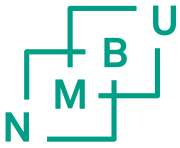
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Abstract

DNA methylation is a prominent type of epigenetic modifications. Honey bees (*Apis mellifera)* serve as a model organism to study epigenetics as they have an epigenetic machinery homologous to mammals. However, the anatomical aspects of DNA methylation in the adult and the developing bee brain are not yet known.

In this study, DNA methylation patterns in the honey bee brain are examined. The specific aims of the study were to find out if all cells in the adult bee brain were methylated, and whether there were different methylation patterns between cell types, calyx regions and pupal stages. Immunostaining for DNA methylation (5meC) as well as confocal microscope and ELISA were used to study the methylation patterns.

The results from the immunostaining and microscopy show that neurons as well as glia cells in the adult bee brain are methylated. Further, I show that glial cells seem to be more methylated than neurons in the early pupae stage. However, there are no obvious differences in the global methylation patterns between pupal stages or between different calyx regions.

These results indicate that the establishment methylation of most cells happens in the earlier stages. Therefore, future methylation studies should have a specific focus on earlier developmental stages or the specific methylated loci.

Sammendrag

DNA metylering er en vanlig form for epigenetisk modifikasjon. Honningbien (*Apis mellifera*) er en modellorganisme for å studere epigenetikk fordi de har et epigenetisk maskineri som er homologt med pattedyrs. Men de anatomiske aspektene av DNA metylering i den voksne og utviklende biehjernen er ikke kjent enda.

I dette studiet blir DNA metyleringsmønstrene i biehjernen forsket på. De spesifikke målene med studiet var å finne ut om alle celler i hjernen til voksne bier er metylert, og om det er forskjellige metyleringsmønstre mellom celletyper, calyx regioner og puppestadier. Immunofarging for DNA metylering (5meC) i tillegg til konfokalmikroskopi og ELISA ble benyttet for å studere metyleringsmønstrene.

Resultatene fra immunofargingen og mikroskopering viser at neuroner i tillegg til gliaceller i den voksne biehjernen er metylert. Videre viser jeg at gliaceller ser ut til å være mer metylert enn neuronene i det tidlige puppestadiet. Men det er ingen åpenbare forskjeller i globalt metyleringsmønster mellom puppestadier eller mellom forskjellige calyx regioner.

Disse resultatene indikerer at etableringen av metylering i de fleste celler skjer i tidlige stadier. Derfor bør fremtidige metyleringsstudier ha ett spesifikt fokus på tidlige utviklingsstadier eller det spesifikke metylerte loci.

Abbreviations

5caC	5-carboxylcytosine
5fC	5-formylcytosine
5hmC	5-hydroxymethylcytosine
5meC	5-methylcytosine
AL	Antennal lobe
BrdU	5-Bromodeoxyuridin
BSA	Bovine Serum Albumin
CI	Chloroform:isoamylalcohol
CLSM	Confocal laser scanning microscope
CNS	Central nervous system
DAPI	4’,6-diamidino-2-phenylindole
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
dsDNA	Double- stranded DNA
ELISA	Enzyme linked immunosorbent assay
HRP	Horseradish peroxidase
HSD	Honest significant difference
IHC	Immunohistochemistry
MB	Mushroom body
NEB	Newly emerged bee
OL	Optical lobe
PBS	Phosphate- buffered saline
PCI	Phenol:chloroform:isoamylalcohol
PFA	Paraformaldehyde

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1. Introduction

1.1 DNA methylation

Epigenetics is a vast scientific field. There are multiple definitions of epigenetics. One well used definition is that epigenetics considers changes in gene expression, without affecting the DNA sequence (Herb et al. 2012). However, a popular definition is that epigenetics is a structural adaptation of chromosomal regions to register, signal or perpetuate altered activity states (Bird 2007). Epigenetic changes affect, among other things, blood pressure, insulin resistance, maternal nutrition, behavior, metabolism and aging (Maleszka 2008). The most common mechanisms of epigenetics are DNA methylation and histone modification (Chittka & Chittka 2010). The present study will only focus on DNA methylation.

DNA methylation is the addition of a methyl group (-CH_3) to the 5' position of a cytosine in DNA, resulting in 5-methylcytosine (5meC), a robust but reversible marking of the DNA. The methylation is initiated by DNA methyltransferases (DNMTs) (Day & Sweatt 2010). There are two different DNMTs. DNMT1 are responsible for post- replication maintenance and thereby makes sure that the methylation status of the DNA is copied to the newly synthesized strand. DNMT3 methylate DNA *de novo. De novo* methylation is the addition of methyl groups to previously unmethylated DNA (Bird 1999; Lyko & Maleszka 2011). Methylated cytosine has a covalent carbon-carbon bond, which is extremely stable. A large amount of energy is required for demethylation, and direct demethylation is therefore unlikely. However, active demethylation can occur. The mechanism that is gaining most consensus is the oxidation of 5-methylcytosine (5meC) to 5- hydroxymethylcytosine (5hmC). 5hmC is then further oxidized to 5-formylcytosine (5-fC) and 5-carboxylcytosine (5caC) (He et al. 2011; Ito et al. 2011). The base excision repair machinery then replace the oxidized base. All the oxidation steps are carried out by Ten Eleven Translocation enzymes (Nabel & Kohli 2011).

To understand epigenetic regulation and the significance of DNA methylation in epigenetics, insects models are often used (Lyko & Maleszka 2011). Insect models have small, sparsely methylated genomes, which makes them easier to study. The honey bee (*Apis mellifera)* is optimal for studying the brain, behavior, aging and other aspects associated with epigenetics (Hunt & Page 1995; Keller & Jemielity 2006; Lyko & Maleszka 2011; Wolschin et al. 2009). They have a full complement of DNMTs, unlike *Drosophila melanogaster*. Furthermore, their

DNA is methylated similar to mammalian species except that in honey bees, only gene bodies are methylated (Lyko & Maleszka 2011; Maleszka 2008; Wang et al. 2006).

1.2 The honey bee

The honey bee is an eusocial insect (Winston 1987). Eusociality involves cooperative brood care and often a division of labor into reproductive (queen) and non-reproductive (worker) groups (Wilson & Hölldobler 2005). Furthermore, bees have a complex communication system and a very organized division of labor, with the workers further subdivided into nurses and foragers (Winston 1987). The workers transit between different tasks as they age, which is called temporal polyethism (Dolezal & Toth 2013). Temporal polyethism is one of the most striking aspects of eusocial insects. Epigenetic changes are associated with the transition between worker castes. For example, Herb et al. (2012) reversed the natural transition from nursing to foraging and studied the changes in distribution of methylation in the brain. When they had forced foragers back into nurse tasks they observed different methylation patterns between nurses, foragers and reverted workers. This supports the theory that nurses and forages differ in brain loci methylation patterns, and that these methylation patterns are reversible. The honey bee is not only a good model because of the epigenetics, it is also interesting to study because it is an essential pollinator which is important for the global ecology (Weinstock et al. 2006).

An important aspect of the honey bee is its life development. The honey bee is a holometabolous insect, which means that its development include four life stages: egg, larvae, pupae and adult. The development from egg to newly emerged bee (NEB) takes two to three weeks. Larval stages last eight to nine days. During the first two days all individuals are fed royal jelly. Thereafter only queen larvae are fed royal jelly. One hypothesis is that the royal jelly influences the epigenetic status, which then result in the different phenotypes between workers and queens. A study done by Lyko et al. (2010) support this theory. They silenced DNMT3 in larvae, and the resulting phenotype was similar to that of larvae fed royal jelly.

During the pupal stages, the bees undergo reorganization of tissue. The pupation period vary between the castes, e.g. normal workers have a pupal period of 12 days (Stone 2007). The larval and pupal stages can further be divided into substages, representing how far in the development the bee has come. In this work the classification system of Amdam et al. (2010) is used. Figure

1 shows the three pupal stages that will be studied further, and appendix 1 shows the max and min individuals within these stages.

Figure 1: The three pupal stages of interest: P1 (early pupae), P4 (middle pupae) and P7 (late pupae).

1.3 The honey bee brain

The honey bee brain develops as the bee goes through the larva and pupae stages. Brandt et al. (2005) made a digital map of the adult honey bee brain which shows the major brain regions (Fig. 2). Well-studied, major brain regions are the mushroom body (MB), antennal lobe (AL) and optical lobes (OL). The OL process visual information. When light enter the retinal cells, the OL transmit the created nerve impulses to higher order brain regions with functions in motor reactions and perceptions of light (Winston 1987). The AL process olfactory information and forms an interface between the central nervous system (CNS) and the incoming signals of antennal sensory neurons (Flanagan & Mercer 1989).

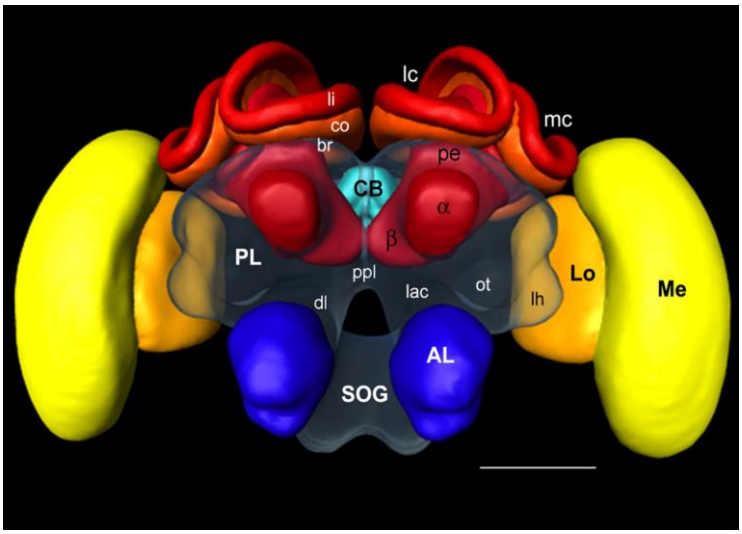

Figure 2: Digital map of the bee brain, from Rybak et al. (2010). Scale bar: 300 μm. PL: protocerebral lobe, ppl: posterious protocerebral lobe, Lo: lobula, Me: medulla, li: lip; co: collar, br: basal ring, lh: lateral horn, ot: optic tubercle, lac: lateral accessory lobe, mc: median calyx, lc: lateral calyx, pe: peduncle, α: alpha-lobe, β: beta-lobe, SOG: subesophageal ganglion

MBs are a most conspicuous structure in the brain from a morphological and physiological point of view, and it is also the most studied structure (Roat & Landim 2010). The MBs are center for learning and memory, and thereby play important roles in behavior, memory, learning and decision-making (Kaneko et al. 2013; Strausfeld 2002). MBs are paired, protocerebral neuropils, and each has two cup-like structures called calyces. The MBs receive input from optical neuropils and AL (Farris et al. 1999). The main cell types in the MBs are neurons, intrinsic Kenyon cells and projection neurons from the antennal lobes. Within the calyx, most neuronal somata belong to Kenyon cells. This area, filled with neuronal somata, is called soma cortex. The calyx itself consists of axons and dendrites, and it is called neuropile. The calyx is divided into three parts (Fig. 3). The lip region receives mainly olfactory input from the antennal lobe, the collar receives predominant visual input and the basal ring receives mixed olfactory and visual input (Farris et al. 1999; Mobbs 1982).

