

Chapter 7

Flow Cytometric and Laser Scanning Microscopic Approaches in Epigenetics Research

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Abstract

Our understanding of epigenetics has been transformed in recent years by the advance of technological possibilities based primarily on a powerful tool, chromatin immunoprecipitation (ChIP). However, in many cases, the detection of epigenetic changes requires methods providing a high-throughput (HTP) platform. Cytometry has opened a novel approach for the quantitative measurement of molecules, including PCR products, anchored to appropriately addressed microbeads (Pataki et al. 2005. *Cytometry* **68**, 45–52). Here we show selected examples for the utility of two different cytometry-based platforms of epigenetic analysis: ChIP-on-beads, a flow-cytometric test of local histone modifications (Balint et al. 2005. *Mol. Cell Biol.* **25**, 5648–5663), and the laser scanning cytometry-based measurement of global epigenetic modifications that might help predict clinical behavior in different pathological conditions. We anticipate that such alternative tools may shortly become indispensable in clinical practice, translating the systematic screening of epigenetic tags from basic research into routine diagnostics of HTP demand.

Key words: Chromatin immunoprecipitation (ChIP), flow cytometry, ChIP-on-beads, laser scanning cytometry (LSC).

1. Introduction

Epigenetic changes associated with gene regulation play a major role in the establishment of altered differentiation states (3). Specific modifications often correlate with gene activation or repression; for instance H3K4ac and H3K4me3 are permissive for gene activation whereas H3K9me2, H3K27me3, and methylation of CpG islands in promoter regions correlate with transcriptional silencing. Often, activating and repressive marks co-exist at gene start sites, reflecting perhaps epigenetic heterogeneity among otherwise similar cells, establishing a fine balance that could determine the gene expression patterns in the tissue.

49 The ‘epigenetic code’ has become an indispensable concept in
50 basic research, and its principles are also utilized to develop drugs
51 and diagnostic tools. Several genes being epigenetically misregu-
52 lated have been shown to associate with different kinds of cancer,
53 highlighting the role of the ‘language’ of covalent modifications in
54 tumorigenesis (4, 5). For instance, based on the patterns of mod-
55 ifications, two disease subtypes with different risks of tumor recur-
56 rence have been characterized in prostate cancer patients,
57 independently from tumor stage, preoperative prostate-specific
58 antigen levels, and capsule invasion (6).

59 The chromatin of cancer cells often exhibits both an overall (glo-
60 bal) DNA hypomethylation and hypermethylation of specific regions,
61 leading to ‘DNA methylation imbalance’ (7). The recurrence of global
62 DNA hypomethylation in many types of human cancer is suggestive of
63 its significant role in carcinogenesis, perhaps by inducing genomic
64 instability and/or activating oncogenes (8, 9). However, global hypo-
65 methylation is subject to a high degree of variability, unaccounted for
66 by our current level of understanding (10, 11). In addition to neoplas-
67 tic transformation, problems of epigenetic regulation, including CpG
68 methylation disorders are also involved in a wide range of pathological
69 phenomena (12, 13). In most eukaryotes, methylation of DNA occurs
70 at the cytosine residues of cytosine-phospho-guanine (CpG) dinucleo-
71 tides. The enzymes responsible for the production of 5-methylcytosine
72 (5-mc) involving the fifth carbon atom of cytosine in CpG dinucleo-
73 tides are the DNA methyltransferases DNMT1, DNMT3a, and
74 DNMT3b, of which the first is involved in the maintenance of methy-
75 lation during DNA replication, while all appear to be important in the
76 establishment of methylation patterns in most physiological and patho-
77 logical settings (14–16).

79 **1.1. Flow- and Laser** 80 **Scanning Cytometry in** 81 **Epigenetics Research**

82 Our understanding of epigenetics has been transformed in recent
83 years by a succession of technological innovations. Approaches
84 involving microarrays and, most recently ultra-high throughput
85 (deep) sequencing technology have been applied to map cytosine
86 methylation, chromatin modifications, and ncRNAs across entire
87 genomes. Genome-scale studies of histone modifications and
88 other aspects of chromatin structure typically rely on an immuno-
89 logical procedure, chromatin immunoprecipitation (ChIP) (17),
90 in which specific antibodies are used to enrich chromatin. ChIP is a
91 powerful tool in epigenetics; however, in many cases the detection
92 of epigenetic changes or transcription factor binding associated
93 with the regulation of certain genes would require ChIP-based
94 methods that provide high-throughput (HTP) potential. Moni-
95 toring local as well as global changes of epigenetic markers could
96 be extremely useful in diagnostics as well as in basic research.

