CHAPTER

13 CometChip: Single-Cell Microarray for High-Throughput Detection of DNA Damage

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CHAPTER OUTLINE

Methods in Cell Biology, Vol 112 0091-679X
http://dx.doi.org/10.1016/B978-0-12-405914-6.00013-5

ABSTRACT

DNA damage promotes cancer, and ironically, at high doses DNA damaging agents are also often used to treat cancer. Despite its importance, most assays for DNA damage are very low throughput, as little has been done to introduce engineering principles. Here, we present a novel platform for high throughput analysis of DNA damage in human cells. Based upon the well-established single cell gel electrophoresis assay (a.k.a. the comet assay), the CometChip enables robust, high throughput and objective DNA damage quantification. Single cells are captured by gravity into an agarose microwell array. Arrayed cells can then be directly assayed, or challenged with exposure to DNA damaging agents prior to analysis. The microarray maximizes real estate and normalizes distribution, enabling analysis of 96 samples of widely varying cell concentrations to be processed in parallel. The platform is compatible with both the alkaline conditions (detecting single strand breaks, abasic sites and alkali sensitive sites) and neutral conditions (detecting double strand breaks). Analysis of 96 samples in parallel greatly reduces sample-to-sample variation and can be completed in one day. Through integration of biological and engineering principles, the CometChip provides the necessary throughput and sensitivity for a wide variety of applications in epidemiological studies, in the clinic and in drug development.

1 PURPOSE

DNA damage can lead to mutations and toxicity, which contribute to cancer and premature aging. The "CometChip" is a newly engineered platform for high-throughput analysis of DNA damage in mammalian cells. On the basis of single-cell electrophoresis assay, also known as the comet assay, this assay detects a wide range of DNA lesions, including base damage, abasic sites, single-strand breaks, double-strand breaks and interstrand crosslinks. (Collins, 2004; Olive, 2006; Fortini, 1996; Collins, 2001). The assay is compatible with almost all mammalian cell types and can also be used to analyze the DNA damage levels of cell aggregates. Only ~10,000 cells are required for each condition being tested. The CometChip, therefore, has broad utility as a tool in epidemiology, medicine and drug development.

2 THEORY

The single-cell electrophoresis or "comet" assay is based on the principle that damaged DNA migrates more readily than undamaged DNA when a cell is electrophoresed. DNA is normally highly supercoiled and thus remains compact, even under electrophoresis. DNA damage can lead to strand breaks that release the superhelical tension and/or fragment the DNA. These DNA loops and fragments migrate farther in an agarose gel than undamaged DNA, and can be detected by microscopic examination. (Collins, 2004; Olive, 2006; Ostling, 1984; Singh, 1988).

The two basic approaches are to use an alkaline condition and a neutral condition. The alkali comet assay detects strand breaks, abasic sites and alkali-sensitive sites. On the other hand, when the assay is performed at neutral pH, DNA double-strand breaks can be detected (Collins, 2004; Olive, 2006). The level of DNA damage is proportional to the distance and the amount of DNA that migrates away from the nucleoid during electrophoresis. The comet assay has been used in a variety of applications, including basic research on DNA damage and repair, genotoxicity testing, epidemiology and environmental health. (Collins, 2004; Olive, 2006; Brendler-Schwaab, 2005; Witte, 2007; Valverde, 2009; Moller, 2005; Dusinska, 2008; Dhawan, 2009; McKenna, 2008; Cadet, 2008). It is a straightforward and inexpensive method to directly analyze DNA damage. Wider acceptance of the comet assay has been limited, however, by its low throughput, poor reproducibility, and laborious data processing and analysis procedures.

The CometChip overcomes problems with throughput, bias, and noise by exploiting single-cell patterning (Wood, 2010). Using a microfabricated mold that is pressed into molten agarose, an array of microwells is created, each with a diameter large enough to capture one or a few cells. The resulting spatial encoding increases throughput and consistency. In addition, the format has been adapted to a standard 96-macrowell format (each with hundreds of microwells at its base), which can be integrated into high-throughput screening (HTS) tools, including fully automated imaging and analysis. The CometChip enables high-throughput assessment of DNA damage and repair, thus providing a valuable tool for researchers, clinicians and epidemiologists.