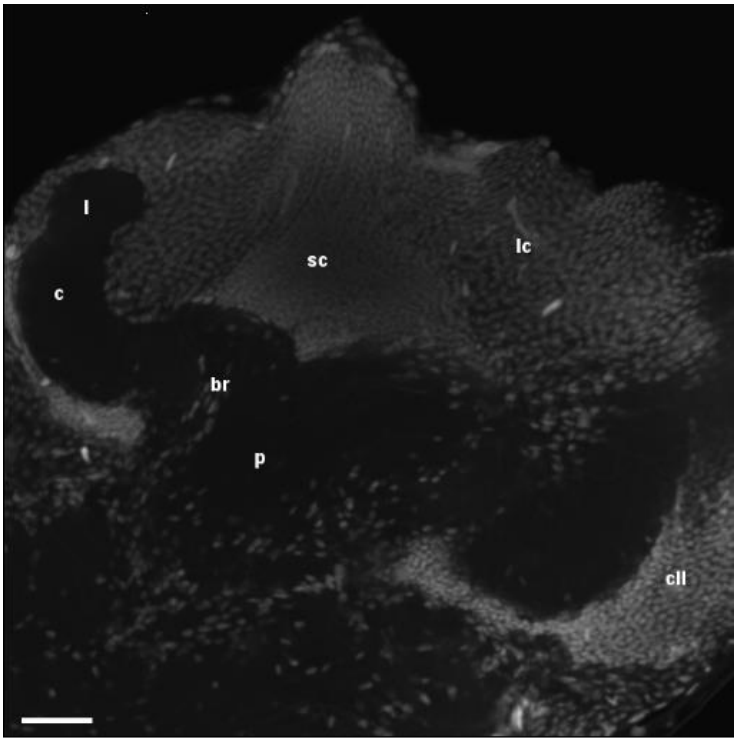

Figure 3: Overview of a calyx in a DAPI stained NEB. The picture visualize the position of cell bodies (light) and neuropiles (dark). The brain was sectioned in longitudinal direction and the section is 40 μm thick. l: lip, c: collar, br: basal ring, p: pedunculus, sc: small class I Kenyon cell, lc: large class I Kenyon cell, cII: class II Kenyon cell. Scale bar: 50 μm

The two main cell types in the brain are glial cells and neurons. The most common neuronal cell type in the MBs are the Kenyon cells. Each Kenyon cell consists of a cell body and a neurite that divides and forms input branches within the calyces. These axon like branches are sent to output areas of the mushroom body (Fahrbach 2006). The branches have both pre- and postsynaptic specialization, and form synapses both with MB extrinsic neurons and with each other (Farris et al. 2004). There are 170 000 Kenyon cells inside each MB. Furthermore, there are 14 000 Kenyon cells outside each MB as well (Fahrbach 2006). This means that there are more than 340 000 Kenyon cells in each bee brain, which add up to one third of the entire neuronal population of the brain (Ganeshina et al. 2000).

The Kenyon cells can be divided into four classes based on cell body size and morphology of arborizations in calyces and projections (Fig. 3) (Fahrbach 2006; Farris et al. 1999; Kaneko et al. 2013; Strausfeld 2002). Authors name the classes differently. I choose to use the classification and naming system used by Kaneko et al. (2013) (Table 1). The class I Kenyon cells are located in the inner core of the calyx. Small class I Kenyon cells are located as a central stack above the basal ring. They are compact and provide dendrites to the basal ring. Large class I Kenyon cells are located inside along the edges of the calyx, and they are non-compact. Large class I provide dendrites to the lip and collar of the calyx (Strausfeld 2002). Recently a new class I Kenyon cell was discovered by Kaneko et al. (2013), the middle class I Kenyon cell. Class II Kenyon cells are located at the periphery of the calyx. They are small and compact, as the small class I. Class II Kenyon cells have dendrites in the lip and collar of the calyx (Strausfeld 2002).

The different Kenyon cells develop at different stages. Class II Kenyon cells are the oldest of the Kenyon cells and develop first, which can be observed because they are as far from the production area as possible. The large class I cells follow them, and the small class I are the youngest of the Kenyon cells.

Table 1: Different Kenyon Cell types in the MB

Kenyon Cell	Compact/ non-compact	Location	Detailed location	Dendrites	Age
Small Class I	Compact	Inside calyx	central stack above basal ring	Basal ring	Youngest
Middle Class I		Inside calyx			
Large Class I	Non-compact	Inside calyx	Along the edges of the calyx	Lip, collar	
Class II	Compact	Outside calyx		Lip, collar	Oldest

The other cell type in the calyx is the glial cells. Glial cells construct guidance structures for migrating neurons or outgrowing axons, they support neuronal survival and differentiation by release of growth factors. They are required for maintenance of structural integrity of nervous system, and they play an important role in patterning the central nervous system (CNS) (Hähnlein & Bicker 1997).

Identification of glial cells are based on morphology and location. In the present study a classification system which distinguish between 3 types of glial cells are used (Freeman & Doherty 2006; Ito et al. 1995). The first, surface glia, are closely associated with the CNS outer surface, and they ensheath and support peripheral nerves. The second, cortex glia, lies among the neuronal cell bodies in the cortex and extend their membranes around the neuronal cell bodies, and they are in close contact with neurons. The third, neuropile glia, are sheath like membrane structures around target axons or bundles of axons and thereby promote neuropile survival. Neuropile glia are subdivided into two types. The first, ensheating glia (type I), is localized inside the neuropile. The second, astrocyte- like glia (type II) is localized in the interface between the soma cortex and the neuropile (Awasaki et al. 2008; Doherty et al. 2009).

1.4 Antibody

Cells have to be stained to be localized in a confocal microscope. Antibodies are often used for staining samples. For staining with antibodies a primary and a secondary antibody is necessary. The primary antibody is specific to a ligand, while the secondary antibody is specific to the animal the primary antibody were raised in. Secondary antibodies are therefore specific to the primary antibody. In this study I investigate methylation patterns, and to do that I need antibodies that recognize 5meC. Antibodies against 5meC have been raised (Gu et al. 2011; Inoue & Zhang 2011; Iqbal et al. 2011).

1.5 Aim of study

The importance of DNA methylation and epigenetics is now evident from recent research. Studying DNA methylation in mammals has its challenges because of the dynamic and complex nature of their methylation patterns (Lyko & Maleszka 2011). Therefore, the honey bee is becoming an important model organism. The aim of the present study is to investigate the DNA methylation in the honey bee brain. During my research I want to answer four questions by use of a confocal microscope:

1. Are all cells in the brain of newly emerged bees methylated?
2. Are there different methylation patterns between the calyx regions in newly emerged bees?
3. Are there different methylation pattern between cell types in newly emerged bees?
4. How does the methylation pattern changes during pupal development?

2. Materials and Method

2.1 Sampling and fixation for immunohistochemistry (IHC)

All experiments were conducted during fall 2013 and winter 2014 at the Norwegian University of Life Sciences (NMBU[1]). The sampling was conducted during October and November 2013. For sampling of NEBs, the brood frames were taken from the beehive and put in an incubator at 34.5 °C. NEBs were sampled between emergence and 24 hours after they had emerged. For sampling of pupae, frames were taken out of the beehive, and pupae in stages P1, P4 and P7 (Fig. 1) were sampled directly. Criteria described by Amdam et al. (2010) were used to determine the specific developmental stages (Appendix 1). For fixation, the heads were removed and transferred to 4% paraformaldehyde (PFA) in phosphate- buffered saline (PBS) and incubated overnight at 4 °C. To facilitate tissue perfusion with PFA, an incision around the head of the NEBs was made before fixation. The other stages had a soft enough cuticle for the PFA to penetrate without cutting. Subsequently, the heads were washed 3x10 min in PBS. For long- term storage, the brains were incubated in 30 % sucrose in PBS overnight, embedded in Tissue-Tek O.C.T compound (Sakura Finetek Europe B.V., The Netherlands) and snap frozen in liquid nitrogen, before they were stored at -80°C.

2.2 Dissection and dehydration/rehydration

The fixed brains were removed from the head capsule, and adjacent tissues, such as glands, were cut away. The brains were washed 4x10 min in PBS, dehydrated in an increasing ethanol series (50%, 70%, 90%, 95%, 100%, each for 10 min) and rehydrated in a decreasing ethanol series (100%, 95%, 90%, 70%, 50%, each for 10 min). The dehydration/rehydration in ethanol series is done to improve permeability for antibodies by removal of lipids (Nässel 1996). Subsequently, the brains were washed 2x10 min in PBS, microwaved for 3x2 min at 800W in ice water to prevent excessive heat, which was followed by a new PBS wash for 2x10 min.

2.3 Sectioning

The brains used in all the experiments were embedded in 5 % low gelling temperature agarose (Sigma) and cut with a Leica VT 1000S vibratome (Leica Biosystems, Nussloch, Germany).