Flow-cytometric analysis provides a novel means for the quanti-
tative measurement of molecules also in cell-free solutions, anchoring
them to appropriately addressed microbeads. The utility and power

of this approach has been demonstrated in the case of various assays of molecular diagnostic value: immunoassays, sensitive measurement of protease or nuclease activity, detection of deletion/insertion of sequences by heteroduplex analysis, etc., that could all be adapted to a 'lab-on-beads' platform, i.e., the flow-cytometric analysis of microbead-captured macromolecules (1, 18, 19). Many samples can be simultaneously analyzed in a FACSarray instrument using fluorescent dyes matching its optical channels.

Beyond lending a HTP platform for the analysis of gene-specific epigenetic markers, cytometry also makes global analysis of epigenetic changes possible, most conveniently in its on-slide format, by microscope-based cytometers. Laser scanning cytometry (LSC) provides a robust method for analyzing single-cell events on slides (20, 21). It generates quantitative fluorescence data similar to flow cytometry, but the analyzed cells are attached to the surfaces of microscopic slides or culture chambers. The main advantages of LSC are that (i) the possible correlation between the simultaneously measured parameters is detected at the individual cell resolution, i.e., with a sensitivity surpassing that of flow cytometry; (ii) the instrument is able to relocate each cell for additional measurements, thus the analysis of functional features of live cells can be combined with measurements that require fixed cells; and (iii) measurements can be performed in an automated fashion, pre-programmed for several slides.

Examples highlighted in this review demonstrate the value of two different HTP platforms for epigenetic analysis, namely ChIP-on-beads and assessment of global epigenetic traits by LSC. These methods might help introduce systematic screening of different epigenetic tags into clinical practice, especially of those that correlate with therapeutic success. It will be shown that sequence-specific capture of PCR-amplified ChIP-fragments on microbeads allows a robust detection of histone-tail modifications in the promoter region of a well-characterized gene, tissue transglutaminase type 2 (*TGM2*). We also assess the prospects of laser scanning cytometry for the analysis of epigenetic changes involving the whole genome via the example of a global DNA methylation test.

1.2. High-Throughput Screening of Local Epigenetic Changes by ChIP-on-Beads

We have investigated the cellular levels of H4K acetylation and H3K4 methylation of the histone tails at the promoter of the *TGM2* gene, to test whether these covalent modifications can be detected using a flow-cytometric platform. As shown earlier (2) and briefly recapped herein, the flow-ChIP method, nicknamed ChIP-on-beads, can be easily implemented in a routine flow-cytometric clinical laboratory without relying on real-time QPCR. In the ChIP-on-beads assay, a standard ChIP is performed and then this DNA is used as template in an end-point PCR reaction. The sense and anti-sense primers are tagged at their 5' ends with fluorescent dyes (e.g., Fam, Cy3) and biotin,

respectively. Small aliquots of the Fam/biotin-ended PCR products are then bound to streptavidin-conjugated microbeads and quantified by flow cytometry. Of note, PCRs must be stopped in the linear phase to ensure reliable quantification; this should be initially determined in pilot QPCR experiments. The similarity of data obtained by QPCR and by flow cytometry has been shown (2).

As shown in Fig.7.1A, the fluorescence intensity of the microbeads increases linearly with the quantity of the fluoresceinated PCR products added, allowing the expression of ChIP-PCR