3 EQUIPMENTS

PDMS stamp to create the microwells in molten agarose (provided by the Engelward Lab)
Square petri dish (100 mm × 100 mm × 15 mm)
Bottomless 96-well plate
Tweezers
1.5″ binder clips
Glass plate
4 °C refrigerator
37 °C and 43 °C incubator
Electrophoresis chamber and power supply

4 MATERIALS

Normal-melting-point (NMP) agarose powder
Low-melting-point (LMP) agarose powder
Dulbecco's phosphate buffered saline (PBS) 1×
GelBond® film

Crystalline NaCl
Crystalline Na_2EDTA
Crystalline Tris (base)
Crystalline Tris (acid)
Crystalline N-lauroylsarcosine
Triton X-100
Dimethyl sulfoxide (DMSO)
Crystalline NaOH
Hydrochloric acid
Crystalline boric acid
Distilled H_2O
Fluorescent DNA stain

4.1 Solutions and Buffers—Step 1 Preparing the CometChip

Component	Final concentration	Stock	Amount L^{-1}
1% Normal-Melting-Point Agarose			
NMP Agarose Powder	1%	In powder	10 g
PBS	1×	1×	1 L

4.2 Solutions and Buffers—Step 2 Loading Cells

Component	Final concentration	Stock	Amount L^{-1}
1% Low-Melting-Point Agarose			
LMP Agarose Powder	1%	In powder	10 g
PBS	1×	1×	1 L

4.3 Solutions and Buffers—Step 4 Lysis

Component	Final concentration	Stock	Amount L^{-1}
Alkaline Lysis Stock Solution pH 10			
Crystalline NaCl	2.5 M		146.1 g
Crystalline Na_2EDTA	100 mM		37.22 g
Crystalline Tris (base)	10 mM		1.211 g
Distilled H_2O			~1 L
Neutral Lysis Stock Solution pH 9.5			
Crystalline NaCl	2.5 M		146.1 g
Crystalline Na_2EDTA	100 mM		37.22 g
Crystalline Tris (base)	10 mM		1.211 g

Component	Final concentration	Stock	Amount L^{-1}
Crystalline N-lauroylsarcosine	1%		10 g
Distilled H_2O			~1 L

4.4 Solutions and Buffers—Step 5 Alkaline Comet Assay

Component	Final concentration	Stock	Amount L^{-1}
NaOH Stock Solution			
Crystalline NaOH	5 M		199.5 g
Distilled H_2O			~1 L
Na2EDTA Stock Solution			
Crystalline Na_2EDTA	0.2 M		74.5 g
Distilled H_2O			~1 L
Alkaline Electrophoresis Buffer			
NaOH Stock Solution	0.3 M	5 M	60 mL
Na_2EDTA Stock Solution	1 mM	0.2 M	5 mL
Distilled H_2O			935 mL

4.5 Solutions and Buffers—Step 6 Neutral Comet Assay

Component	Final concentration	Stock	Amount L^{-1}
Neutral Electrophoresis Buffer (TBE) pH 8.5			
Crystalline Na_2EDTA	2 mM		0.744 g
Crystalline Tris (base)	90 mM		10.9 g
Crystalline boric acid	90 mM		5.56 g
Distilled H_2O			~1 L

4.6 Solutions and Buffers—Step 7 Fluorescent Imaging

Component	Final concentration	Stock	Amount L^{-1}
Tris Stock Solution pH 7.5			
Crystalline Tris (acid)	1 M		157.6 g
Distilled H_2O			~1 L
Neutralization Buffer pH 7.5			
Tris Stock Solution	0.4 M	1 M	400 mL
Distilled H_2O			~600 mL

