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Measuring Topology of Low-Intensity DNA Methylation Sites for High Throughput Assessment of Epigenetic Drug-Induced Effects in Cancer Cells

Arkadiusz Gertych^{1,2,*}, Daniel L. Farkas¹, and Jian Tajbakhsh^{1,*}

¹Translational Cytoomics Group, Department of Surgery, Cedars-Sinai Medical Center, Los Angeles California.

²Bioinformatics, Department of Surgery, Cedars-Sinai Medical Center, Los Angeles California.

Abstract

Epigenetic anti-cancer drugs with demethylating effects have shown to alter genome organization in mammalian cell nuclei. The interest in the development of novel epigenetic drugs has increased the demand for cell-based assays to evaluate drug performance in pre-clinical studies. An imaging-based cytometrical approach that can measure demethylation effects as changes in the spatial nuclear distributions of methylated cytosine and global DNA in cancer cells is introduced in this paper. The cells were studied by immunofluorescence with a specific antibody against 5-methylcytosine (MeC), and 4,6-diamidino-2-phenylindole (DAPI) for delineation of methylated sites and global DNA in nuclei. In the preprocessing step the segmentation of nuclei in three-dimensional images (3-D) is followed by an automated assessment of nuclear DAPI/MeC patterns to exclude dissimilar entities. Next, low-intensity MeC (LIM) and low-intensity DNA (LID) sites of similar nuclei are localized and processed to obtain specific nuclear density profiles. These profiles sampled at half of the total nuclear volume yielded two parameters: $LIM_{0.5}$ and $LID_{0.5}$. The analysis shows that zebularine and 5-azacytidine - the two tested epigenetic drugs introduce changes in the spatial distribution of low-intensity DNA and MeC signals. $LIM_{0.5}$ and $LID_{0.5}$ were significantly different ($p < 0.001$) in 5-azacytidine treated ($n=660$) and zebularine treated ($n=496$) vs. untreated ($n=649$) DU145 human prostate cancer cells. In the latter case the LIM sites were predominantly found at the nuclear border, whereas treated populations showed different degrees of increase in LIMs towards the interior nuclear space, in which a large portion of heterochromatin is located. The cell-by-cell evaluation of changes in the spatial reorganization of MeC/DAPI signals revealed that zebularine is a more gentle demethylating agent than 5-azacytidine. Measuring changes in the topology of low-intensity sites can potentially be a valuable component in the high throughput assessment of demethylation and risk of chromatin reorganization in epigenetic-drug screening tasks.

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*Corresponding authors: Arkadiusz Gertych PhD: gertycha@cshs.org; Jian Tajbakhsh PhD: tajbakhshj@cshs.org, Translational Cytoomics Group, Department of Surgery, Cedars-Sinai Medical Center, 8700 Beverly Blvd, Davis Building, Room #6067, Los Angeles, California 90048. Tel: 310-423-0543, fax 310-423-7707.

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Competing Interests

The authors declare that they have no competing interests.

Keywords

DNA methylation; epigenetic drug screening; 3-D image analysis; heterochromatin reorganization; 5-azacytidine; zebularine

INTRODUCTION

DNA methylation plays a key role in cellular differentiation, and has recently become one of the most studied gene regulation mechanisms in carcinogenesis. Since aberrant methylation of cytosine in the human genome is correlated with cancer development and progression the resulting epigenetic alterations of the DNA fall into two categories: (i) gene-specific hypermethylation of gene promoters in gene-rich genomic regions termed CpG-islands, and (ii) genome-wide hypomethylation, a large percentage of which occurs in repetitive DNA elements. Global hypomethylation is assumed to occur at late stages of cancer whereas local hypermethylation of promoter regions is generally plausible for early stages of cancer. Aberrant global methylation patterns are a result of changes in the activation status of up to 50% of genes, including oncogenes and tumor-suppressor genes, which are regulated by methylation and demethylation of CpG islands [1–2].

The reversibility of epigenetic modifications, such as cytosine methylation, has gained much attraction in cancer therapy, with an emphasis on the development of anti-cancer drugs with demethylating potential to primarily reestablish tumor suppressor gene functionality. A number of DNA methylation inhibitors of different categories have been designed. 5-azacytidine (AZA) and its analogues being the most prominent and approved for treatment [3–4] and recently 1- β -D-ribofuranosyl-2(1H)-pyrimidone, known as zebularine (ZEB), that has shown promising properties for oral administration. ZEB seems to be less toxic to cultured cells than the other two azanucleosides, and has a significantly stronger anti-proliferative effect on cancer cells than on normal cells in culture [5–6]. Epigenetic drugs with demethylating effects have also been shown to alter genome organization within mammalian cell nuclei. The underlying processes usually involve large-scale DNA demethylation and chromatin reorganization that can be visualized by light microscopy [7–13].