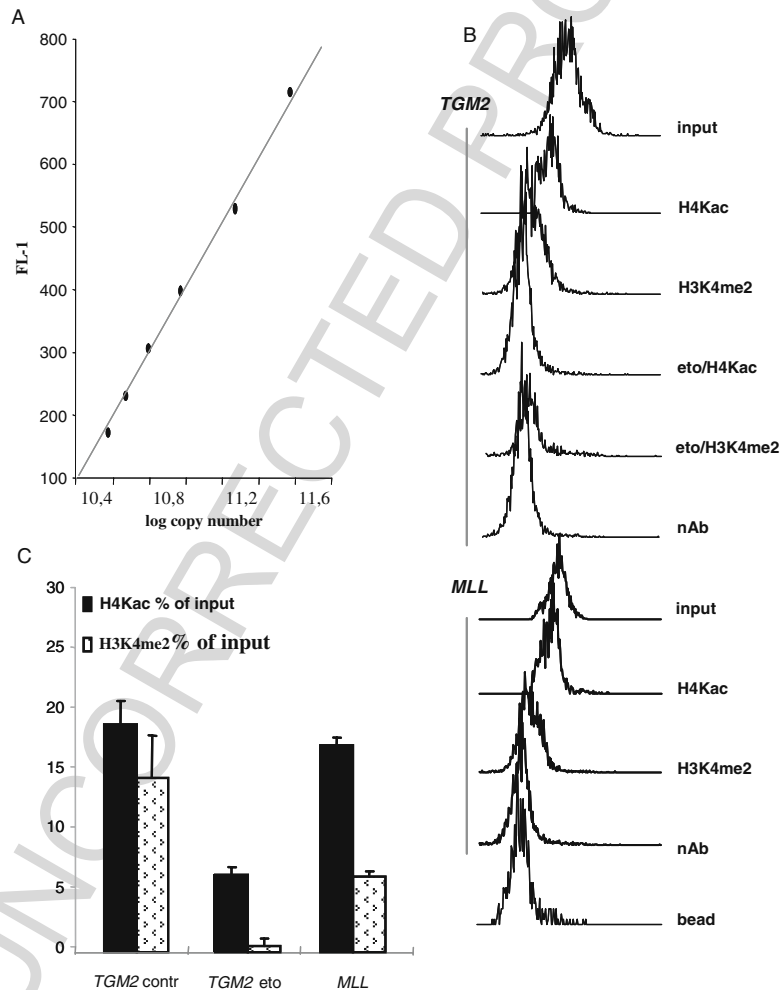


Fig. 7.1. Analysis of gene specific histone modifications by ChIP-on-beads. (A) Calibration curve (FL-1 vs. log copy number) based on a dilution series of known quantities of Fam/biotin-tagged PCR products. *TGM2* copy numbers of ChIP-PCR samples were determined by reference to this standard curve. (B,C) ChIP-on-beads analysis of H4Kac and H3K4me2 histone modifications at the *TGM2* gene promoter and at exon 9 of the *MLL* gene, in Jurkat cells. Apoptosis was induced by etoposide treatment (Eto). (B) Flow-cytometric fluorescence distribution histograms of Fam/biotin-labeled ChIP-PCR samples captured on streptavidin-conjugated microbeads. (C) The level of modified histones within the *TGM2* and *MLL* genes are expressed as percent of input values (Y axis), based on the means of fluorescence distribution and after subtracting the background (i.e., no-antibody % of input values). Panels (B) and (C) were reproduced from (2).

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yields as absolute copy numbers. The flow-cytometric fluorescence distribution means are used to calculate the fraction of DNA copy numbers in the ChIP samples relative to the input DNA (Fig.7.1B). Comparing control and early-apoptotic Jurkat cells for changes in the level of H4Kac and H3K4me within the promoter of *TGM2*, we observed a significant decrease in both histone modifications (Fig.7.1C), suggestive of the closure of chromatin structure early upon apoptosis. In comparison, the observed histone modifications at exon 9 of the *MLL* gene, used as positive control, were in accordance with its known histone-code profile (22); in contrast, the β -globin gene, used as negative control, gave <0.1% Ab/input ratios (not shown).

1.3. Testing Global Epigenetic Changes by Laser Scanning Microscopy: Studies on DNA Methylation

It is often important to consider in what global context local epigenetic changes occur (23). Moreover, global changes of certain epigenetic modifications may have their independent diagnostic value, especially when analyzed in correlation with other phenotypic markers, an opportunity offered by up-to-date laser scanning microscopic systems (20, 21). Development of antibodies and chimeric methyl CpG-binding antibody-like proteins (24–27), both recognizing CpG with high specificity, has opened novel perspectives for the diagnostic analysis of global methylation states. Anti-5mC antibodies are commercially available through various sources (e.g., Abcam and Biocarta US).