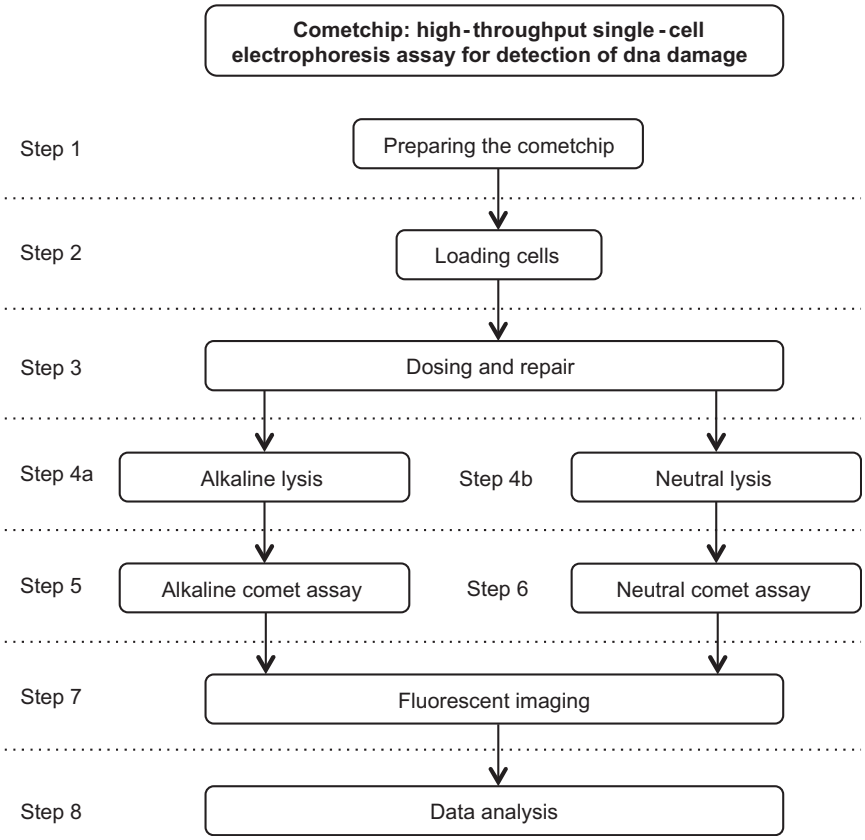

FIGURE 1

Overview of protocol.

5 PROTOCOL

Duration		Time
	Preparation	1–7 days, depending on the source of cells
	Protocol	2–4 days

Preparation	Grow the cells to be studied in the experiment. Approximately 1×10^6 cells are needed for a 96-well plate.

See Fig. 1 for the flowchart of the complete protocol.