[1] The university changed its Norwegian name from UMB January 1, 2014

The brains used to study anti-5meC staining, calyx regions and cell types were sectioned in longitudinal direction with a thickness of 40 μm. Each section should contain both calyx and antennal lobe. To achieve this, the sections were contrast- stained with Stevenel's Blue and observed with a Wild Stereomicroscope M3C (Wild Heerbrugg, Switzerland). When both calyx and antennal lobes were visible, alternating sections were put in two Eppendorf tubes with PBS, before proceeding to immunohistochemistry (IHC).

The brains used to study the pupal stages were sectioned in transverse direction with a thickness of 40 μm. The whole brain was sectioned, and the sections were put in Eppendorf tubes with one individual per tube.

2.4 Immunohistochemistry

For the experiments on adult bee brains, each individual had to be stained with an antibody, which stains DNA methylation by binding to 5meC. The 5meC antibody binds to partially denatured DNA. To this end, the sections were incubated 15 min in 4N HCl. Subsequently, the sections were incubated 30 min in blocking solution (5 % Bovine Serum Albumin (BSA) + 0,1 % TritonX in PBS), and 48 hours in primary antibody 5meC (mouse, monoclonal 33D3 Diagenode lot. GF-003) diluted 1:200 in blocking solution. To my knowledge, 5meC antibody has not been used for studies on honey bee brains previously. However, the 5meC antibody is expected to be specific because it binds to 5meC, which is present in the honey bee (Anier et al. 2010; Gu et al. 2011). The sections were washed 6x20 min in PBST (0,1 % TritonX in PBS) and incubated 24 hours in Cy3-conjugated goat anti- mouse secondary antibody (Jackson ImmunoResearch), diluted 1:200 in PBST. Finally, the sections were washed 6x10 min in PBST and incubated 30 min in 30 % glycerol (Sigma-Aldrich).

As a reference for cell nuclei, a DNA stain (4',6-diamidino-2-phenylindole (DAPI)) was used. DAPI is a fluorescent dye that stains double- stranded DNA (dsDNA) (Kapuscinski 1995). DAPI binds to AT rich regions in the minor groove of DNA. However, DAPI only binds dsDNA and is therefore not compatible with the acid treatment (Lewis & Errington 1997). Hence, alternating sections in each individual were stained with DAPI and antibody. The sections stained with DAPI were treated much the same way, with the exception that the incubation steps in 4N HCl and antibody were omitted. However, they were incubated 20 min in DAPI (Sigma), diluted 1:5000 in PBS, between the last washing step and incubation in 30 % glycerol. All sections were mounted in 50 % glycerol. The cover slip was sealed with nail polish.

The brains for studying pupal stages were only stained with antibody. The sections therefore followed the same IHC procedure as described above for the antibody stained sections.

2.5 Injections

BrdU was planned to be used to study the possible methylation differences between proliferating and non-proliferating cells in different pupal stages. Therefore, samples were injected with BrdU. In brief, the procedure was as follows: After sampling, the individuals were put in the fridge for 10 minutes to anesthetize the bees (Wang et al. 2010). Subsequently they were immobilized by crossing needles between thorax and abdomen. For the BrdU incorporation, 2,5 µl of 6,25 % BrdU (Sigma) in bee saline (in mM: 130 NaCl, 6 KCl, 2 $MgCl_2$, 7 $CaCl_2$, 160 glucose, 10 HEPES, pH 6,7 (Malun et al. 2003)) were injected in the neck between head and thorax. The controls were injected with bee saline. All individuals were incubated in room temperature for 1 hour before the heads were removed and fixated as described. Even though the BrdU injections did not work out, the control samples were used for the studies on pupae development.

2.6 Confocal microscopy

A confocal laser scanning microscope (CLSM) is a light microscope with fluorescence and a confocal part. The CLSM works by two principal ideas. Firstly, it has a point-by-point illumination of the sample and secondly it rejects out of focus light. The aim of confocal microscopy is to explore relations in three dimensions. This is done by exploring structure and structural relations along the x-y plane, but also along the z (optical) axis. Only one point of the sample is observed at any moment, and therefore a computer reconstructs the 2D image of the plane. To achieve a 3D image all the 2D images of different depths are combined (Prasad et al. 2007). In the present study, a confocal microscope was used because it has the advantage of high resolution and multiple channels, and it gives the possibility of observing the pictures in 3D.

The sections were viewed by using a Leica TCS SP5 confocal microscope (LSCM, Leica Microsystems, Wetzlar, Germany). For all experiments the xy format 1024x1024 was used, and all pictures had a line average of 2. Laser power and detector sensitivity were kept constant to be able to compare the images, but also to limit the technical variability.

A laser line with 405 nm wavelength was used for excitation of the DAPI stain and a 561 nm laser for the Cy3- conjugated secondary antibody. For detection of emitted signals, detection filters were set to 410 – 480 nm for DAPI and to 570-626 nm for detecting Cy3. A 20x oil objective (HCX PL APO CS 20, numerical aperture 0.7) was used for studies in adults, and a 40x oil objective (HCX PL APO lambda blue 40, numerical aperture 1.25) was used to study pupae development. Smart offset was -0.4 % for all pictures in all experiments. Z- step size was 1 μm in all images for studying 5meC distribution in the adult brain and 0.5 μm for studying pupal development.

2.7 Image processing and statistics

ImageJ (v1.47, US National Institutes of Health, Bethesda, MD, USA) was used for image processing and P1 analysis. All pictures were processed with smoothing edges (Gaussian Blur, Sigma = 1) to reduce noise and stack collapse (z-projection, max intensity). Light adjustment was limited to simple tresholding and no automatic contrast was used.

For analysis of cell types in early pupal stages, each cell had to be distinguishable. Therefore, a stack collapse of four pictures was made. A stack of more than four pictures would make it difficult to tell apart the cells. Surface glia, neuronal glia, class II Kenyon cells and large class I Kenyon cells were analyzed in each picture by outlining each cell. ImageJ then calculated the signal intensity. For each cell type, the cells to be analyzed were chosen by fixed rules. The cell most to the left in each picture were chosen first, and a straight line was drawn to the nearest cell on the right. Thus, by always going from the left to the right, the line would not go in circles and the chosen cells would not be from only one cluster. A Post-hoc analysis was performed using the Tukey honest significant difference (HSD) test. $P < 0.001$ was assessed as significant.

2.8 Enzyme-linked immunosorbent assay (ELISA)

ELISA is a quantifying technique used to determine if and how much of a particular substance of interest is present in a given sample. There are two variations of ELISA. It can be used to determine how much antibody it is in the sample, and it can be used to determine how much protein is bound by antibody. ELISA is usually performed on a 96 wells plate, and standards made of negative and positive controls are included for quantification. Inside each well an enzyme reaction occurs. A plate reader determines the optical density for each well to determine the amount of primary antibody (Campbell 2002).

In this study ELISA was used for quantification of DNA methylation in NEBs and P1 pupae.

2.8.1 Sampling and DNA extraction

Sampling for the ELISA experiment was conducted in February 2014 similarly to other experiments (see 2.1). However, only P1 of the pupal stages were sampled. Whole individuals were directly collected into Eppendorf tubes, snap- frozen in liquid nitrogen and stored at -80 °C.

The brains were dissected and put in Eppendorf tubes with 200 µl PBS. For NEB and P1 two and three brains were pooled together, respectively. To help dissolve extracellular matrix and to deactivate nucleases that otherwise could degrade the DNA, 20 µl proteinase K (Sigma-Aldrich) was added to each tube and mixed in. Subsequently 200 µl AL buffer (lysis buffer, Qiagen) was added to break open the cells and nuclear membranes using the same method. The samples were incubated for 16 hours at 56 °C with 400 rpm shaking on a Thermomixer comfort (Eppendorf, Hamburg, Germany). Afterwards, the samples were vortexed and chilled for 2 min at room temperature before the supernatant was transferred to a clean Eppendorf tube. dH_2O was added until a final volume of 500 µl was reached. An equal volume of phenol:chloroform:isoamylalcohol (PCI) (invitrogen) was added to separate DNA from proteins and other cell components. Then the samples were vortexed and centrifugated (5804 R, Eppendorf, Hamburg, Germany) at 15 000 x g for 5 min at room temperature. Subsequently, the aqueous phase was transferred to a clean Eppendorf tube. To destroy the RNA, 15 µl RNase A (PureLink, invitrogen) was added. Then the samples were incubated for 30 min at 37 °C with 550 rpm shaking. Afterwards, the supernatants were transferred to a clean Eppendorf tube and PCI was added at equal amount as supernatant, and the samples were vortexed and centrifuged as before. The same steps were then repeated once with chloroform:isoamylalcohol (CI) (Sigma). CI binds to proteins and lipids and thereby help separate them from the DNA. 400 µl of the aqueous phase was collected after centrifugation, and 40 µl 3M NaAc (Sigma-Aldrich), 5 µl linear acrylamide (AM9520, Ambion) and 1 ml ice cold absolute ethanol (VWR Chemicals) was added. The salt neutralize the charge on the DNA and make the molecule less hydrophilic. The ethanol lower the dielectric constant, and thereby force precipitation of DNA, while linear acrylamide is a coprecipitant which enhance recovery of small amounts of DNA. The samples were vortexed and percipitated at -80 ºC for at least 2 hours. Afterwards the samples were centrifuged at 20 000 x g at 4 ºC for 15 min and washed twice with ice-cold 70

% ethanol with centrifuging at 20 000 x g at 4 ºC for 8 min in each wash. Subsequently the ethanol was removed and the pellet was left to airdry for about 10 min. The DNA was then dissolved in 30 µl dH_2O.

The DNA yield was checked using a Qubit dsDNA fluorometer kit (Invitrogen). The kit consists of a qubit reagent and a qubit buffer, which were used to make a qubit working solution, and two standards. The samples were stored at -20 °C.