In experiments using the recombinant mCpG-binding antibody-like MBD-Fc protein (26–28), the overall level of CpG methylation has been quantified in the HCT116 cell line (Fig.7.2). As shown by confocal laser scanning microscopy

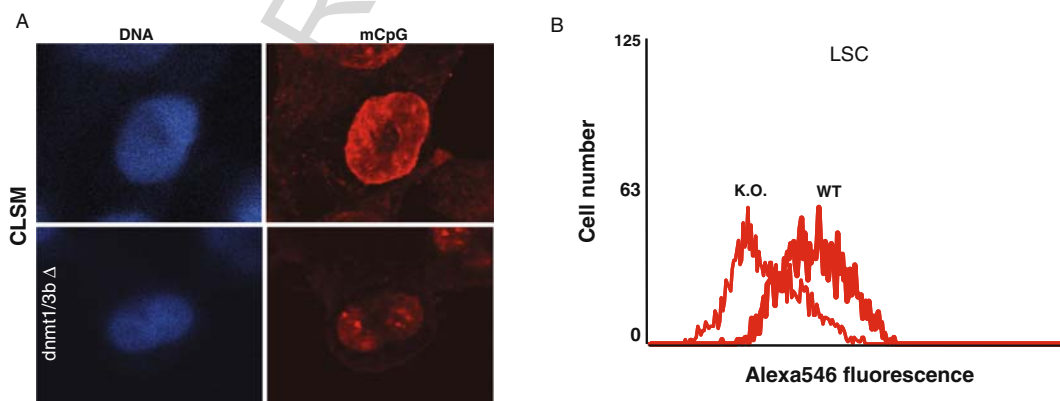


Fig. 7.2. Global DNA methylation analyzed by confocal laser scanning microscopy and laser scanning cytometry. *WT*: wild-type *DNMT1/DNMT3b* HCT116 cells immunolabeled with the MBD-Fc fusion protein. *K.O.*: *dnmt1/dnmt3b* knock-out HCT116 cells immunolabeled with the MBD-Fc fusion protein. *Left slides*: DNA stained by Hoechst. *Right slides*: methylated DNA (mCpGs). (A) Methylated CpG dinucleotides visualized by confocal laser scanning microscopy (CLSM). (B) Sample analyzed by laser scanning cytometry (LSC). MCpG (red) fluorescence was quantified in the slide-attached cells ($n > 400$) and presented (in arbitrary units) as fluorescence distribution histograms.

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(CLSM), mCpGs have been efficiently labeled by indirect immunofluorescence in *DNMT1/3b* wild-type and, to a lesser extent, *DNMT1/3b* double knock-out cells. The level of mCpGs has been quantified in a sizable population of cells by an iCys laser scanning cytometer and iCyte 2.6 software (CompuCyte, USA). As shown in **Fig.7.2**, the fluorescence distributions of the Alexa546-labeled mCpGs are significantly different in the *DNMT1/3b*^{+/-} cells; this result demonstrates the utility of LSC for the fine assessment of global methylation states in different cell types (e.g., differentiated vs. stem cells) or in a specific cell type (e.g., in human peripheral lymphocytes isolated from blood samples) before and after drug treatment or chemotherapy. Since LSC can be performed in an automated fashion, such studies could be made on large sets of biopsy material so as to establish the exact role of global DNA methylation in human pathological diagnosis of various diseases.

Data presented herein have demonstrated that if combined, flow cytometry and conventional PCR offer a powerful tool in the quantitative analysis of ChIP results. We have found high levels of H4Kac and H3K4me at the *TGM2* gene core promoter (**Fig.7.1**). These levels significantly decreased upon apoptosis and this was accompanied by the down-regulation of *TGM2* mRNA expression (2), suggesting that this enzyme does not contribute to the early manifestations of apoptosis in Jurkat cells. Differences in the global level of DNA methylation in HCT116 wild-type and methylation defective cells have been revealed by LSC, the on-slide version of flow cytometry (**Fig.7.2**). Both assays can be easily implemented, and readily applied in a HTP format. We envisage the utility of these platforms primarily in clinical screening efforts addressing one, or a few, epigenetic markers in many samples simultaneously, depending on cost/time considerations and availability of instrumentation/expertise.

Although the epigenetic changes are heritable, they appear to be readily reversed by specific drug treatments as opposed to gene mutations. We expect that the epigenetic silencing of, e.g., tumor suppressor genes will soon become a frequent target of HTP screening studies because these mechanisms may be as important in carcinogenesis as the inactivating mutations. Drugs targeting the enzymes that remove or add these chemical tags are at the forefront of research: diseases to be targeted include cancer, imprinting disorders, autoimmune diseases, certain neurological disorders, diabetes, cardiopulmonary diseases, in which mis-steps in epigenetic programming have been directly implicated. Pharmaceutical companies have set up programs on histone deacetylases (HDACs) and DNA methyltransferases (DNMTs) and their inhibitors, as they have the potential to re-activate specific tumor suppressor genes; clinical trials being on the way are promising the prospect of eliciting tumor regression by modulation of epigenetic regulation.