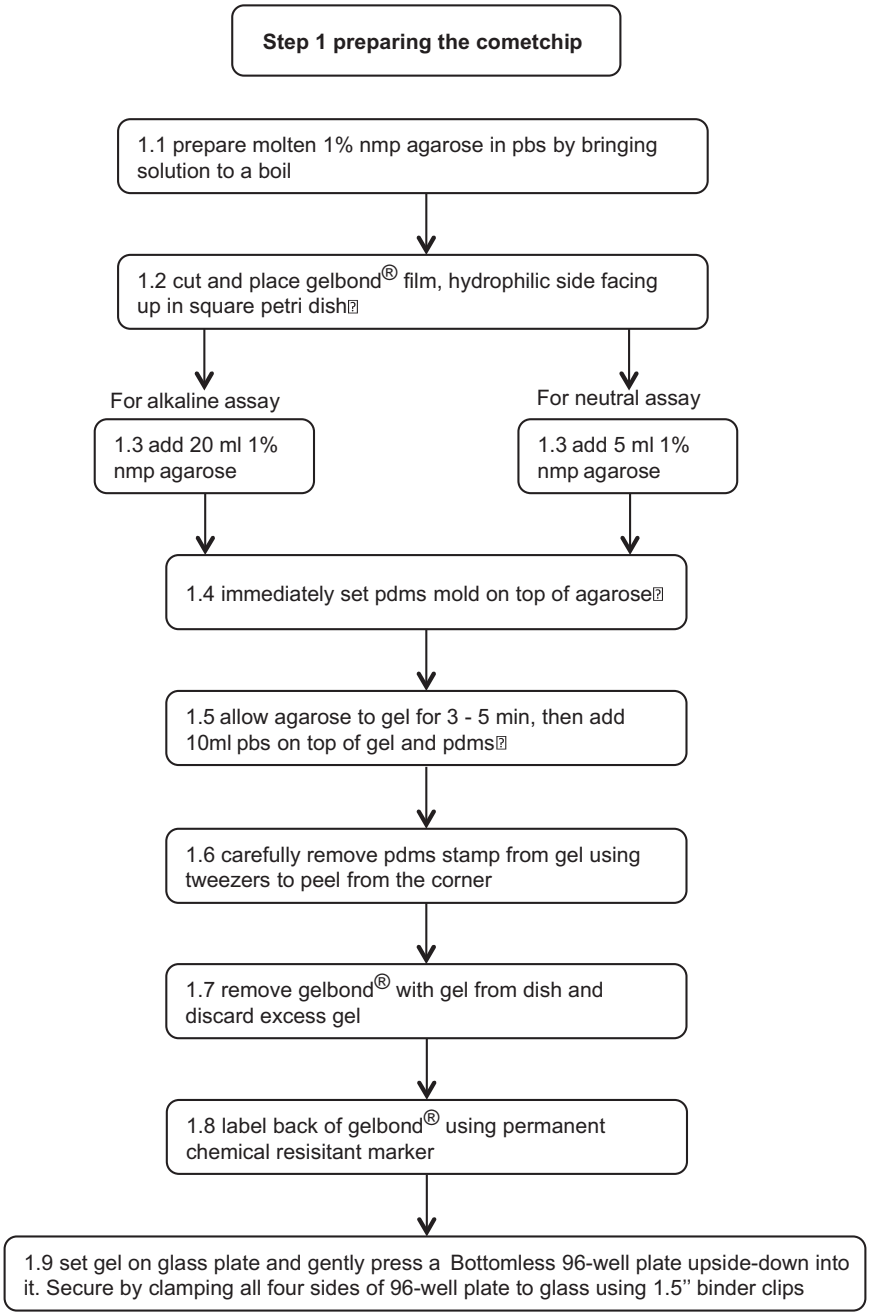

FIGURE 2

Overview of Step 1: preparing the CometChip.

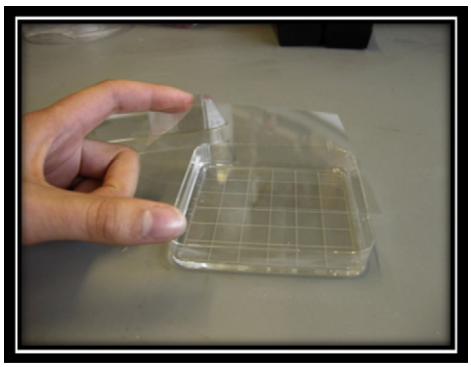

FIGURE 3

Placement of GelBond® film in square petri dish. For color version of this figure, the reader is referred to the online version of this book.

