Epigenetic Optical Mapping of 5-Hydroxymethylcytosine in Nanochannel Arrays

Tslil Gabrieli[1&], Hila Sharim[1&], Gil Nifker[1], Jonathan Jeffet[1], Tamar Shahal[1], Rani Arielly[1], Michal Levi-Sakin[1], Lily Hoch[1], Nissim Arbib[2], Yael Michaeli[1]* and Yuval Ebenstein[1]*

[1]School of Chemistry, Center for Nanoscience and Nanotechnology, Center for Light-Matter Interaction, Raymond and Beverly Sackler Faculty of Exact Sciences, Tel Aviv University, Tel Aviv, Israel; [2]Department of Obstetrics and Gynecology, Meir Hospital, Kfar Saba, Israel & Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel.

Supporting information

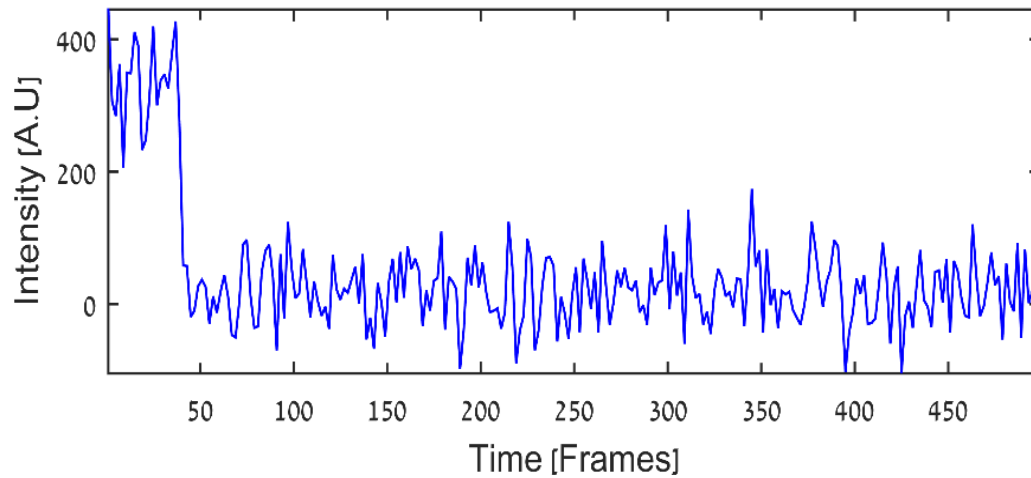**Quantification of 5-hmC sites per detected label by measuring photobleaching steps.**

Figure S1. Time traces for photobleahching steps of 5-hmC labelling. 500 frames, 20 ms, 60 W/cm^2, 637/ 666 nm.
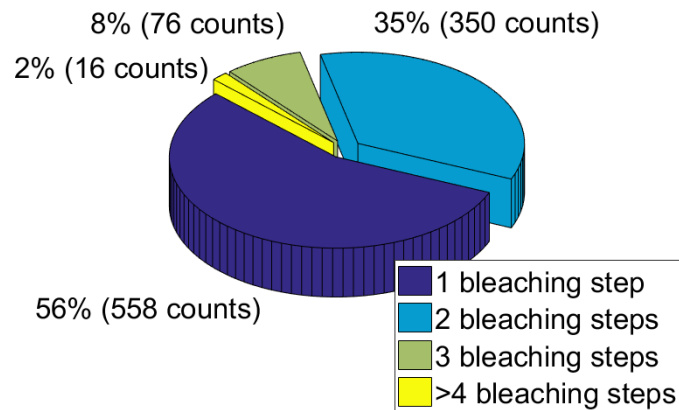

Figure S2. Pie chart representation of the amount of photobleaching steps per Intensity-time trace. It is clear that in most cases (>90%) there are less than 3 photobleaching steps per time trace.
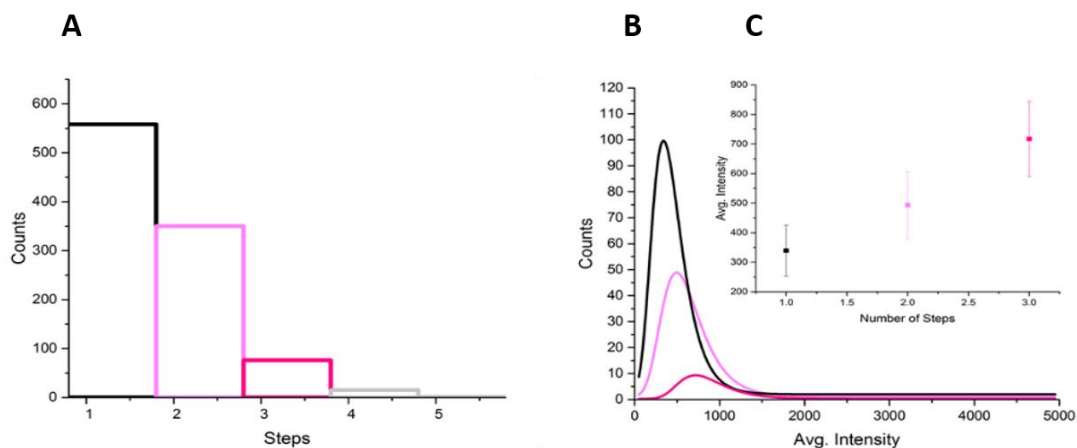
Figure S3. **A**. 5-hmC photobleaching steps distribution. **B**. Intensity distribution. **C.** Average intensity measured per number of steps.