The interest in the development of novel epigenetic drugs has increased the demand for cell-based assays to evaluate drug performance in the important pre-clinical phases of drug screening. This process can greatly benefit from high-resolution microscopic imaging, an indispensable tool for measuring the spatial and temporal distribution of molecules and cellular components that are vital to understanding the activity of drug targets at the cellular level [14]. Logically, the new assays need to aim at the quantitative characterization of global DNA methylation changes in a high-throughput and cell-by-cell fashion to verify unwanted effects such as increased heterochromatin hypomethylation and reorganization, which can induce chromosome instability and cause severe adverse reactions of cells. A recently introduced image-cytometrical approach has demonstrated that the codistribution of MeC and DAPI fluorescence signals can be utilized as signatures for the characterization of stem cells in differentiation and mouse cancer cells upon treatment with demethylating agents [8,12]. The algorithm succeeded in tracking and quantification of genome-wide MeC/DAPI signals in mouse tumor cells which represent high concentrations of MeC sites largely overlapping with clusters of centromeric and pericentromeric heterochromatin. In comparison, human cancer cells show a much finer granulation of methylated and global DNA. To address this issue an upgraded methodology with additional features for the interrogation of human cells by 3-D quantitative DNA methylation imaging (3D-qDMI) is proposed in this paper. It can automatically process multichannel high-volume images of

cells and considers the characterization of individual nuclei as well as the entire cell population for a more comprehensive pattern analysis of nuclear methylated cytosine vs. global DNA. In this work 3D-qDMI is used to study drug-induced demethylation in DU145 human prostate cancer cells. The methodology extracts two parameters ($LIM_{0.5}$ and $LID_{0.5}$) which reflect the spatial distribution of low-intensity MeC and DAPI sites in individual nuclei. Statistically significant differences were obtained between measures of these parameters in cell populations treated in parallel by two epigenetic drugs, AZA and ZEB, and in comparison to untreated control cells.

MATERIALS and METHODS

Cell Culture, Drug Treatment and Immunofluorescence

Cells (ATCC) were grown in Dulbecco's modified Eagle's medium (Cellgro) supplemented with 10% newborn calf serum, and 1% antibiotic/antimycotic (1000 units/ml penicillin G sodium, 10 mg/ml streptomycin sulfate) (Gemini Bio-Products), in 5% CO₂, 37°C. Cells were plated at 1×10^5 cells onto coverslips in multi-well plates in replicates, and allowed to attach for 24 hrs. Then, wells were divided into two groups: (i) control population that was not treated for 72 hrs, and (ii) populations of cells treated with either 5 μ M of 5-azacytidine or 200 μ M of zebularine for 72 hrs. These drug concentrations have been described as being compatible with normal cell viability and division [5,15–18]. For all cells, medium was changed every 24 hrs and drugs were freshly mixed into replacement medium prior to their application to cells. On day fifth cells were fixed and processed for immunofluorescence. In order to preserve the 3-D structure, cells cultured on coverslips in 12-well microplates were fixed with 4% paraformaldehyde/phosphate buffered saline (PBS) (Sigma-Aldrich) [19–20], and processed for immunofluorescence with a monoclonal mouse anti-5-methylcytosine primary antibody (EMD Biosciences) and a secondary antibody Alexa 488-conjugated donkey anti-mouse polyclonal IgG (Invitrogen) as described in [8,12]. The specimens were then counterstained with 4,6-diamidino-2-phenylindole. The above procedure yielded the following samples: untreated (UT-DU145), AZA-treated cells (AZA-DU145) and zebularine-treated (ZEB-DU145) cells respectively.

Image Acquisition

Serial optical sections were collected at increments of 250 nm using high-resolution supercontinuum confocal laser scanning microscope (TCS SP5 X, Leica Microsystems Inc.) with a Plan-Apo 63 $\times$ 1.4 glycerol immersion lens. Pinhole size was 1.0 airy unit. The imaging of DAPI and MeC fluorescence was performed sequentially to avoid cross-talk between channels. The typical size of a 2-D optical section was 2048 $\times$ 2048, with a respective voxel size of 120nm $\times$ 120nm $\times$ 250nm (*x*-, *y*-, and *z*-axis) resulting in an imaged field of view of 246 μ m $\times$ 246 μ m $\times$ 6 μ m with dynamic intensity range of 12 bits/pixel. Signals from optical sections were recorded into separate 3-D channels I_{MeC} and I_{DAPI} , respectively. All images were acquired under nearly identical conditions and modality settings. The drift of the settings during acquisition was considered minimal and neglected.