Table 1 Volume of NMP Agarose for Alkaline and Neutral Comet assay

Alkaline	Neutral
20 mL	5 mL

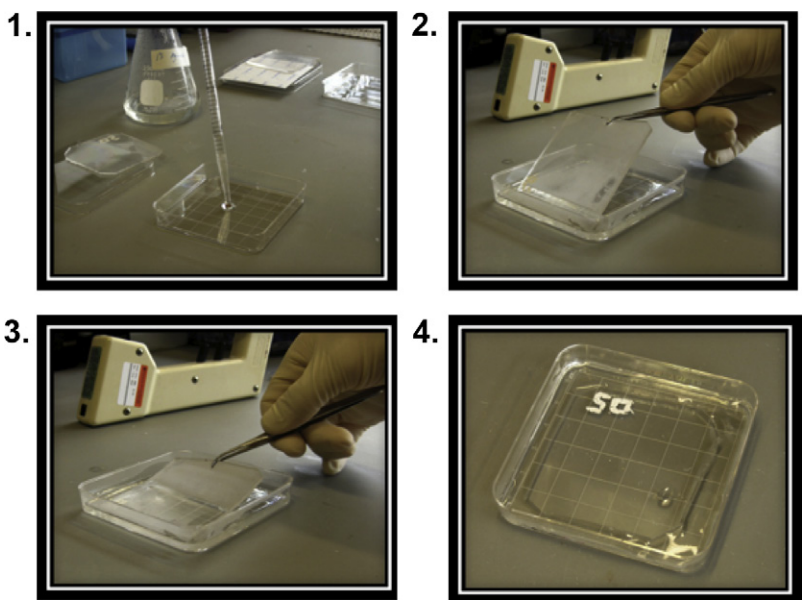

FIGURE 4

Pouring of agarose and setting PDMS mold. For color version of this figure, the reader is referred to the online version of this book.

5.1 Step 1—Preparing the CometChip

Overview	This step prepares the agarose chip with microwells for cells to load in.
Duration	10–20 min.

See Fig. 2 for the flowchart of Step 1.

Procedure

1.1	Prepare molten 1% NMP agarose in PBS by bringing solution to boil.
1.2	Cut and place GelBond film, hydrophilic side facing up in square petri dish (Fig. 3).

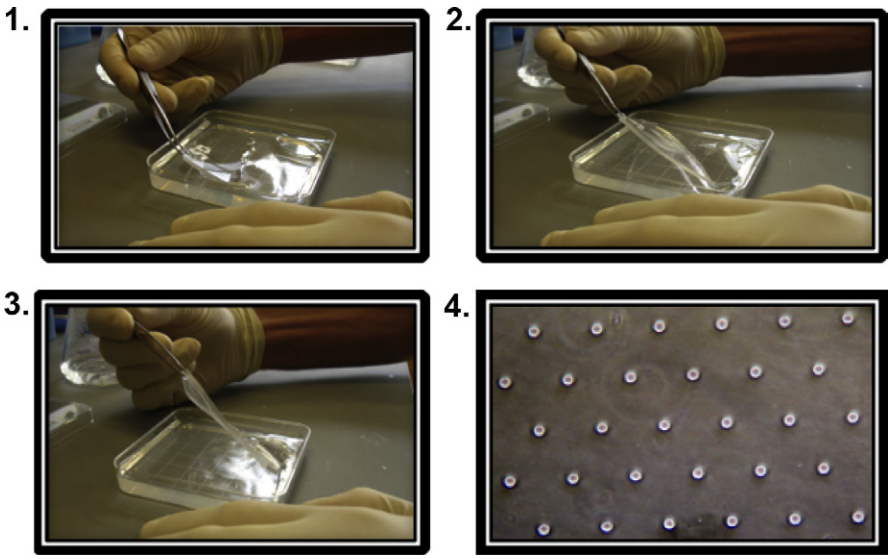

FIGURE 5

Removal of PDMS mold. For color version of this figure, the reader is referred to the online version of this book.

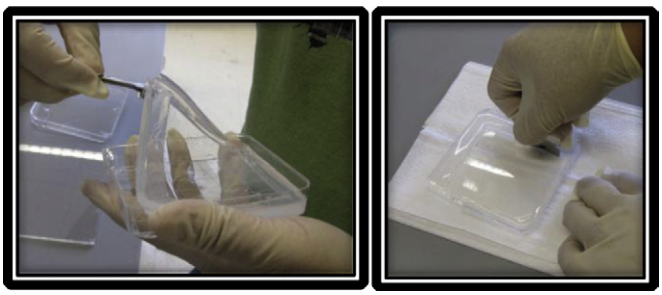

FIGURE 6

Removal of gel. For color version of this figure, the reader is referred to the online version of this book.

