and ligands (optional). The flow setup, used to apply physiological fluid shear stress conditions, limits the cell number per condition. Therefore, the harvesting procedure is the most challenging step. Cutting open the channels needs hands-on training as shown in Methods video S2. Also, trypsinization needs to be tightly controlled, since over-trypsinization drastically reduces cell viability. This then introduces problems during the next steps such as over-tagmentation (Figure 5B). Furthermore, the overall low cell numbers of the flow setup limit the application to one sequencing-based read out such as ATAC-seq at a time and is not easily scaled up. Furthermore, the application of FSS to cell types other than the described ECs has to be established accordingly in regard of cell numbers, attachment strength and ramp phases. Fluid Shear Stress regimes would have to be adapted based on the respective vascular bed. Due to the laborious harvesting procedure, FSS exposure time resolution is limited to hours rather than minutes.

Table 1. Exemplary quantifications per sample in the different steps: cell counts per channel, DNA concentrations after tagmentation and after library generation

Sample condition	Step 28: Cell count (cells/mL)	Step 42: [DNA] (ng/μL) after tag	Step 73: [1:4 dil] (ng/μL)	Step 74: V_add (μL)	4×[1:4 dil] = [Lib] (ng/μL)
Static	1.90×10^5	6.4	6.33	15.99	25.32
Static+BMP9	1.75×10^5	4.3	1.93	2.79	7.72
FSS	6.00×10^4	6.7	11.2	30.6	44.8
FSS+BMP9	1.35×10^5	5.3	3.03	6.09	12.12
Average	1.34×10^5	*5.68*	*5.62*		*22.49*

TROUBLESHOOTING

Problem 1

Cell viability impaired due to silicone rubber inserts (step 11, 12 and 17 to 20 in before you begin).

Potential solution

- The silicone rubber elastomer might contain residual soluble components such as monomers from the two components A and B. Try repeating step 11 and 12 and test cell viability again.
- In case repeated cooking does not help, try a different batch of silicone rubber or choose a comparable biocompatible elastomer.

Problem 2

Cells lost in flow setup (step 17).

Potential solution

- Air bubbles flushing over the cells might destroy the monolayer. Remove all air bubbles before attaching the channel slides. While running the "remove bubbles" program from the perfusion system, flick all connectors and check for the release of bubbles. Only then attach the slides.
- Too long switching times will lead to an empty running of the reservoirs, introducing air into the perfusion system. Shorten the switching time so there is always a minimum of 2 mL of medium in both reservoirs.
- Starting the flow program from a state of unequally filled reservoirs can lead to the introduction of air into the perfusion system. Always start from equilibrated reservoirs when initially starting the flow.

Note: When using the pause function, the system will automatically go on with the correct direction and timing. There is no need to equilibrate the reservoirs.

- Perfusion at high flow rates without equilibration can disturb the cell monolayer. When applying high shear rates, use a ramp phase to let the cells adjust to the mechanical stress.
- In subconfluent monolayers, cell adhesion is not as strong as in confluent cell layers. Make sure cells reach confluence before applying FSS (Figure 2). You can either increase cell numbers when seeding or prolong the cultivation in the channel slides until a confluent monolayer is formed.
- Contamination, even if not visible, strongly impairs cell attachment. Always make sure to handle open perfusion sets, reservoirs and channels under sterile conditions. Transfer the setup in a closed state to the incubator and run decontamination programs on a regular basis. Placing of flow setups tend to facilitate contamination of incubators due to long opening times during the setup.
- For attachment, ECs rely on binding motifs such as arginyl-glycyl-aspartic acid (Arg-Gly-Asp protein sequence, RGD) provided by protein coating with gelatin or other extracellular matrix (ECM) molecules. Make sure your cell type recognizes the abundant motif and verify a consistent coating.

Problem 3

Not enough cells after harvesting (step 28).