Measuring 5-hmC levels by mass spectrometry

In order to verify the 5-hmC density obtained from optical mapping, we performed LC-MS/MS measurements on DNA extracted from the same PBMCs sample.

Extraction and preparation of genomic DNA

DNA was extracted from PBMCs using archive pure DNA cell kits (5Prime), according to the manufactures' protocol. Extracted DNA was hydrolyzed following denaturation at 98 °C for 5 min using the following enzymes: Nuclease S1 (Promega), phosphodiesterase (Affymetrix) and Antartic phosphatase (New England Biolabs). Denatured DNA was incubated with Nuclease S1 (100 U/ 5 µg DNA) for 3 h at 37 °C and then with a mixture of phosphodiesterase (5 U/ 5 µg DNA) and phosphatase (0.1 U/ 5 µg DNA) in the presence of the enzymes' related buffers. The resulting nucleoside mixture was cleaned by an AMICON ULTRA 0.5ML10K filter (Mercury) and 10 µL of the filtered samples were injected into the LC–MS/MS system.

Standards for Calibration curve

The percentage of 5-hmC in the sample was interpolated from a calibration curve. The standards for the calibration curve were prepared by the hydrolysis of 1 kbp PCR product prepared by forward and reverse primers containing one 5-hmC each. Mixing 5-hmC-labeled 1 kbp PCR products with non-labeled 1 kbp products yields standards with different percentages of 5-hmC in the range of 0.001-0.01% (for example: 10% 5-hmC 1 kbp product mixed with 90% non-labeled 1 kbp product results in a 0.01% 5-hmC standard). PCR was performed by amplification of Lambda DNA (New England Biolabs) using 0.4 µM of the following primers: forward primer, 5'-CTC ATG CTG AAA A/i5HydMe-dC/G TGG TG-3'; reverse primer, 5'-GGA CAG GAC /i5HydMe-dC/AG CAT ACG-3'. A typical reaction was performed in 50 µl volume containing 200 ng of template DNA and MyTaq Red Mix (Bioline). PCR amplification was conducted according to the following protocol:

incubation at 95 °C for 1 min as an initial step, followed by 30 cycles of 30 sec at 95 °C, 60 sec at 53 °C, and 60 sec at 72 °C, and finally 5 min at 72 °C.

LC-MS/MS measurements

The chromatographic separations were performed on an Acquity UPLC system (Waters) using an Xselect HSS T3 XP column (2.5um 2.1 x 75 mm, Waters), which was maintained at 40 °C. The mobile phases used in the current study were 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (ACN) (solvent B). The separations were performed at a flow rate of 0.3 ml/min with the following gradient elution conditions: 100% of solvent A from 0 min to 1 min, linear increase to 4.0% solvent B from 1 min to 2 min, linear increase to 9% solvent B from 2 min to 4 min, from 4 min to 5 min linear increase to 20% solvent B which was maintained up to 5.5 min. At 5.51 min the composition was returned to 100% solvent A, and held under these elution conditions up to 8.0 min. The UPLC instrument was coupled to a Xevo TQD mass spectrometer (Waters), which was operating in the positive ion electron spray ionization (ESI⁺) mode. The target nucleosides were monitored by a multiple reaction monitoring (MRM) method according to previously published procedure with minor variations[2].

Calculation of 5-hmC percentage

The percentage of 5-hmC in DNA samples was calculated based on the ratios between the peak area of the 5-hmC MRM transition signal and the sum of peak areas of C,G,A and T MRM transition signals for the sample. This approach allowed normalization to differences in the amounts of DNA in each sample and accounted for the variability in GC content between PCR products and genomic DNA. The ratiometric data was plotted as a function of known 5-hmC content in the standard samples in order to build a calibration curve (Figure S4, blue dots). The percentage of 5-hmC in genomic PBMCs DNA was calculated by interpolating the ratiomatric data obtained for this sample from the calibration graph (Figure S4, red dot).