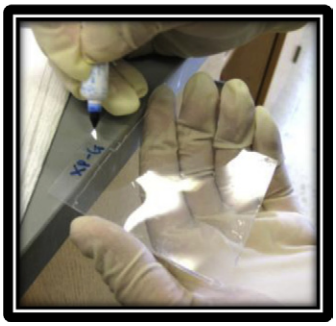

FIGURE 7

Labeling of gel. For color version of this figure, the reader is referred to the online version of this book.

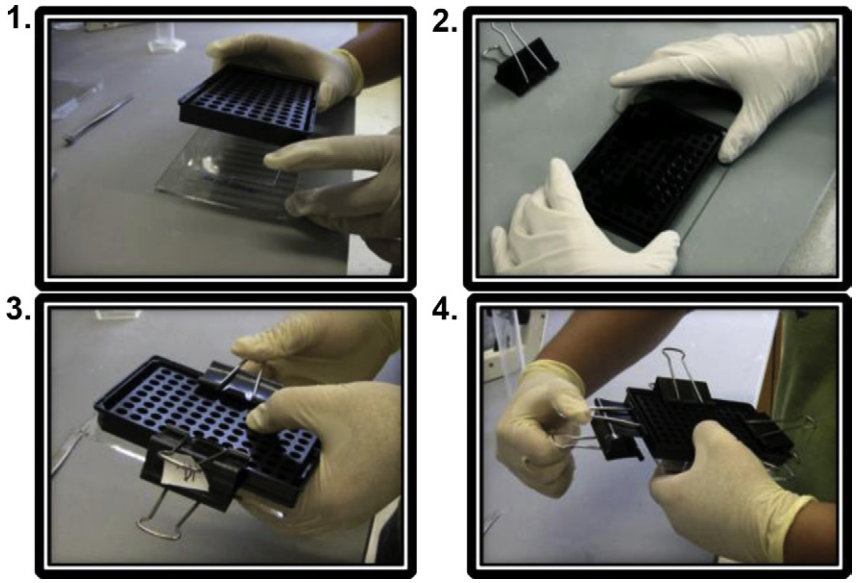

FIGURE 8

Clamping of 96-well plate. For color version of this figure, the reader is referred to the online version of this book.

Tips	(1) Use approximately 2 mL of 1% NMP agarose to seal the GelBond to the dish surface.
	(2) Water will bead on surface of hydrophobic side.
1.3	Add molten 1% NMP Agarose. See Table 1 to determine the amount of NMP agarose.
1.4	Immediately set PDMS mold on top of the agarose (Fig. 4).

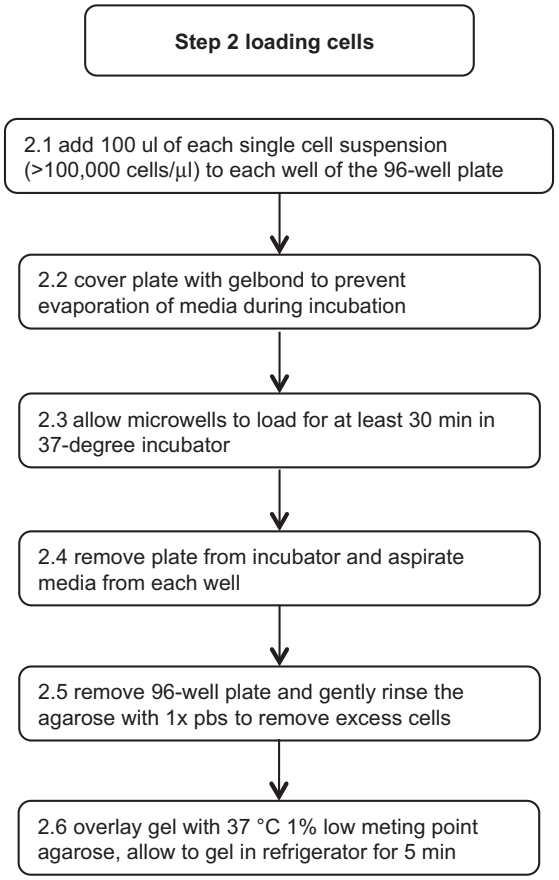

FIGURE 9

Overview of Step 2: loading cells.