Preprocessing

Image files of untreated and treated cells originally saved in Leica format (*.lif) were converted to a series of TIFFs using the open source ImageJ package [21]. Output files were sequentially analyzed by two in-house developed MATLAB-based software modules (preprocessing and in depth analysis) according to the sequence of analytical steps in Fig. 1.

The first module delineates individual 3-D nuclear regions of interest (ROI) in DAPI images using adaptive seeded watershed segmentation. Next, 2-D intensity histograms are formed from DAPI and MeC voxels within each ROI. For better clarity these histograms are called

nuclear MeC/DAPI codistribution patterns. These patterns are evaluated (soft-qualified) by means of Kullback-Leibler (K-L) divergence measured between individual MeC/DAPI pattern and a reference pattern being the sum of all patterns in the analyzed population. An example of MeC/DAPI pattern is shown in Fig. 1. Based on the K-L divergence value each cell is assigned to one of the four classes: *similar* $KL \in [0,0.5)$, *likely similar* $KL \in [0.5,2)$, *unlikely similar* $KL \in [2,4.5)$, and *dissimilar* $KL \in [4.5, \infty)$. Each class is also false-colored for easier readability (Fig. 1). More details on nuclei segmentation and MeC/DAPI pattern soft-qualification can be found in [8]. *Dissimilar* nuclei were discarded as outliers from further analyses, whereas nuclei with similar profiles were subjected to topological quantification of MeC and DAPI signals by the second 3D-qDMI module.

3-D Topological quantification of MeC/DAPI sites

The main aim of the in depth analysis is to provide quantities which go beyond the capabilities of K-L-based evaluation of pattern similarities. In this step the topological analysis is based on the assumption that recorded signal intensities in the MeC and DAPI channels are proportional to the spatial concentrations of methylated cytosine and global DNA in a volume unit of a ROI. According to this, the local concentration of MeC or DAPI-rich sites will yield a stronger fluorescence signal. Thus two types of site categories can be distinguished: high-MeC concentrations and low-MeC concentrations, and analogously high- and low- concentrations of DAPI, which can be seen as local perturbations of the signal amplitude in the cross-sectional intensity profiles of nuclei (Fig. 2).

The application of demethylating agents results in the presence of: i) a strong reduction of MeC signal amplitude and an increase of low-intensity MeC (LIM) sites, and ii) to a lesser extent the occurrence of low-intensity DAPI (LID) sites, to be detected and quantified by the proposed approach. Since the steps for LIM and LID quantification are identical from the analytical point of view, the methodology is described using the detection of LIM sites as an example. First a thresholding function for MeC signal was defined, by which LIM sites can be isolated in the nuclear ROIs:

$$I_{LIM} = [1 \text{ if } t_{bcg} \leq \overline{I_{MeC}} < t_Q, 0 \text{ otherwise}], \quad (1)$$

where: t_{bcg} is the threshold value for the background, t_Q separates high-amplitude from low-amplitude components of MeC intensities, $\overline{I_{MeC}} = [I_{MeC} - \min(I_{MeC})] / [\max(I_{MeC}) - \min(I_{MeC})]$ is the normalized input image, I_{MeC} is the raw 3-D image data recorded in the MeC channel and I_{LIM} is the output binary image. The threshold t_{bcg} serves as a noise signal cut-off level, and is defined as the mean image intensity measured in the space outside of the nuclear ROIs in the MeC channel. The high-intensity threshold (t_Q) is obtained from the Rose criterion determining distinguishable image features at 100% certainty [22]. Thus t_Q is set to $5t_{bcg}$ to separate high-intensity MeC nuclear patterns with sufficient confidence.