2.8.2 ELISA

ELISA was done according to the protocol made by the manufacturer, Zymo Research group (CA, USA) (Appendix 2). The same protocol were used to generate standards with known 5meC. The standards were made prior to denaturation and treated the same way as the samples. DNA from the DNA extraction were mixed with 5-mC Coating buffer, denatured at 98 ºC for 5 min and incubated for 1 hour at 37 ºC. Subsequently the wells were washed three times with 5-mC ELISA buffer and incubated for 30 min in 5-mC ELISA buffer at 37 ºC to rinse the plates between the reagent additions. An antibody mix was added to each well and the plate was incubated for 1 hour at 37 ºC. Further, the wells were washed three times with 5-mC ELISA buffer and horseradish peroxidase (HRP) developer was added to each well. HRP is an oxidoreductase that use hydrogen donors to reduce hydrogen peroxide. This yields a color change that is detectable by spectrophotometric methods, e.g. ELISA (Ryan et al. 1994). The absorbance was measured using a SPECTROstar Nano (BMG Labtech, Ortenberg, Germany).

3. Results

The objectives of this study were to use the honey bee brain to investigate anatomical aspects of DNA methylation in the brain, to study cell- specific differences of methylation patterns and to see if there are any developmental differences of methylation.

3.1 Anti-5meC staining in NEB

An image of a whole DAPI stained NEB brain sectioned in longitudinal direction is shown in Figure 4A. This figure gives an overview of the organization of the honey bee brain. The white square surround a part of the calyx, and an enlarged picture of this calyx is shown. This study focused on the calyx, an area of the brain which has been extensively studied for its relevance in e.g. memory formation.

NEBs were sectioned in longitudinal direction and stained with DAPI and 5meC antibody to determine if all cells are methylated. Figure 4 B, D are stained with DAPI as a reference for cell nuclei. Figure 4 C, E are stained with anti- 5meC. The data suggest that 5meC antibody stains similarly to DAPI, which indicates that all cells are methylated.

The different brain regions were studied briefly. At first glance, there were no obvious differences in methylation pattern between the different regions (data not shown). However, the different regions were not studied further due to time constrains. Therefore, only the calyces of the MBs were studied here (Fig. 4 B-E). There does not seem to be any differences between anti-5meC and DAPI staining in the calyx regions.

There are two main cell types that have their bodies in the calyx of the MBs: the neurons, mostly Kenyon cells, and glia cells. The arrows in Figure 4 B-E depict different cell types. All cell types are visible in both DAPI and antibody stained adult individuals, and no obvious intensity differences between them was observed. The exception is the small class I Kenyon cells. Small class I Kenyon cells have a higher intensity than the other cells for all individuals. This observation was made in both DAPI- and anti-5meC stained individuals. The data indicate that all cells, both neurons and glia, are methylated. Furthermore, the images suggest that all the different cell types have the same amount of methylation. Kaneko et al. (2013) found a third class I Kenyon cell, called middle class I Kenyon cell. It was not possible to distinguish this type from the other class I in these images.

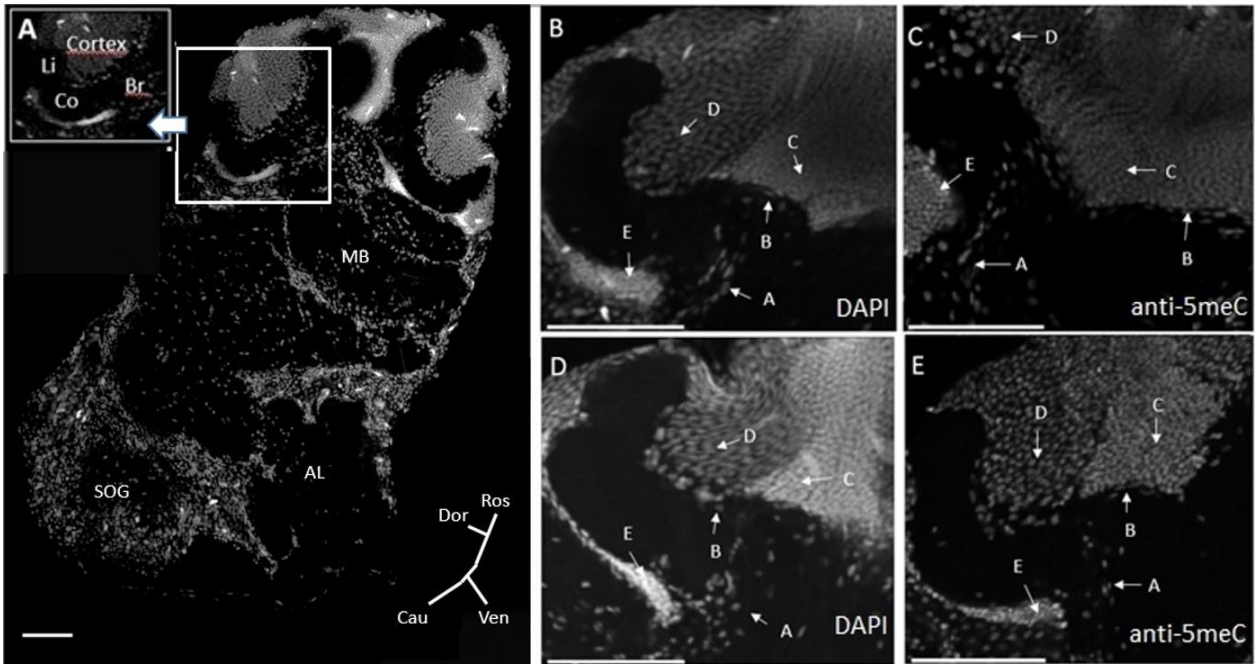

Figure 4: DAPI and anti-5meC staining of longitudinal sections of NEBs. A) Overview of a DAPI stained section of the whole brain. The square shows a part of the calyx. The neuroaxis is shown as white lines in bottom right corner. Ros: rostral, Dor: dorsal, Ven: ventral, Cau: caudal. Li: lip, Co: collar, Br: basal ring, MB: mushroom body, AL: Antennal lobe, SOG: suboesophageal ganglion. B,D) Part of calyx of two individuals, both DAPI stained. C, E) Part of the calyx for two individuals, both anti- 5meC stained. B and C are sections from the same individual, and D and E are from the same individual. For B-D the arrows points towards different celltypes, and all the cell types are visible in both DAPI and anti-5meC stained sections. The cell types are: A) type I (ensheating) neuropile glia, B) type II (astrocyte-like) neuropil glia, C) small class I Kenyon cells, D) large class I Kenyon cells, E) class II Kenyon cells. All images are taken with a 20x oil objective. Scale bar: 100 μm.

3.2 Methylation patterns through pupal development

Three different pupal stages (P1, P4 and P7, shown in Fig. 1) and NEBs were used to study the methylation patterns between developmental stages. The calyx of two individuals is shown for each stage in Figure 5. There is a lot of variation between the images, and as much difference between the stages as between the individuals within the same stage. Therefore, it is not possible to conclude that there are developmental differences. Furthermore, the quality of the pictures are not good enough for detailed comparisons (see discussion 4.3).

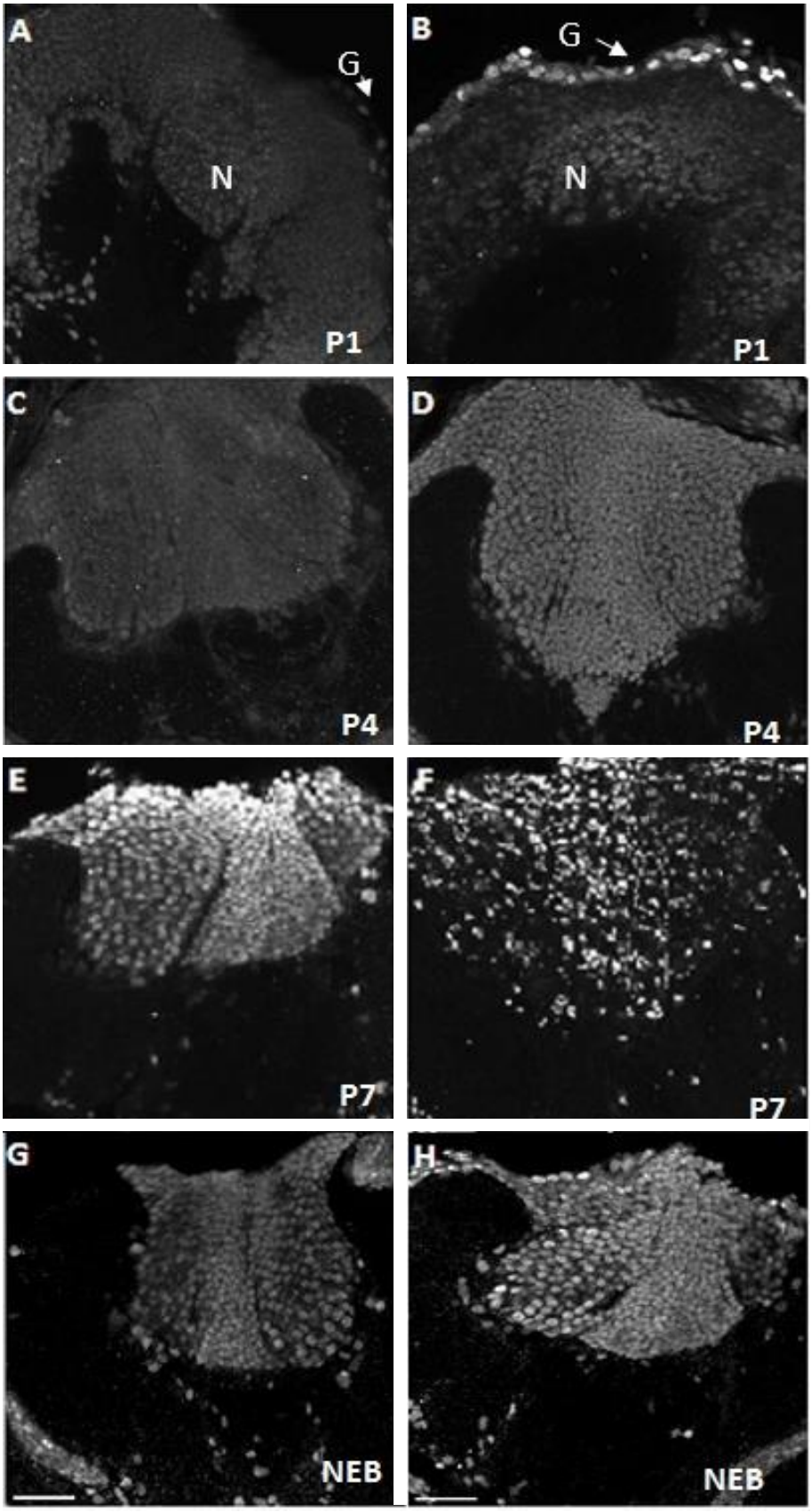

Figure 5: Anti- 5meC stained sections for studying methylation pattern during pupae development. The pictures represents three pupal stages, P1 (A,B), P4 (C,D) and P7 (E,F), and NEB(G,H). Note the different staining intensities for glial (G) and neuronal (N) cell bodies in P1 (A, B). Four individuals and eight pictures represented each stage in total. Here two images from each stage is shown, and each image represents an individual stained with anti- 5meC. All images are taken in transverse direction, with a 40x oil objective, stack collapse of 20 images (10 μm) and scale bar: 40 μm