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289 Based on the above, we anticipate that epigenetic analysis will
290 enter routine diagnostic practice whenever monitoring epigenetic
291 markers can help predict clinical behavior. When large sets of
292 samples are to be assessed, high-throughput platforms for the
293 accurate evaluation of the ChIP results are of general interest. In
294 view of the fact that most routine techniques can be adapted to
295 flow cytometry which exceeds more conventional methods in
296 sensitivity and reproducibility, the approaches shown can provide
297 a universal platform for almost any kind of lab purposes. Whether
298 ChIP-QPCR, ChIP-on-beads, or LSC-based assays of global epi-
299 genetic changes will be selected as the approach of choice for such
300 screening projects will be determined by the particular task under-
301 taken, and the capabilities of the clinical laboratories. We believe
302 that these alternative ChIP platforms can help bring epigenetic
303 analysis within reach for routine laboratories, especially for those
304 involved in clinical diagnostics.

308 2. Materials

311 2.1. Cell Culture

- 312 1. McCoy's medium (Sigma-Aldrich).
- 313 2. Solution of trypsin: stock solution at 0.5%, working solution
314 at 0.05% in 1X phosphate buffered saline (PBS); store at -
315 20°C.
- 316 3. Glutamine: stock solution at 200 mM, final concentration at 2
317 mM in ddH₂O; store at -20°C.
- 318 4. Etoposide (Sigma-Aldrich): stock solution at 40 mM, work-
319 ing concentration at 40 μM.

321 2.2. Detection 322 of Methylated CpGs by 323 Immunofluorescence

- 324 1. 1X PBS: 1.37 MNaCl, 27 mM KCl, 100 mM Na₂HPO₄, 18
325 mM KH₂PO₄; adjust to pH 7.4 with HCl if necessary.
- 326 2. Labeling solution: 1X PBS/10%BSA; store at -20°C.
- 327 3. Primary antibody (1.9 mg/mL): MBD-Fc, a recombinant
328 antibody which was made of human MBD domain (methyl
329 binding domain) fused with an Fc fragment of a human
330 IgG1 and expressed in *Drosophila* S2 cells (26-28); store
331 at 4°C.
- 332 4. Secondary antibody (2 mg/mL): Alexa546-conjugated anti-
333 human IgG (Invitrogen); store at 4°C.
- 334 5. Hoechst 33342 (Invitrogen): stock solution: 1 mM, working
335 solution: 4 μM, final concentration: 2 μM, diluted in 1X PBS;
336 store at -20°C.
6. Prolong Gold (Invitrogen).

2.3. ChIP-on-Beads

1. Nucleus isolation buffer: 5 mM pipes, pH 8.0, 85 mM KCl, 0.5% NP-40, protease inhibitors (Sigma-Aldrich, cat no. P8340).
2. Sonication buffer: 1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.0, protease inhibitors.
3. IP buffer: 0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 20 mM Tris-HCl pH 8.0, 167 mM NaCl, protease inhibitors.
4. Blocked protein A/G Sepharose (Upstate, cat. no. 16-157).
5. Antibodies (Upstate): anti-H4Kac, 2 µg/IP (cat. no. 06-866), anti-H3K4me2, 5 µg/IP (cat. no. 07-030).
6. Wash buffer (WB) A: 0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.0, 150 mM NaCl, protease inhibitors.
7. WB B: 0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.0, 500 mM NaCl, protease inhibitors.
8. WB C: 0.25 M LiCl, 1% NP-40, 1% Na-deoxycholate, 1 mM EDTA, 10 mM Tris-HCl, pH 8.0, protease inhibitors.
9. 1X TE: 10 mM Tris-HCl, pH 7.5, 1 mM EDTA.
10. QIAquick PCR Purification Kit (Qiagen).
11. Primers: forward 5'-Fam-GAGACCCTCCAAGTGCAGAC-3', reverse 5'-Biotin-CCAAAGCGGGCTATAAGTTA GC-3'.
12. Streptavidin-coated microbeads (6 µm, Polyscience).