According to (Equ. 1) the LIM voxels in the binary image I_{LIM} are turned to one. The concept of topological analysis is based on a consecutive integration of I_{LIM} granules in nuclear shells across a nuclear ROI starting from the ROI periphery towards the center (Fig. 1). A nuclear shell can be distinguished as a sub-volume of a ROI by means of morphological erosion with the anisotropic structuring element defining shell thickness in the ROI [23]. The geometrical size of the structuring element was empirically adjusted according to the average volume of nuclei to assure that gradually progressing ROI erosion entails a complete shell-by-shell reduction of the ROI volume. The mean volume of $15.7\mu\text{m} \times 15.4\mu\text{m} \times 3.5\mu\text{m}$ was obtained from 35 ROIs randomly selected from one 3-D image cube of UT-DU145 cells. A constant voxel-rate of $1.32\mu\text{m} \times 1.32\mu\text{m} \times 0.25\mu\text{m}$ in x , y and z

direction was obtained and converted to the structuring element with of size of $11 \times 11 \times 1$ pixels. Each shell was superimposed onto a respective area in the $\overline{I_{MeC}}$ image and used to determine the local density of LIM sites. This process was continued until the complete erosion of ROI, and a normalized cumulative density profile D_{LIM} was constructed for each individual nucleus:

$$LIM(j) = \frac{LIM_j}{LIM_{tot}} = \frac{\sum_i^n I_{LIMj}(i)}{\sum_j^N \sum_i^n I_{LIMj}(i)} \quad (2)$$

where: i -th voxel (with binary value 0 or 1) in the j -th shell I_{LIM} in represents undetected or detected LIM sites in the 3-D shell space, and n is the number of voxels within a shell.

$\sum_j^N \sum_i^n I_{LIMj}(i)$ reflects the number of all I_{LIM} -positive sites and serves as a normalizing constant. The number of shells N varies depending on the ROI volume.

Since the removal of the first few shells results in approximately 50% reduction of total nuclear volume, the outer 50% of the ROI was defined as the periphery and the remaining 50% as the interior of each nucleus. According to this, each of the LIM profiles was sampled at the rate of 50% to yield a specific parameter ($LIM_{0.5}$) used to characterize drug induced demethylation in the whole cell population. The $LID_{0.5}$ measurement was obtained in an identical manner.

The two analytical modules were integrated into one component and implemented on a two-processor workstation platform with parallel computing capabilities. A pseudo-code for LIM $LIM_{0.5}$ and calculations is outlined in Scheme 1. Calculations of $LID_{0.5}$ were removed from the pseudo-code for clarity. The main loop (steps A–E) can be run in parallel meaning that each ROI can be processed independently (one ROI by one CPU core, with eight cores available). Twelve 3-D fields of view (approximately 10G bytes of raw image data) were analyzed for each type of cells.

RESULTS

Measuring codistribution of methylated cytosine and global DNA in cell nuclei

Utilizing 3D-qDMI a total of 649 nuclei of UT-DU145, 496 nuclei of AZA-DU145 and 660 nuclei of ZEB-DU145 cells were analyzed. All cells originally derived from one culture were split for parallel treatment with subsequent immunofluorescence, 3-D imaging and analysis. Following the algorithm workflow in Fig 1, the MeC- and DAPI-specific fluorescence signals were extracted from 3-D nuclear ROIs and assessed by K-L divergence, in an automated fashion. Nuclei with a K-L value >4.5 respectively constituting 15.1%, 10.3% and 6.7% of cells in UT-DU145, ZEB-DU145, and AZA-DU145 populations, were marked as *dissimilar* and were excluded from the in-depth analysis. These results agree with observations made in the previous study, in which naïve mouse pituitary tumor cell populations have shown a higher portion of dissimilar cells comparing to the cells that were treated with DNA demethylating drugs including AZA [8]. Nuclei with a K-L-value lower than 4.5 were considered as similar and further analyzed. The nuclei from Fig. 2 B, E and F fell into this category, and their MeC/DAPI codistribution patterns are presented in Figure 3A, C and E, respectively.

The MeC/DAPI codistribution patterns of ZEB-treated cells closer resemble the patterns of untreated cells rather than AZA cells, i.e. show a lower degree of demethylation of global DNA. In comparison, AZA-DU145 cells show a much different (flatter graph) MeC/DAPI

codistributions suggesting stronger demethylation of heterochromatic sites. A color-coded mapping of K-L based cell similarity evaluation and outlier removal constituting the output of the first 3D-qDMI module is shown in Figure 4.