3.3 Cell- type specific methylation patterns

In the early pupal stages (P1, Fig. 5A, B) the glial cells (G) seems to have higher intensity than the neurons (N). For the other stages, I did not observe similar intensity differences. Therefore, an analysis of intensity differences between glial cells and Kenyon cells in pupale stage P1 was performed. Quantitative analyses were performed for two individuals, each represented by two images that were of sufficient quality for quantification (see discussion). Furthermore, the number of cells analyzed for each cell type varied. Figure 6 shows the mean intensity of the cell types surface glia ($N_{S.glia} = 35$), neuropile glia ($N_{N.glia} = 70$), small class I Kenyon cells ($N_{KC\ I} = 121$) and class II Kenyon cells ($N_{KC\ II} = 139$). The figure suggests that surface glia have more methylation than the other cell types, and that the two Kenyon cell types have similar amount of methylation. Staining intensity for neuropile glia seems higher than what was observed for Kenyon cells, however there was more variation within the neuropile glia group.

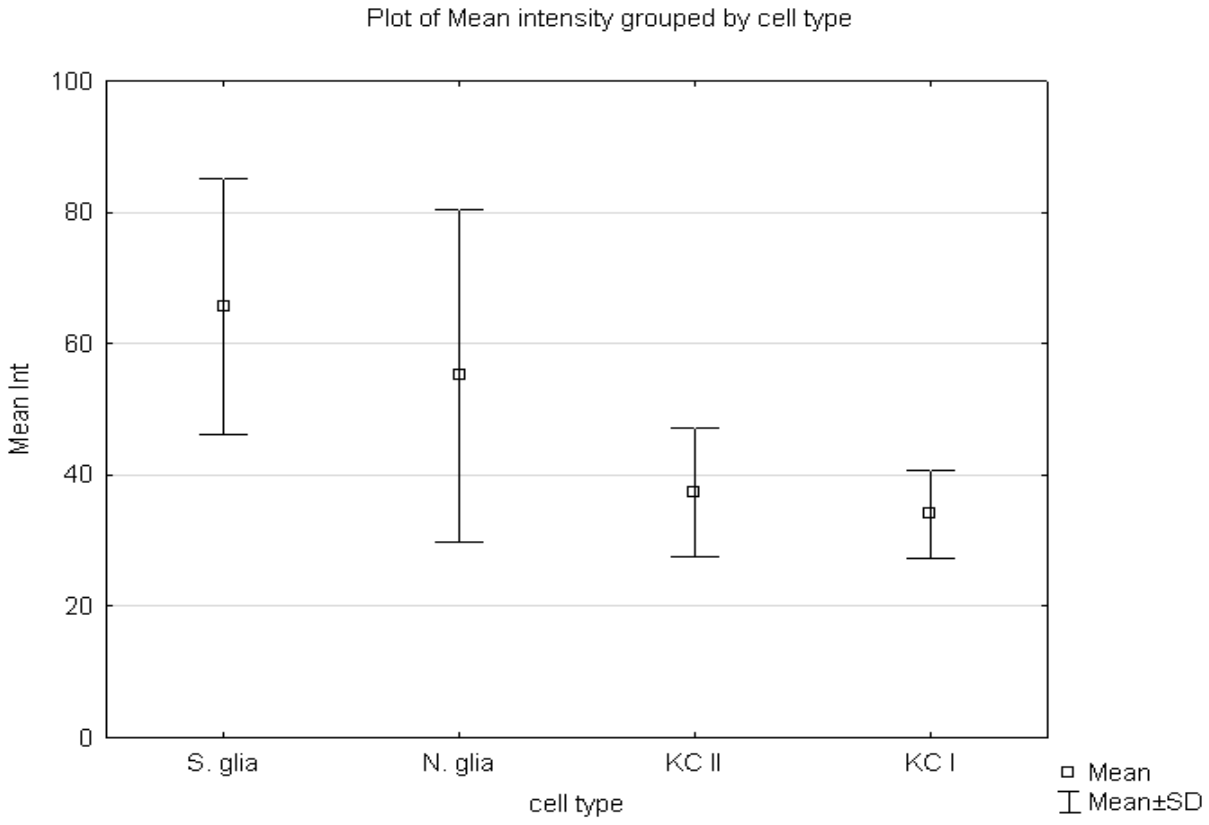

Figure 6: Mean intensity differences between cell types in stage P1. Surface glia (S. glia), neuropile glia (N. glia), class II Kenyon cells (KC II) and large class I Kenyon cells (KC I) were analyzed. Number of cells analyzed varied between cell types. Four images, from two individuals, were analyzed by use of ImageJ, ANOVA and a post-hoc test.

To assess potential differences in methylation pattern between cell types, I performed a main factor ANOVA test. Anti- 5meC staining intensity was the dependent variable, and cell type and image (replicate) independent factors. The ANOVA test confirms a significant main effect for cell type ($F_{df=3,\ N=358} = 71$, $P < 0.001$). However, a replicate effect was detected as well confirming that staining intensity varies between images ($F_{df=3,\ N=358} = 42$, $P < 0.001$). The post-hoc analysis showed significant differences between all cell types, except between the two types of Kenyon cells (Tukey HSD, see Table 2). The Tukey HSD test showed $P_{KC1\ vs\ KCII} > 0.001$.

Table 2: Results from the Tukey HSD test, variable mean intensity. There were significant differences ($P < 0.001$) between all cell types except between the two types of Kenyon cells.

	S. glia	**N. glia**	**KC II**	**KC I**
S. glia	-	< 0.001	< 0.001	< 0.001
N. glia	< 0.001	-	< 0.001	< 0.001
KC II	< 0.001	< 0.001	-	0.1426
KC I	< 0.001	< 0.001	0.1426	-

3.4 Differences in amount of methylation between NEB and early pupae

ELISA was used to assess the differences in amount of DNA methylation between NEBs and P1 pupae. DNA samples from the DNA extraction was used to perform the ELISA. A series of standards with known 5meC content were made to create a standard curve (Fig. 7). The standard curve was used to be to quantify the percentage of 5meC in the DNA samples from NEB and P1 pupae.

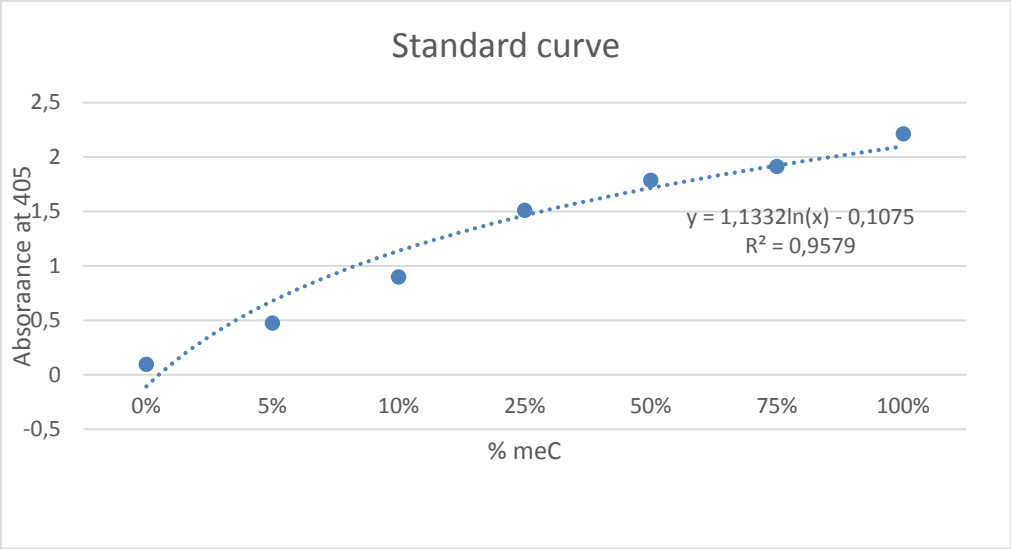

Figure 7: Standard curve made from mixtures with known 5meC content.

The percentage of 5meC in the DNA samples was quantified by use of a logarithmic second-order regression provided by the manufacturer (Appendix 2). The regression equation used for quantification was made from the standard curve (Fig. 7) and the absorption for the samples in each well. Figure 8 shows the calculated average percentage of methylated DNA in the brains from each stage. Again, no significant differences in methylation pattern between the stages were detected (t-test, $T_{df=1,\ N=10} = 0.3340$; $P = 0.7481$).

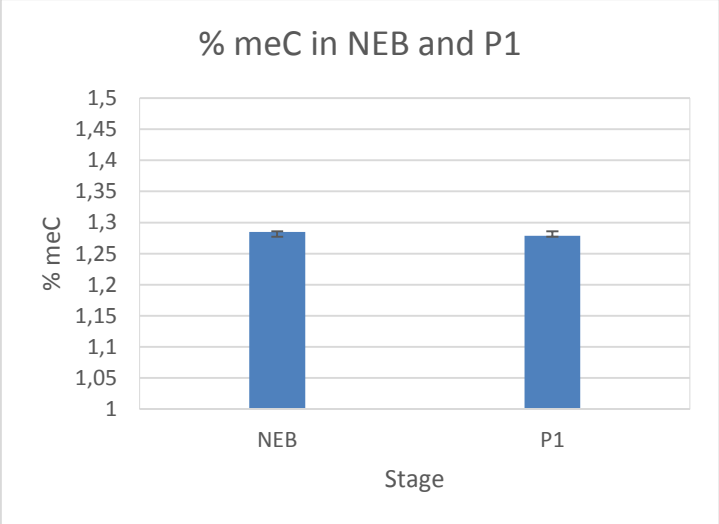

Figure 8: Percentage 5meC in NEB and P1. The percentage is calculated from absorption values and the standard curve (Fig. 7) by use of a regression equation. Each bar represents the mean value from ten DNA samples, including duplicates. Standard deviation is shown for each bar.