3. Methods

3.1. ChIP-on-Beads

1. Treat exponentially growing Jurkat cells with 40 µM etoposide (eto) for 3 h at 37°C to induce apoptosis.
2. Fix cells with 1% formaldehyde for 10 min at room temperature. Stop fixation by adding 2.5 M glycine to a final concentration of 0.67 M, for 5 min at room temperature. Wash cells twice in ice-cold PBS.
3. Resuspend cells in 1 mL of nucleus isolation buffer and incubate them for 10 min on ice. Vortex tubes in every 2–3 min.
4. Centrifuge isolated nuclei at 500g for 3 min, at 4°C. Resuspend pellet in 500 µL sonication buffer.
5. Sonicate chromatin to an average fragment size of 500 bp using a Bioruptor (Diagenode); 0.5 min ON/0.5 min OFF pulses for 2 × 12 min usually produces the desired size distribution.

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- 385 6. Centrifuge sheared chromatin samples at maximum speed for
386 20 min. Keep supernatants (leave 50 μ L on the bottom of the
387 tubes). Freeze in liquid nitrogen and store samples at -80°C
388 (or proceed immediately).
- 389 7. Thaw samples on ice and centrifuge them at maximum speed
390 for 10 min at 4°C . Transfer supernatants into clean tubes (do
391 not disturb pellet on the bottom of the tubes).
- 392 8. Dilute chromatin samples 1:10 in IP buffer as follows: 100 μ L
393 chromatin 900 μ L IP buffer.
- 394 9. Pre-clear samples by incubating them on a rotating wheel with
395 30 μ L of blocked protein A/G Sepharose for 30 min at 4°C .
396 Spin samples at 500g for 3 min at 4°C . Keep supernatants.
397
- 398 10. Perform immunoselection for >12 h on a rotating wheel by
399 adding the following antibodies to the samples: anti-H4Kac
400 and anti-H3K4me2; as negative control, omit specific Ab but
401 add a specific IgG protein from the same isotype to one of the
402 pre-cleared samples.
- 403 11. Preserve 10 μ L from the 'negative control' as 'input' DNA
404 and store it at -20°C . Collect immune complexes by adding
405 40 μ L of blocked protein A/G Sepharose to each sample and
406 incubate them for 45 min on a rotator. Spin samples at 500g
407 for 3 min.
- 408 12. Wash the pelleted immune complexes as follows: $2 \times$ WB A,
409 $2 \times$ WB B, $2 \times$ WB C, $1 \times$ TE. Resuspend pellets in 500 μ L
410 TE. At this point thaw input DNA and dilute it to 500 μ L;
411 process it together with the IP samples.
- 412 13. Reverse cross-links by incubating the samples at 98°C for
413 10 min. Put samples on ice.
- 414 14. Digest residual RNAs with 200 $\mu\text{g}/\text{mL}$ RNase A for 30 min at
415 37°C .
- 416 15. Digest proteins by 0.5 mg/mL proteinase K for at least 2 h at
417 55°C .
- 418 16. Purify DNA on PCR clean-up columns (Qiagen). Immuno-
419 precipitated DNA samples (input, negative control, H4Kac/
420 H3K4me2, respectively) are ready to be tagged by *Fam/*
421 *biotin* PCR.
- 422 17. In the *Fam/biotin* PCR, use primers listed in **Section 2.3**.
423 Perform PCRs under standard conditions and stop after
424 15–20 cycles, i.e., in the linear phase. Validate by QPCR
425 (2). Purify the 5'-*Fam/biotin* labeled ChIP-PCR products
426 on PCR clean-up columns.
427
- 428 18. Carry out flow cytometry on a Becton-Dickinson FACScan
429 flow cytometer as follows: 5 μ L of the *Fam/biotin*-tagged
430 ChIP-DNA was added to 10,000 streptavidin-coated, plain
431
432

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433 beads in 50 μ L PBS. Incubate samples for 15 min at room
 434 temperature, wash in 1 mL PBS, and run at high speed. Set
 435 laser power to 15 mW and detect fluorescence signals through
 436 the 530/30 interference filter of the FL1 channel in logarithmic
 437 mode. Evaluate results using the BDIS CELLQUEST 3.3
 438 (Becton-Dickinson) software. *TGM2* copy numbers are deter-
 439 mined by reference to a standard curve obtained from a dilu-
 440 tion series of known quantities of Fam/biotin-tagged PCR
 441 products (Fig.7.1A). Express ChIP yields as percentage of
 442 input after subtracting background (no antibody (nAb) % of
 443 input).
 444