Nuclear topology of low-intensity MeC and DNA signals in cells

Nuclei with similar MeC/DAPI patterns were subjected to the automated analysis of spatial LIM/LID density distribution. To obtain the low-intensity DNA signal components, the LIM and LID sites were defined as voxels with signal amplitudes between t_{bcg} and t_Q (Fig 5 A, D, G). The thresholding was followed by a complete erosion of each nucleus to define all contained shells. This procedure yielded LIM and LID density profiles for each nucleus (Fig 5 B, E, H) which are one of the key outcomes of the topological analysis. Each profile sampled at half of the nuclear volume yielded two specific quantities $LIM_{0.5}$ and $LID_{0.5}$ related to demethylation and organization of DNA.

Large majority of untreated cells (Fig. 5B) had rim-like LIM and LID ($LIM_{0.5} = 0.75$, and $LID_{0.5} = 0.82$) sites detected at or close to the nuclear border with a few small areas in the nuclear interior (~20% of all sites respectively). In treated cells the nuclei showed an increased portion of interior LIM sites: ~40% in ZEB-DU145 cell and more than 60% in AZA-DU145 cell localized in the nuclear interior (Fig. 5B, E and H). Furthermore, the appearance of LID sites was quite similar to the localization of LIMs in the nuclei of untreated cells, but slightly different in treated cells. First, the low-intensity DNA signals occurred less frequently in the nuclear interior compared to the same regions marked as LIMs. Second, treated cells showed an increase of 30% (40% interior LIDs) compared to untreated cells (~10% of interior LIDs), indicating that a potential reorganization of DAPI-intense sites occurs along with demethylation mostly at the nuclear periphery and less in the interior areas, with no significant difference noted between the two drugs at the applied concentrations. Also, a lesser density of LID sites was observed in the area adjacent to the nuclear envelope comparing to the density of LIM sites.

The histograms (Fig. 5 C, F and I) reflect the frequency of $LIM_{0.5}$ and $LID_{0.5}$ values in all analyzed populations: untreated, ZEB-, and AZA-treated cells. The $LIM_{0.5}$ histogram peaks respectively at 0.62, 0.53, and 0.41 showed remarkable differences which positively correlate with the individual $LIM_{0.5}$ samples in the nuclei (Fig. 5B, E, and H), and with the observation of the global loss of methylation reflected by individual and global MeC/DAPI codistributions in Figure 3. Furthermore, the most frequent occurrence of $LIM_{0.5}$ in AZA-DU145 treated population (Fig. 5I) was nearly identical with the measurement obtained for the pattern in Fig. 5H. The measurements of $LIM_{0.5}$ in ZEB- and UT-DU145 cells in Fig. 5E and B were within one standard deviation from their mean values. The distributions of $LID_{0.5}$ parameter demonstrated that the most frequent occurrence of $LID_{0.5}$ in ZEB-DU145 and AZA-DU145 treated cells does not differ much and is located nearly $LID_{0.5} = 0.55$. The respective peak from UT-DU145 cells is located at 0.75 indicating that low-intensity DAPI signals in the untreated cells is in most cases found exclusively at the nuclear periphery.

Statistical analysis using a Kolmogorov-Smirnov test performed for each pair of data showed a significant difference of $LIM_{0.5}$ and $LID_{0.5}$ samples collected from untreated and treated cells. Table 1 shows the results as well as parameters of the respective distributions. In general, the location and shape of a histogram peak reflects the efficacy of drug response within a cell population: the narrower the peaks are the higher homogeneity in MeC or DAPI distribution in treated cells. These results indicate that the $LIM_{0.5}$, $LID_{0.5}$ histogram can be used as another quantitative marker of global demethylation effects measured on a population level.

DISCUSSION

Epigenetic drugs with demethylating effects have shown to alter genome organization within mammalian cell nuclei [4–6,24–25]. In this study the fine granularity of nuclear patterns of two classes of nucleic acids representing regions of the genome that can potentially be affected by drug induced demethylation was visualized and quantitated by image analysis. DU145 human prostate cancer cells were used as a model for proof-of-principle.