Potential solution

- Validate an intact cell monolayer under a microscope after stopping the flow. In case of detachment see problem 1.
- When flushing the cells off the cutout plastic stripe, make sure to remove all cells from the surface. To do so, place the cutout into a 10 cm dish, the cells previously covered with cells facing the dish. Residual PBS will provide enough liquid to ensure a temporary attachment by capillary forces. Check the sample under a microscope for remaining cells. If necessary, repeat PBS wash (no FCS!) and Trypsin treatment with subsequent flushing with 10% FCS in PBS (cold).
- Make sure the channels are washed with enough PBS to completely remove the medium. Residual FCS will block Trypsin activity.
- Viability is crucial for collecting sufficient cell numbers. Several parameters, such as foaming while pipetting, high centrifuge speeds and prolonged trypsin exposure, drastically reduce cell survival during the harvesting process. When counting cells, use trypan blue or other appropriate markers to quantify viability.

Problem 4

Low DNA concentrations after tagmentation (step 42).

Potential solution

- Digitonin is a critical agent for the perforation of the nuclear envelope, facilitating the diffusion of transposed DNA fragments out of the nucleus. Due to its unstable nature, keep thaw-freeze cycles of the digitonin solution at a low number. In case of doubt freshly prepare the proper dilution.
- Some cells need stronger or longer lysis/permeabilization procedures due to their ECM composition. Make sure all reagents are prepared in the correct concentrations and did not exceed their shelf life. Optionally, longer incubation times or higher concentrations of detergents can be tested, verifying permeability using trypan blue.

Problem 5

Strong primer-dimer peaks at ≅ 125 bp (step 75).

Potential solution

- When performing bead purification, test different bead/sample ratios for the best cutoff without removing any library fragments. Reducing the amount of AMPure XP beads in the step with 0.9× sample volume of bead solution raises the cutoff size for small fragments.
- The provided primers have a high affinity to the tagmented fragments. Hence, reducing primer concentrations might still lead to sufficient library amplification while reducing primer-dimer contamination.

Problem 6

No nucleosomal peaks visible (step 75, Figure 5B).

Potential solution

- As previously described, the ratio between cell number and Tn5 transposase strongly influences tagmentation efficiency. In case of too high Tn5 concentrations, transposition efficiency is too high and nucleosomal fragments get further digested, resulting in a smeared, peakless size distribution. To avoid this, make sure to keep the timing on point. Additionally, you can test lower Tn5/cell ratios by adjusting the volume of transposase solution accordingly.

Problem 7

The first, inter-nucleosomal peak, is very low (step 75, Figure 5C).

Potential solution

- The amount of open chromatin is variable. Hence, the peak heights are subject to variation. However, if the first peak appears very low, your size selection might have cut off the small fragments of the library. Check bead/sample ratios and increase the amount of bead solution in the step when adding 0.9× the sample volume of bead solution.

Problem 8

Large amounts of big fragments visible (step 75, Figure 5D).

Potential solution

- Destroyed cells release their DNA into the surrounding liquid. The highly accessible DNA will also be tagmented and contaminate your library. When harvesting and collecting cells for tagmentation, ensure gentle centrifugation and pipetting conditions to increase viability.
- When performing size selection of your libraries, large amounts of big fragments might saturate the binding capacity of your beads. Either increase the bead solution volume in the 0.55× step or repeat the removal of big fragments. Make sure to validate the ratios before performing cleanups on your actual libraries.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Petra Knaus (petra.knaus@fu-berlin.de).

Technical contact

Technical questions on executing this protocol should be directed to and will be answered by the technical contact, Lion Raaz (raaz@molgen.mpg.de).

Materials availability

This study did not generate new unique reagents.

Data and code availability

The published article[1] includes all ATAC-seq data generated and analyzed during this study.

The ATAC-seq data analyzed during this study is available at GEO: GSE227588. The regulatory regions track from ReMap[11] (RRs, see Figure 6) was added in the UCSC browser[6] directly, using the following filter option:

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*EC
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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2024.102859.

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AUTHOR CONTRIBUTIONS

L.R. developed the method and drafted the manuscript and figures. P.-L.M. co-developed the method and commented on the manuscript. J.J. designed the graphical abstract and commented on the manuscript. S.M. advised on the experimental design and commented on the manuscript. P.K. advised on the experimental design and commented on the manuscript. L.R. and J.J. designed the experiments, discussed all data, and wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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