4. Discussion

Immunohistochemistry and confocal microscope were used to examine methylation patterns in the honey bee brain. To my knowledge, this is the first study to provide an anatomical description of methylation patterns in the honey bee brain. I found that all cells in the adult bee brain are methylated. Furthermore, I found indications of methylation differences between neurons and glial cells in the early pupale stage (P1). My results do not indicate differences in methylation patterns between calyx regions or between cell types in adult individuals. Further, the results do not support large differences between pupal stages.

4.1 Methylation patterns in the adult and developing bee brain

4.1.1 The adult bee brain

In the present study, images of DAPI and anti- 5meC stained adult individuals were compared. The present study showed that all cells in the honey bee brain are methylated. My data support the findings from a study on the whole honey bee genome which discovered that there were methylation in specific fractions of the bee genome (Lyko et al. 2010). However, Lyko et al. (2010) did not assume this to be true for all cells. Another much studied insect is *Drosophila,* which is often used as a model organism. However, it is not a good model for the study of DNA methylation. The methylation level is low and often under the limit for bisulphite sequencing (Capuano et al. 2014), and it does not have a full complement of DNA methylation relevant enzymes, i.e. DNMTs (Field et al. 2004). Analysis of other insects genomes show a similar pattern with a lack of canonical DNMTs (Elango et al. 2009). In contrast, social insects, e.g. the wasp *Nasonia vitripennis,* might have a full complement of DNMTs similarly to the honey bee (Lyko & Maleszka 2011). However, these social insects are not much studied, and little is known about their methylation patterns. The methylation patterns in vertebrates are much studied. A similarity between mammals and honey bees is that both have three similar main classes of DNMTs (Okano et al. 1999; Wang et al. 2006). Therefore, the honey bee is deemed a good model organism for studying DNA methylation.

The brain is important in the study of epigenetics. The mushroom bodies are often referred to as the learning center in the honey bee brain, and multiple studies suggest that they have a key role in learning and memory (Fahrbach 2006; Farris 2008; Strausfeld et al. 1998). My results suggest that the DNA of all cells in the MBs is methylated, and my data does not support large differences between calyx regions. A study performed by Lockett et al. (2010) showed that

DNA methylation has a critical role in learning and both long and short term memory processing in honey bees. It has to be noted that another study conducted on honey bees found that DNA methylation is associated with long term memory, but not with short term memory (Biergans et al. 2012). Their study thereby partly contradicts the study performed by Lockett et al. (2010). The two studies use different protocols, which might be a reason for the different results. Even though the studies partly disagree about the role of DNA methylation in memory processing, they both say that DNA methylation has a role in learning and memory. In vertebrates, studies have shown that DNA methylation is implicated in learning and memory. For example, Miller and Sweatt (2007) found that DNA methylation is crucial in memory formation in rats. Thus, studies in rats and honey bees as well indicate a role for DNA methylation in learning and memory. In the present study I showed that all MBs seem to have the potential to be methylated during learning and memory formation. Thus, these studies support a role for methylation in learning and memory, similar to my data.

4.1.2 Pupal development

The MBs were studied through pupal development. No changes in methylation patterns during development were observed. However, there might be changes that I did not detect, because loci specific methylation changes could not be detected by use of IHC. The similar methylation patterns between the pupal stages may indicate that most DNA methylation is already established before pupation. This is supported by the DNMT1 activity being restricted to dividing cells, which means most methylation happens during early development (Miller & Sweatt 2007). However, cell proliferation in the MBs continue until pupale stage P4 (Ganeshina et al. 2000), which means that some methylation most likely happens during pupation. That methylation happens in developmental stages is further supported by a study on rats where it was discovered that all the DNMTs are highly expressed during development, and drop significantly by adulthood (Simmons et al. 2013). It is a possibility that the same applies to the honey bee, where initial DNA methylation patterns are already established before pupation.

Developmental studies on the honey bee have been conducted. Elango et al. (2009) suggests that the activity of genes with roles in development can be differentially methylated. This is supported by a study performed by Cameron et al. (2013). They examined the differences in methylation patterns between queen and worker larvae, and suggest that the methylation differences arose from events that take place in mid to late larvae stages. Therefore, methylation

probably happens in the early developmental stages. These studies further support my results. For investigating when and where the DNA methylation happens, larval stages would probably have to be studied.

4.1.3 Cell types

The present study indicates that there are differences in DNA methylation between glial cells and neurons in the early pupale stage (P1). My results shows that glial cells are more methylated than neurons in P1. In the other stages, no apparent differences between cell types were observed. However, I cannot exclude that there were differences. To my knowledge there are no other studies investigating the methylation patterns between cell types in insect brains. Thus, I will compare the results with work done on mammals. Differences in methylation patterns between cell types have been studied in the human brain. In the human brain, the methylation pattern differ between neuronal and non- neuronal (mostly glial) cell types (Iwamoto et al. 2011). Iwamoto et al. (2011) found that neurons showed lower methylation than non-neuronal cell types. This is similar to my results, which suggest that glial cells have higher methylation than neurons. However, my data only show these findings in P1, while the study conducted by Iwamoto et al. (2011) observe methylation differences between neurons and non-neurons in adult individuals.

Neurons, and especially Kenyon cells, are much studied in insect species. The classes of Kenyon cells appear in sequential order during development of many insects, e.g. *Periplaneta americana* (cockroach), *Acheta domesticus* (cricket) and *Drosophila* in addition to the honey bee (Malaterre et al. 2002). In *Drosophila*, neurons and glia cells develop randomly (Bayer & Altman 1991). In mammals, however, glial cells develop after neuronal cells (Miller & Gauthier 2007). The Kenyon cells are established first followed by a matching of glia numbers and position. However, bees have most methylation within genes while mammals have most in promoters and repeat sequences. The gene body methylation in bees are not repressive (Flores et al. 2012), and comparison of gene expression between honey bees and mammals are thereby probably not informative.

4.2 Methylation differences between NEB and P1

ELISA was used as an alternative approach for studying global methylation differences between NEB and pupale stage P1. The method is simpler than IHC and microscopy, and makes it possible to quantify methylation differences between the two stages. The ELISA results showed no differences in global methylation between NEB and early pupae. The individual differences were larger than the differences between the stages. It might be that there are no gross methylation differences between the stages. However, it might also be that the methylation during development only affect a few genes, which would not be distinguishable by use of ELISA. The percentage of methylated DNA in bees is low, and therefore the method might not be sensitive enough to pick up small changes. Therefore, the ELISA results does not prove that the amount of methylation is similar between the stages.

During the DNA extraction for ELISA the brains had to be pooled. For NEB two brains were pooled together, while three brains were pooled together for P1. More brains had to be used for P1 because their brains are smaller than the NEB brains, and only two brains would not yield enough DNA. Therefore, inter-individual variation in P1 may have been compressed because more brains were pooled, which may affect the statistical analyzes.

4.3 Methodological considerations

The pupae were sampled directly from the comb, and stages had to be determined by appearance only. Therefore, it was difficult to determine the exact pupal stage. Furthermore, the early pupae develop fast and hours in age difference may be enough for substantial differences to occur (Fahrbach et al. 1995). There are also individual differences, with pupae of the same age that develop at different speed, which also has to be taken into consideration (Appendix 1). However, these uncertainties in staging probably only have minor impact on the observed methylation patterns because of the comparably larger developmental time between the stages chosen for sampling.

Results from main effect ANOVA and a post-hoc (Tukey HSD) support that surface glia have higher intensity than the other cell types. The Tukey HSD test showed that there were significant differences between all cell types except between class II and large class I Kenyon cells. However, the initial goal of this study was to detect developmental differences, not cell-type specific differences. The developmental studies demanded that the scanning settings were

kept constant. Therefore, the images were not optimally using the full dynamic intensity range, and only four images from two individuals were of sufficient quality to be analyzed. Sufficient quality implies that the images had to be bright enough, had to have sufficient intensity depth and the pixel intensity should cover almost the full 8- bit range (0…255). Even though the images were not optimal for investigating cell-type specific differences, differences between cell types were observed in the early pupale stage. Due to the insufficient image quality, there might be cell-type specific differences in the other stages that I did not detect. Furthermore, the number of cells analyzed for each cell type in P1 differed. Surface glia was represented by less than half as many cells as the other cell types. Because of the small number of replicates and images analyzed in this first study, future studies with more individuals should be conducted to address cell-type specific methylation.

It is a possibility that other factors could explain the found staining differences. In fact, another DNA specific staining, DAPI, can also show staining differences. In the present study, small Class I Kenyon cells were brighter than the other cell types in both DAPI and anti- 5meC stained individuals. In the case of DAPI staining, differences are often explained by differences in DNA compaction (Buckingham 2012). DNA compaction can in principle also affect other cell types, although I did not observe this. However, I cannot exclude that part of the differences in anti-5meC staining between cell types can also be explained by DNA compaction. To reduce the impact of such influences, small class I Kenyon cells were excluded from the analysis of cell types. Since they showed different staining from the other cell types in all individuals, it is possible to assume that it has nothing to do with methylation. However, it is not possible to rule out that factors other than DNA compaction may contribute to the detected differences. The pupal stages were not reference stained with DAPI, but the methylation patterns between pupae and adults are otherwise similar. Therefore, it is possible to assume that the small class I Kenyon cells would have higher staining intensity with DAPI in pupae as well.

Bees injected with bee saline were used to study pupale development. These bees were supposed to be control individuals for a BrdU experiment. BrdU incorporation was tried out to study proliferation in the honey bee brain during development. Proliferation would have been interesting to examine, especially because there seem to be methylation differences between cell types in P1. Staining with anti-BrdU could show if the glia cells had more proliferation than Kenyon cells. However, the experiment did not succeed and were not taken any further.