The imaging-based cytometrical approach proposed in this paper combines previously developed image processing routines such as the segmentation of nuclei, MeC/DAPI pattern extraction, and similarity assessment of cell populations with the newly added topological integration of low-intensity MeC and DAPI signals (a consequence of drug-induced demethylation) in consecutive nuclear sections defined by morphological erosion in 3-D. The system readout includes combined quantities of specific MeC and DAPI signal measures. The approach provides two new parameters in the evaluation of demethylating drug effects: (i) region-specific changes in MeC load, and (ii) alterations in density distributions of global DNA. Both parameters yielded highly differential values for the three types of cell populations used in this study. Two interesting observations were made: 1) in treated and untreated cases the highest value of LIM density was observed in the nuclear periphery and 2) the degree of demethylation was concordant with an increase in LIM density beyond the nuclear border into the interior of the nucleus, meaning that the stronger the demethylating effect of the drug was the more LIM sites could be registered within the inner shells of the nucleus. This becomes apparent when comparing ZEB- and AZA-treated cells. AZA-DU145 nuclei display significantly higher LIM densities even in the areas deep inside the nuclei compared to cell ZEB-DU145 cells. As the interior of the nuclei harbors a large portion of the highly compact constitutive heterochromatin, it is assumed that these areas of the genome have been largely demethylated by AZA but not as much by ZEB.

Both drugs seem to also affect global DNA organization as shown in Fig. 2 and 5. The fluctuation of the DAPI signal in ZEB-DU145 and AZA-DU145 nuclei is stronger than in untreated cells. Moreover, the results in Fig. 3 (MeC/DAPI codistributions) are correlated with the topological findings in Fig. 5. By projecting the codistributions (not shown) from Figure 3 onto the Y- and X-axes it is also becomes more evident that low-intensities in MeC and DAPI channels occur more frequently in the treated populations. Unfortunately the codistribution patterns themselves cannot provide any topological information. Measuring topology of low-intensity MeC signals as a subset of total MeC can resolve the differences in demethylation effects between the two drugs in the human cancer cell model in a comparative way. Although fluorescent MeC- and DNA-specific staining produces measurable signals in nuclei that can be extracted from individual 2-D optical sections or projections of 3-D image data, the signals do not usually generate quantitative and reproducible patterns of exact geometrical positions that are shared by all the cells. Also, due to the high variability and limitations of current imaging modalities it is challenging to precisely localize DNA signals and other similar nuclear structures [26]. Based on the dynamic self-organization of the genome, the spatial distribution of different classes of DNA (referred to as chromatin texture) is a descriptive feature in the differential characterization of cells and tissues in diverse states, as experienced in basic science [27–28] and translational medicine [29–30]. Thus, a new concept that proposes to detect low-intensity MeC and DNA zones, as a consequence of drug-induced demethylation is introduced. The results show that in the case of DU145 cells the demethylation progresses from the nuclear border into the nuclear interior. The results are concordant with the outcome of studies involving molecular methods showing that zebularine is a milder demethylator than AZA [18]. The image-cytometric approach proposed could confirm these observations by delivering a topological picture: in ZEB-treated cells the vast majority of low-intensity

signals are confined to periphery, whereas AZA-treated cells also display a significant portion of LIMs in the nuclear interior. This discrepancy may have some impact on the reorganization of the heterochromatic regions, as there were more low-intensity DAPI sites found in the same interior areas, however there was no difference observed between the two agents at the applied concentrations.

These results provide some hints that measuring of low-intensity sites in cancer cells can serve as a potent indicator in the quantitative assessment of demethylating effects to evaluate in particular the targeted (DNA demethylation) and accompanying effects (chromatin reorganization) of such therapeutic manipulations. Both effects are considered in therapy as perturbation of the higher-order chromatin organization and need to be tested for eventual risks of causal genome instability in targeted cells [31]. The enhanced performance of the improved cytometrical approach is a step forward furthering the development of 3D-qDMI as an automated image-based high throughput screening method for profiling of drugs that target the epigenetic make-up of cells. The interrogation of an algorithm that localizes low-intensity MeC sites delivers an actual (phenotype) map of differential demethylation in the nucleus alongside with accompanying changes in the organization of global DNA. The information on LIM and LID topology can be used to support the assessment of risks associated with genome-wide demethylation. 3D-qDMI is scalable, and thus the new feature can be used in high throughput cell-based assays. Furthermore the approach described can be supportive to molecular methods by adding more information to the (epi)genotype. Global DNA methylation could be first analyzed by 3D-qDMI, which provides a holistic estimate of DNA methylation changes in a cell-by-cell mode and then individual target cells or groups of cells that share a phenotype can be selected for high-resolution methylation-specific genotyping with a variety of existing molecular methods including PCR-based approaches, whole-genomic tiling arrays and massively parallel sequencing [32–33]. The cell similarity assessment feature implemented in 3D-qDMI is therefore extremely valuable in two ways: (i) drug efficiency can be estimated from the degree of cellular response based on the homogeneity of the cell population in spatial MeC distribution, and (ii) the selection of phenotypically similar cells enables the generation of molecular data with higher confidence. The accuracy and performance of this technique is only constrained by parameters of imaging modalities such as the spatial resolution, point spread function, and the speed of computational unit. However, through automation of imaging, mostly practiced in drug discovery and development, imaging modalities can be kept much more consistently than in research environments, and computational capacity is usually ramped up to compensate for high-volume parallel throughput.