The calibration curve is linear with an R-squared of 0.9929. The percentage of 5-hmC in DNA extracted from PBMCs was calculated by extrapolation to be 0.0035%. This is in agreement with the mean 5-hmC density measured in the optical mapping experiment (0.0029%).
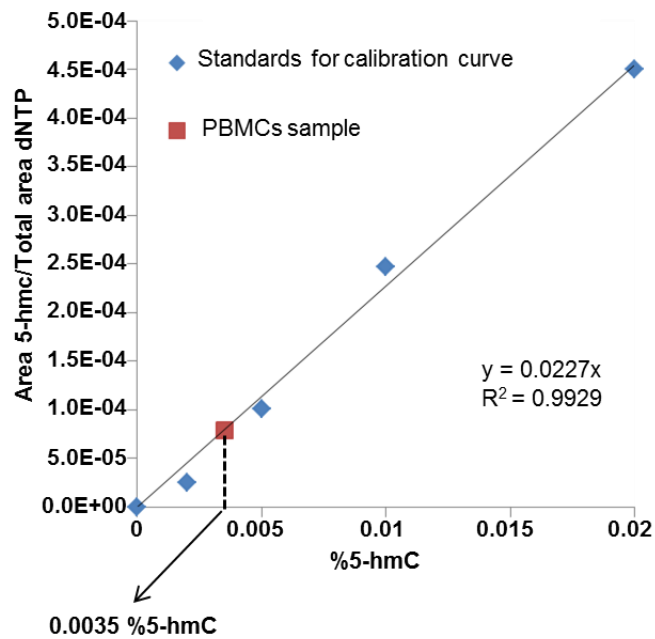
Figure S4. An area-based linear regression calibration curve based on standard samples containing different percentages of 5-hmC (blue dots). For each 5-hmC standard, the ratio between the area of the MRM transition signals of 5-hmC and the one for all the nucleotides were plotted as a function of the nominal percent of 5-hmC in the standard sample. The red dot represents the 5-hmC value in a blood sample.

Determining minimal signal level in hMeDIP experiment by the signal extraction scaling method
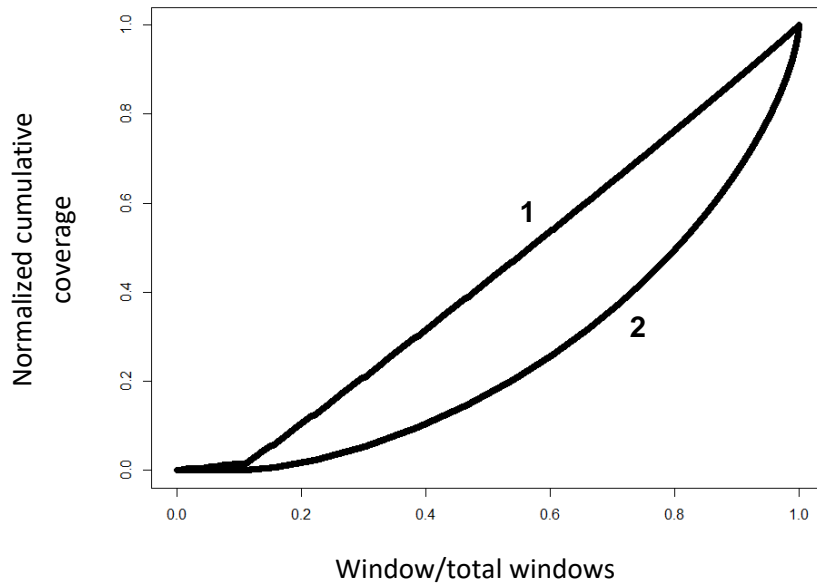

Figure S5. Normalized coverage cumulative sum in 1 kb non-overlapping windows (see methods). 1: input, 2: 5-hmC.
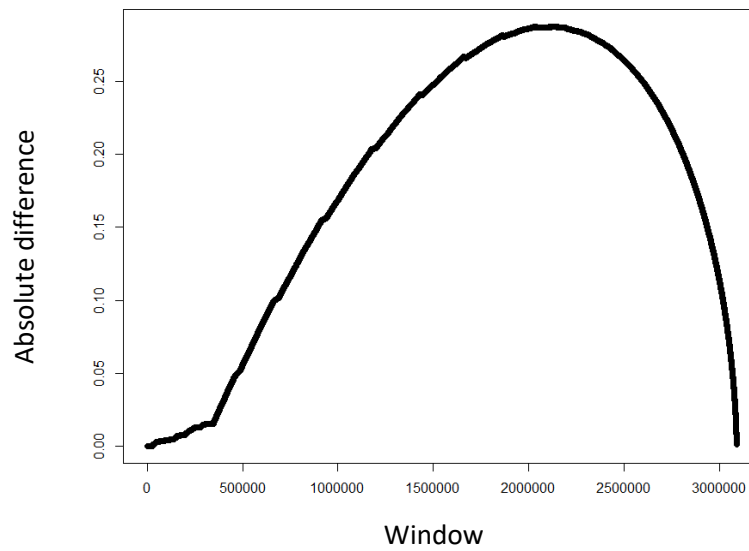

Figure S6. Absolute value of difference between cumulative sums in each window (see methods).