445
 446 **3.2.**
 447 **Immunofluorescence**
 448 **and Laser Scanning**
 449 **Cytometry**
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1. Grow HCT116 *DNMT1/3b* wt and *DNMT1/3b* knock-out cells on coverslips overnight.
2. Wash cells in 200 μ L 1X PBS, 3 \times 3 min.
3. Fix cells in a series of diluted methylalcohol (MetOH) (as shown below); wash cells with 200 μ L of diluted MetOH once for 3 min, for each dilution. Start with the 10 \times dilution. After washes, incubate cells in concentrated MetOH overnight at -20° C.

	1X PBS (μ L)	MetOH (μ L)
10 \times MetOH	900	100
8 \times MetOH	875	125
6 \times MetOH	833	167
4 \times MetOH	750	250
2 \times MetOH	500	500

4. Rehydrate cells in a series of diluted 1X PBS as shown below; wash cells in 200 μ L diluted MetOH for 3 min in each dilution. Start with the 10 \times dilution. After the final rehydration step, wash with 200 μ L 1X PBSs

	MetOH (μ L)	1X PBS (μ L)
10 \times (1X PBS)	900	100
8 \times (1X PBS)	875	125
6 \times (1X PBS)	833	167
4 \times (1X PBS)	750	250
2 \times (1X PBS)	500	500

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- 481 5. In order to relax DNA, place samples into Petri dishes (with-
482 out the cover) in PBS/1% BSA and irradiate them with UV
483 light for 30 min.
- 484 6. Immunolabel samples using the mCpG-specific MBD-Fc
485 fusion protein or a commercially available Anti-5mC as pri-
486 mary antibody for 30 min at room temperature. Wash cells in
487 200 μ L of 1% BSA/PBS, 3 \times for 3 min.
- 488 7. Label samples with an Alexa546-conjugated anti-human IgG
489 secondary antibody, for 30 min at room temperature. Wash
490 cells in 200 μ L 1% BSA/PBS 3 \times for 3 min.
- 491 8. Stain DNA with 50 μ L Hoechst 33342 (2 μ M) and cover with
492 Prolong Gold antifade.
- 493 9. Scan slides (*see Note 1*).

4. Notes



- 500 1. MCpGs have been visualized using a Zeiss LSM 510 confocal
501 laser-scanning microscope using excitation wavelengths of
502 543 and 351/364 nm. Fluorescence emission was detected
503 through 560–615 and 385–470 nm band-pass filters. Images
504 were taken in multitrack mode to prevent cross-talk between
505 the channels. Pixel image (512 \times 512) stacks of 2–2.5 μ m
506 thick optical sections were obtained with a 63 \times Plan-
507 Apochromat oil immersion objective (NA 1.4).

508 The same samples were also analyzed using an iCys laser scan-
509 ning cytometer (CompuCyte). The instrument used in our
510 studies is equipped with a violet-blue diode, an argon-ion, and
511 a HeNe laser (wavelengths 405, 488, and 633 nm, respec-
512 tively). The violet and Ar-ion laser lines were used for excita-
513 tion of Hoechst and Alexa 546 dyes. To identify single nuclei,
514 contouring was based on Hoechst fluorescence detected in the
515 blue channel (460–485 nm). Fluorescence of Alexa 546
516 (MCpGs) was detected in the orange channel (565–585 nm)
517 based on the contour gained in the blue channel. In single
518 nuclei identified by contouring on fluorescence of the nuclear
519 stain, the integral fluorescence related to the MCpGs divided
520 by the area of the contour was used to describe the methyla-
521 tion level. This corrects for differences in nuclear size. Data
522 evaluation and hardware control were performed using the
523 iCys 2.6 software for Windows XP. Using the 4 \times objective
524 to scan an indicated area on a slide, 400–1000 cells were
525 scanned in about 10 min (21). LSC can screen relatively
526 large number of cells on a slide. The cells are distinguished
527
528

529 based on their fluorescence properties like in flow cytometry.
 530 However, as the position of each cell is fixed on the slide and
 531 the instrument saves the positional information, any correla-
 532 tion between the different parameters measured can be
 533 detected in a very sensitive manner. In addition, the cells can
 534 be relocated and visually analyzed or re-scanned after re-stain-
 535 ing with conventional stains or fluorescent markers.
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