CONCLUSIONS

A strategy to automatically quantify the spatial distribution of low-intensity 5-methylcytosine- and global DNA related sites is presented. It can differentiate the topological changes in MeC load as well as DNA reorganization that demethylating agents cause when targeting the human genome. Specifically, this method can detect the more subtle changes of global DNA methylation features caused by milder drugs such as zebularine. Computationally efficient analytical module was developed as a tool to be used in basic epigenetic related research, its projected translation into therapy as well as in epigenetic drug development.

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Figure 1. 3-D image analysis methodology employing topological quantification of MeC and global DNA signals

The process starts with the uploading of 3-D MeC and DAPI-specific image data from the batch of image file. Next, individual ROIs are delineated in mixed MeC and DAPI images. Then, nuclear MeC/DAPI codistribution patterns are extracted from each ROI, and each nucleus is characterized upon its pattern similarity vs. the degree global MeC/DAPI pattern. In the next step all nuclei identified as similar are selected, and their MeC and DAPI signals are subjected to topological quantification. For that, nuclei are eroded in 3-D and low-intensity MeC and DAPI sites are registered within each shell-like nuclear compartment

(outlined by yellow lines). The complete analysis can be automatically performed in parallel for hundreds of cells within an imaged population.

Figure 2. Example optical sections of fluorescently labeled DU145 cancer cell nuclei and their one-dimensional intensity profiles

Untreated cells (top row), ZEB-treated cells (middle row) and AZA-treated cell (bottom row). Nuclear patterns of DAPI-staining (A, D, and G - blue channel) and MeC-staining (B, E, and H - green channel) that were recorded separately depict spatial distributions of respective signals within the nuclei. Intensity profiles (C, F and I) traced across three nuclei from each population showed different spatial concentration of the MeC- and global DNA related signals. A stronger decrease can be seen for the MeC signal amplitude in AZA versus ZEB-treated cells (compared to the naïve cells). The DAPI signal amplitudes do not differ much between the cells, however the signal fluctuation in treated cells is stronger vs. the naïve cells. Interestingly in all three cell types the MeC signal at nuclear border (red dashed lines) is very low, although the high DAPI signal indicates the presence of

concentrated DNA (heterochromatin at the nuclear envelope) in those areas. Signal intensities in the MeC and DAPI channels were normalized, subjected to thresholding using t_Q and t_{bcg} and quantified. DAPI-related signals in C, F and I are arbitrarily scaled.

Figure 3. MeC/DAPI codistributions for untreated, zebularine-treated and AZA-treated DU145 prostate cancer cells

Individual nuclear patterns (A, C, and E) are obtained via automated segmentation of nuclei from 3-D image data and correspond to nuclear ROIs in figures 2B, 2E and 2H. Plots B, D and F serve as reference patterns in K-L similarity analysis and are the sum of individual nuclear patterns in the respective populations. AZA-DU145 (E) shows a much stronger demethylation of global DNA, including highly DAPI-positive heterochromatin than ZEB-DU145 (C), also in comparison with untreated cells (A): the slopes and heights of the individual plots roughly indicate ratios of methylated vs. demethylated global DNA showing highest degree of methylation in (A) vs. lowest degree in (E) and intermediate in (C).

Figure 4. Soft-qualification of cells using K-L divergence: A) untreated, B) zebularine treated, and C) 5-azacytidine treated cell

Following ROI segmentation the *dissimilar* nuclei (red) and those only partially included in the microscopic field of view are excluded from the analysis. Nuclei marked as *similar* (green), *likely similar* (blue) and *unlikely similar* (yellow) were subjected to further analysis.