Proceeding with the experiment would be time consuming, because a new protocol would have to be established to achieve good staining.

4.4 Conclusion and outlook

To my best knowledge, the present study is the first to confirm DNA methylation anatomically for the neuronal and glial population in an insect's learning center. The anatomical aspects of methylation patterns in the honey bee brain have not been much studied previously. By use of confocal microscopy I found that all cells appeared immunopositive for 5meC. Furthermore, I have described and found differences in staining intensity between glia cells and neurons in the early pupale stage (P1). I have also found that there were no apparent methylation differences between calyx regions or through pupal stages, with the exception of the cell type differences in P1. It would be rewarding to study my results further in the future.

Several studies could be performed on this topic. Firstly, the intensity differences observed between surface glia, neuropile glia and Kenyon cells in P1 should be studied further. The experimental setup used in this study was made for detecting developmental rather than cell-type specific differences. A future approach should be optimized to specifically address differences between cell types. The study could be performed by the same method as in this study, or a laser dissection microscope could be used to cut out glial cells and neurons. The cell types could then be analyzed by using a more sensitive method, e.g. LC-MS/MS. Regardless of the chosen method, the study should also show if the intensity differences are observed only in early pupae, or if the NEBs also show it to some degree.

The results from this study tell us that we can direct future methylation studies to earlier developmental stages or specific methylated regions. Several authors (e.g. Dolezal and Toth (2013); Herb et al. (2012)) have suggested that epigenetics are the reason for the switch between nurses and foragers and it would be interesting to see if it is possible to notice the switch, both when and where it happens in the brain.

In this study, it was not possible to see which genes were methylated, or if there is a difference in gene methylation through development, between cell types or in different brain regions. Differential gene methylation would be interesting to study, especially with genes of known function.

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Appendix 1

Pupal stages

	P1	*P4*	*P7*
Min			
Max			

ZYMO RESEARCH

The Beauty of Science is to Make Things Simple

INSTRUCTION MANUAL

5-mC DNA ELISA Kit

Catalog Nos. **D5325** & **D5326**

Highlights

- For high-throughput, detection of global 5-methylcytosine (5-mC) in DNA.
- The streamlined workflow can be completed in less than 3 hours.

Contents

ZYMO RESEARCH CORP.

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Satisfaction of all Zymo Research products is guaranteed. If you should be dissatisfied with this product please call 1-888-882-9682.

Product Contents

5-mC DNA ELISA Kit (Kit Size)	**D5325** (1 x 96 wells)	**D5326** (2 x 96 wells)	**Storage Temperature**
5-mC Coating Buffer	15 ml	15 ml x 2	4 °C
5-mC ELISA Buffer	250 ml	250 ml x 2	4 °C
Anti-5-Methylcytosine (1 µg/µl)	15 µl	30 µl	-20 °C
Secondary Antibody (1 µg/µl)	15 µl	30 µl	4 °C
HRP Developer	15 ml	15 ml x 2	4 °C
Negative Control (100 ng/µl)	50 µl	50 µl	- 20 °C
Positive Control (100 ng/µl)	50 µl	50 µl	- 20 °C
96-well plate (12 x 8-well Strips)	1 plate	2 plates	Room Temp.
Protocol	1	1	-

Note - Integrity of kit components is guaranteed for up to up to six (6) months from date of purchase. Reagents are routinely tested on a lot-to-lot basis to ensure they provide the highest performance and reliability.

Specifications

Sample Sources – Purified genomic DNA, plasmid DNA, PCR amplification products, or DNA fragments in water, Tris-EDTA, or similar.

DNA Quantity – This protocol is optimized for 100 ng input DNA/well. Compatible with DNA in the range of 10-200 ng.

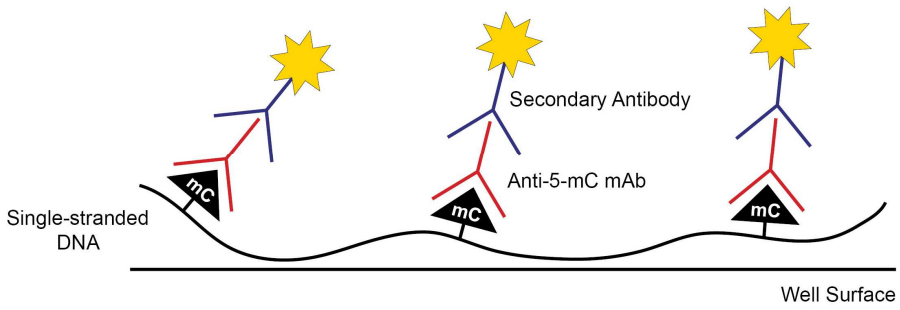
Detection – ≥ 0.5% 5-methylcytosine (5-mC) per 100 ng single-stranded DNA.

Equipment Required – Incubator and ELISA plate reader. A multi-channel pipettor is recommended. An automated plate washer may be used for blocking and wash steps.

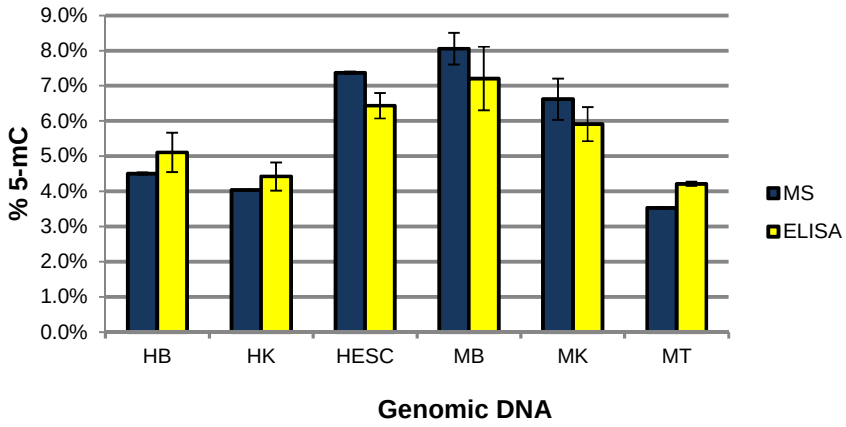
Product Description

The ability to efficiently detect and quantify DNA methylation (i.e., 5-methylcytosine) has become essential for epigenetic-based research. To date, a number of methods have been developed for this purpose including high-performance capillary electrophoresis, bisulfite sequencing, and methylated DNA immunoprecipitation.

The **5-mC DNA ELISA Kit** is a convenient and powerful tool that allows the researcher to accurately quantitate 5-mC in *any* DNA sample in less than 3 hours. The kit features a unique **Anti-5-Methylcytosine** monoclonal antibody that is both sensitive and specific for 5-mC. The assay is compatible with a wide range of input DNA from vertebrate, plant, and microbial sources as well as PCR amplicons and fragmented DNA. Percent 5-mC in a DNA sample can be accurately quantified from a standard curve generated with specially designed controls included with the kit. Also, the fast, streamlined workflow is ideal for high-throughput analyses.



The 5-mC DNA ELISA Kit utilizes the indirect ELISA technique in its workflow. Denatured, single-stranded DNA samples are coated on the well surfaces in **5-mC Coating Buffer**. **Anti-5-Methylcytosine** monoclonal antibody (Anti-5-mC mAb) and the HRP-conjugated **Secondary Antibody** are prepared in **5-mC ELISA Buffer** and added to the wells. Detection of 5-mC occurs after addition of the **HRP Developer**.



The 5-mC DNA ELISA Kit can quantify 5-mC in numerous DNA samples with close correlation to LC-MS/MS-MRM analysis. 100 ng of genomic DNA from human brain (HB), human kidney (HK), human embryonic stem cell (HESC), mouse brain (MB), mouse kidney (MK), and mouse testes (MT). Percent 5-mC was calculated using the second-order regression equation of the standard curve that was contructed with the **Negative Control** and the **Positive Control** (see Appendix, page 5). The percent 5-mC detected in DNA samples by **5-mC DNA ELISA Kit** (ELISA) strongly correlates to mass spectrometry (MS) data of 5-mC found in the respective gDNA sample.

For **Assistance**, please contact Zymo Research Technical Support at 1-888-882-9682 or e-mail tech@zymoresearch.com.

Experimental Considerations

- All DNA *must* be denatured (single-stranded) prior to use with the kit. The protocol is optimized for the detection of 5-mC in 100 ng of single-stranded DNA per well. However, depending on your experimental design, 10 to 200 ng of sample DNA can be used in the assay.
  Note: When using inputs other than 100 ng per well, the amount of control DNA used must be adjusted to equal the amount of sample used. This will ensure accurate % 5-mC quantification.

- The **Negative** and **Positive Controls** consist of double stranded DNA at a concentration of 100 ng/µl, and can be used for the detection/quantification of 5-mC in DNA. For 5-mC detection, both controls should be assayed. For 5-mC quantification, the **Negative Control** should be mixed with the **Positive Control** at different ratios to construct a standard curve (see Appendix, page 5).

- **Secondary Antibody** is a horseradish peroxidase (HRP) conjugate, and supplied at a concentration of 1 µg/µl.

Buffer Storage

- ✓ **5-mC Coating Buffer** is stable at room temperature or 4 °C for extended periods of time.

- ✓ **5-mC ELISA Buffer** should be storage at 4°C and used within 6 months. Alternatively, the buffer may be dispensed into multiple aliquots and kept at -20°C for long term storage. Avoid repeated freeze/thaw cycles.

- ✓ **HRP Developer** must be stored at 4 °C and used within 6 months. Do not freeze. For more rapid color development, bring to room temperature before adding to the wells.

Protocol

This protocol is optimized for 100 ng of DNA per well.
***Duplicate** samples are recommended for accurate 5-mC detection and quantification.*

DNA Coating:

1. Remove the necessary number of well strips[1] to assay DNA samples and controls[2].
2. Add 100 ng of each DNA[3] to a PCR tube and bring the final volume to 100 µl with **5-mC Coating Buffer**.