Figure 5. Topology of low-intensity MeC and DAPI sites

LIM sites detected in the range of $\langle t_{bcg}, t_Q \rangle$ in individual cells (marked in cyan color) are superimposed onto an intermediate optical section in the MeC channel (A, D, and G). The middle column shows an optical mid-section of sample nuclei with typical concentration of low-intensity MeC/DAPI sites (DAPI image is false-colored in blue) and *LIM*/*LID* density curves obtained through morphological erosion. These sites correspond with intensity profiles in Fig 2 and codistribution patterns in Fig. 3 for the same nuclei. The number of graph points in (B, E and H) is associated with the number of detected shells; the value of each point refers to the fraction of all sites found in nucleus up to next shell. The argument $V/V_{tot} = 0.5$ distinguishes all *LIM* sites that are localized in the peripheral half of the nucleus from *LIMs* of the interior half ($V/V_{tot} > 0.5$). V is the shell volume and V_{tot} is the total volume of a nucleus. The third column displays histograms of $LIM_{0.5}$ and $LID_{0.5}$ acquired at $V/V_{tot} = 0.5$ collected from all nuclei for each population type (C, F, I). The diagonal line across each plot in (B, E and H) represents hypothetically equal density of *LIM* or *LID* sites across the nuclear volume. It would also result in the presence of a peak at 0.5 in the respective graphs.

START:

repeat

- A) fetch n-th binary ROI from segmented image,
- B) extract image cubes: MeCvol, from I_{MeC} under spatial location of ROI,
- C) obtain t_{bcg} , t_Q from the analysis of $\overline{I_{MeC}}$
- D) $[LIM_X, LIM_Y] = \text{erodeROI}(\text{ROI}, \text{MeCvol}, \text{struct_el}, t_{bcg}, t_Q);$ obtain LIM densities through [Equ.2](#)
- E) combine LIM_X , and LIM_Y to obtain $LIM(j)$ profile for the n-th ROI,
- F) determine $LIM_{0.5}$. Interpolate LIM_X , and LIM_Y if necessary,

until all nuclear ROIs are processed.

STOP

function $[LIM_X, LIM_Y] = \text{erodeROI}(\text{ROI}, \text{MeCvol}, \text{struct_el}, t_{bcg}, t_Q),$

erROI = ROI; tempROI = ROI; j=1 initialize variables

repeat

- erROI = imerode(erROI, struct_el); compute 3-D binary erosion of ROI mask,
- shell = xor(erROI, tempROI); obtain binary j-th shell by a 3-D exclusive disjunction,
- tempROI = erROI; save eroded ROI,
- volshell_j = count(shell); determine volume of the j-th shell,
- MeCshell = MeCvol(shell); extract all MeC intensities from the j-th shell,
- $LIM_j = \text{count}((\text{MeCshell} > t_{bcg}) \& (\text{MeCshell} < t_Q));$ apply [Equ.1](#) and count LIM voxels in j-th shell,
- j=j+1;

until count(erROI) > count(struct_el)

- $ROI_{tot} = \text{count}(\text{ROI});$ determine, total ROI volume
- $LIM_{tot} = \text{sum}(LIM_j);$ sum up all LIM sites,
- $LIM_X = \text{cumsum}(\text{volshell}_j) / ROI_{tot};$ normalize shell volumes (X axis of a $LIM(j)$ plot),
- $LIM_Y = \text{cumsum}(LIM_j) / LIM_{tot};$ normalize LIM densities (Y axis of $LIM(j)$ plot),

Scheme. 1. A pseudo-code of LIM and LIM_{0.5} routines

Steps A–E in the main loop can be run independently for each ROI yielding a significant acceleration of code execution on a multi-CPU platform.

Table 1
A comparison of low-intensity site statistics in DU145 cancer cells

A significant difference was observed in the distributions of $LIM_{0.5}$ and $LID_{0.5}$ parameters between naïve cells and each of the treated population.

Cell culture	Total number of cells	Number of processed (similar) cells	$LIM_{0.5}$ (mean $\pm$ sd)	$LID_{0.5}$ (mean $\pm$ sd)
UT-DU145	649	551	0.69 $\pm$ 0.12	0.74 $\pm$ 0.10
ZEB-DU145	660	592	0.61 $\pm$ 0.13 ^{*,**}	0.58 $\pm$ 0.10 [*]
AZA-DU145	496	463	0.50 $\pm$ 0.12 ^{*,**}	0.59 $\pm$ 0.12 [*]

* indicates statistical significance between treatment vs. controls $p < 0.001$, and

** a treatment vs. another treatment at $p < 0.001$. The hypothesis that $LID_{0.5}$ parameter in AZA and ZEB treated cells are from the same continuous distributions was not rejected ($p > 0.05$). The significance level in each test (β) was adjusted by Bonferroni correction ($\beta = \alpha/n$, for $\alpha = 0.05$, $n = 3$).