   Example: If the DNA concentration is 20 ng/µl, add 5 µl of DNA to 95 µl of **5-mC Coating Buffer** for a final volume of 100 µl.
3. Denature the DNA at 98°C for 5 minutes in a thermal cycler. After denaturation, transfer immediately to ice for 10 minutes.
4. Add the denatured DNAs to the wells of the plate, cover with foil, and incubate at 37 °C for 1 hour.

Blocking:

1. Discard the buffer from the wells[4].
2. Wash each well 3 times with 200 µl of **5-mC ELISA Buffer**. *Discard the buffer after each wash.*
3. Add 200 µl of **5-mC ELISA Buffer** to each well. Cover the plate with foil and incubate at 37 °C for 30 minutes.

Antibody Addition:

1. Discard buffer from the wells.
2. Prepare an antibody mix[5] consisting of **Anti-5-Methylcytosine** and **Secondary Antibody** in **5-mC ELISA Buffer** according to the following table:

	Dilution	Volume (µl)	Example (18 wells)
5-mC ELISA Buffer	N/A	(# wells + 2) 100	2,000 µl
Anti-5-Methylcytosine	1:2,000	Buffer Vol. / 2,000	1 µl
Secondary Antibody	1:1,000	Buffer Vol. / 1,000	2 µl

3. Add 100 µl of this antibody mix to each well. Cover the plate with foil and incubate at 37°C for 1 hour.

Color Development:

1. Discard the antibody mix from the wells.
2. Wash each well 3 times with 200 µl of **5-mC ELISA Buffer**.
3. Add 100 µl of **HRP Developer** to each well. Allow color to develop for 10-60 minutes[6] at room temperature.
4. Measure absorbance at 405-450 nm using an ELISA plate reader.

Notes:

[1] The well strips should be stored in a clean, dry, dark place for later use.

[2] For more information regarding 5-mC detection and quantification using the **Negative** and **Positive Controls**, refer to the Appendix, page 5.

[3] Make sure that the volume of the DNA added to the **5-mC Coating Buffer** does not exceed 20% of the final volume.

[4] Tap out any remaining buffer onto a paper towel after emptying a well.

[5] The antibody mix can be prepared during the blocking step and kept on ice until it is needed.

[6] The development time will depend on the temperature of the **HRP Developer** (see p. 3).

Notes:

[1] The **Negative** and **Positive Controls** must be included on the same plate as the DNA samples for each assay.

[2] A new standard curve should be generated for each assay.

[3] The number of standard curve mixtures for 5-mC quantification can vary. In the example given in the table, seven mixtures were prepared. Leftover mixtures can be frozen at or below -20 °C for future use.

Appendix - Analysis with Negative and Positive Control DNAs

For 5-mC Detection:

The presence or absence of 5-mC can be determined by comparing the absorbance of samples to **Negative** (0% methylation) and **Positive** (100% methylation) **Controls**[1].

For 5-mC Quantification:

To quantify the percentage of 5-mC in a DNA sample, a standard curve[2] must be generated. This is done by preparing mixtures[3] of the **Negative Control (100 ng/µl)** and **Positive Control (100 ng/µl)** to generate standards of known 5-mC percentage (see table below). These must be prepared *prior to denaturation* and assayed in parallel with the samples. Add 1 µl (i.e., 100 ng) of each mixture to a PCR tube and bring the final volume to 100 µl with **5-mC Coating Buffer**. Proceed with Coating Step 3 of the protocol (p. 4).

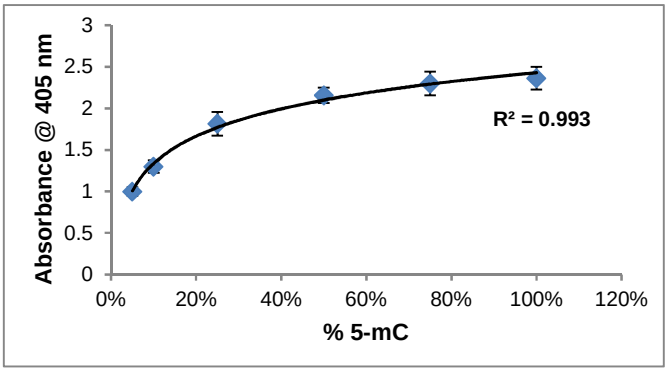
% 5-mC	Negative Control (100 ng/µl)	Positive Control (100 ng/µl)
0%	10.0 µl	0 µl
5%	9.5 µl	0.5 µl
10%	9.0 µl	1.0 µl
25%	7.5 µl	2.5 µl
50%	5.0 µl	5.0 µl
75%	2.5 µl	7.5 µl
100%	0 µl	10.0 µl

Table highlights the preparation of seven mixtures using the **Negative Control** and **Positive Control** to be used to generate a standard curve. Total volume of each is 10 µl at a concentration of 100 ng/µl.

Note: The **Positive** and **Negative Control DNAs** consist of *Escherichia coli* gDNA. The **Positive Control DNA** has been treated with CpG Methylase (Catalog # E2010/11). The density of CpG dinucleotides varies between species and to accurately quantitate the %5-mC simply multiply the calculated %5-mC by the fold difference in CpG density between *E. coli* and the sample species. For example, *E.coli* CpG density/gemone length is 0.075 and mouse CpG density/genome length is 0.0081, therefore, the fold difference between *E. coli* and mouse CpG density is 9.22.

The absorbance for each mixture must be plotted as a function of Absorbance @ 405 nm (Y-axis) vs. % 5-mC (X-axis). Using the equation below, derived from the logarithmic second-order regression, determine the 5-mC percentage for DNA samples (unknowns) based on their absorbance.

$$\% \text{ 5-mC} = e^{\left\{\frac{(\text{Absorbance - y-intercept})}{\text{Slope}}\right\}}$$



Standard curve generated with DNA mixtures. The curve was using the absorbance values of the mixtures indicated in the table above. A logarithmic relationship was observed with a correlation of 0.99.

Ordering Information

Product Description	Catalog No.	Kit Size
5-mC DNA ELISA Kit	D5325 D5326	1 x 96 wells 2 x 96 wells

For Individual Sale	Catalog No.	Amount
5-mC Coating Buffer	D5325-1-15	15 ml
5-mC ELISA Buffer	D5325-2-250	250 ml
Anti-5-Methylcytosine (1 µg/µl)	A3001-15 A3001-30	15 µl 30 µl
Secondary Antibody (1 µg/µl)	D5325-3-15 D5325-3-30	15 µl 30 µl
HRP Developer	D5425-4-15	15 ml
Negative Control (100 ng/µl)	D5325-5-1	50 µl
Positive Control (100 ng/µl)	D5325-5-2	50 µl
96-well ELISA plate (12 x 8-well Strips)	C2020	1 plate

Related Products for 5-mC Analysis:

Additional Products for

Epigenetics Research:

Product Name	Size	Catalog No.
Methylated-DNA IP Kit	10 Rxns.	D5101
***OneStep* qMethyl™ Kit**	1 x 96	D5310
***OneStep* qMethyl™-Lite**	1 x 96	D5311
Zymo*Taq*™ DNA Polymerase	50 Rxns. 200 Rxns.	E2001 E2002
Zymo*Taq*™ PreMix	50 Rxns. 200 Rxns.	E2003 E2004
EZ DNA Methylation™ Kit	50 Rxns. 200 Rxns. 2 x 96 2 x 96	D5001 D5002 D5003 D5004
EZ DNA Methylation-Gold™ Kit	50 Rxns. 200 Rxns. 2 x 96 2 x 96	D5005 D5006 D5007 D5008
EZ DNA Methylation-Direct™ Kit	50 Rxns. 200 Rxns. 2 x 96 2 x 96	D5020 D5021 D5022 D5023
EZ DNA Methylation-Startup™ Kit	50 Rxns.	D5024
EZ Bisulfite DNA Clean-up Kit™	50 Rxns. 200 Rxns. 2 x 96 2 x 96	D5025 D5026 D5027 D5028
Universal Methylated DNA Standard	1 set	D5010
Universal Methylated Human DNA Standard	1 set	D5011
Universal Methylated Mouse DNA Standard	1 set	D5012
Human HCT116 DKO Methylation Standards	1 set	D5014
Human HCT116 DKO Non-methylated DNA Standard	5 µg	D5014-1
Human HCT116 DKO Methylated DNA Standard	5 µg	D5014-2
Bisulfite Converted Universal Methylated Human DNA Standard	1 set	D5015
***E. coli* Non-methylated Genomic DNA**	5 µg	D5016
ChIP DNA Clean & Concentrator™	50 50	D5201 D5205
Anti-5-Methylcytosine Monoclonal Antibody (clone 10G4)	50 µg 200 µg	A3001-50 A3001-200
CpG Methylase (M.SssI)	200 units 400 units	E2010 E2011
5-Methyl dCTP [10 mM]	1 µmol	D1035
5-Methylcytosine dNTP Mix [10 mM]	2.5 µmol	D1030

Product Name	Size	Catalog No.
Quest 5-hmC™ DNA ELISA Kit	1x96	D5425
	2x96	D5426
Anti-5-Hydroxymethylcytosine Polyclonal Antibody	50 µg	A4001-50
	200 µg	A4001-200
Quest 5-hmC™ DNA Enrichment Kit	25 Preps.	D5420
	50 Preps.	D5421
Quest 5-hmC Detection Kit™	25 Preps.	D5410
	50 Preps.	D5411
Quest 5-hmC Detection Kit™-Lite	25 Preps.	D5415
	50 Preps.	D5416
Quest*Taq*™ PreMix	50 Rxns.	E2050
	200 Rxns.	E2051
Human Matched DNA Set	2 x 5 µg	D5018
Mouse ^{5hm}C & ^{5m}C DNA Set	4 x 5 µg	D5019
5-Methylcytosine & 5-Hydroxymethylcytosine DNA Standard Set	3 x 2 µg	D5405
DNA Degradase™	500 units	E2016
	2,000 units	E2017
DNA Degradase Plus™	250 units	E2020
	1,000 units	E2021
5-hmC Glucosyltransferase	100 units	E2026
	200 units	E2027
5-Hydroxymethyl dCTP [100 mM]	10 µmol	D1045
5-Hydroxymethylcytosine dNTP Mix [10 mM]	2.5 µmol	D1040

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