1.5	Allow agarose to gel for 3–5 min, and then add 10 mL PBS on top of the gel and PDMS.
1.6	Carefully remove PDMS stamp from the gel using tweezers to peel from the corner (Fig. 5).
Tips	Inspect microwells under bright field microscope for quality control. Remake if microwells do not look similar to those shown in Fig. 3.
1.7	Remove GelBond® with gel from dish and discard excess gel (Fig. 6).
1.8	Label the back of GelBond® using permanent chemical resistant marker (Fig. 7).
1.9	Set the gel on a glass plate and gently press a bottomless 96-well plate upside-down into it. Secure by clamping all four sides of the 96-well plate to the glass using 1.5″ binder clips (Fig. 8).

5.2 Step 2—Loading Cells

Overview	Cells load into the microwells of the CometChip, which is prepared in Step 1, by gravity. Each well of the 96-well plate can be loaded with a different cell type.
Duration	30–60 min.

See Fig. 9 for the flowchart of Step 2.

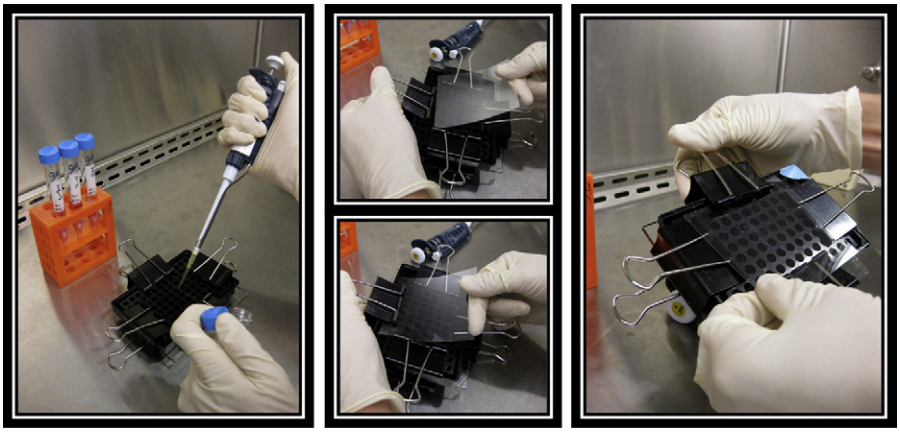

FIGURE 10

Loading of cells in 96-well plate. For color version of this figure, the reader is referred to the online version of this book.

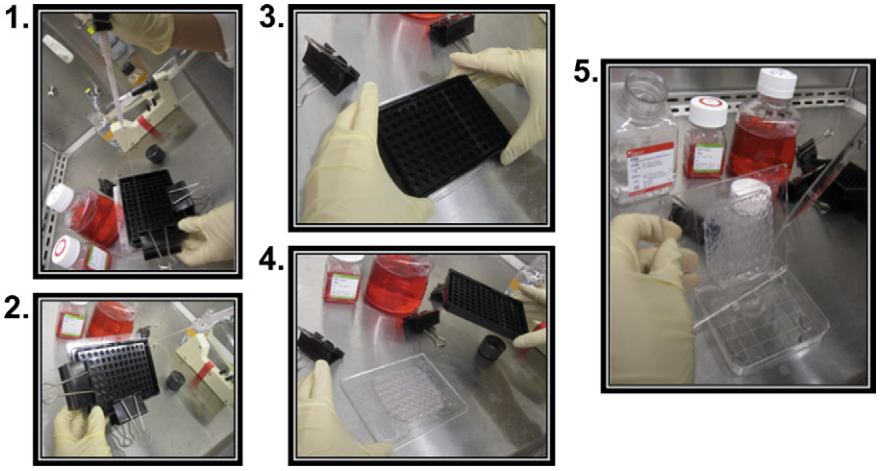

FIGURE 11

Removal of excess cells. For color version of this figure, the reader is referred to the online version of this book.

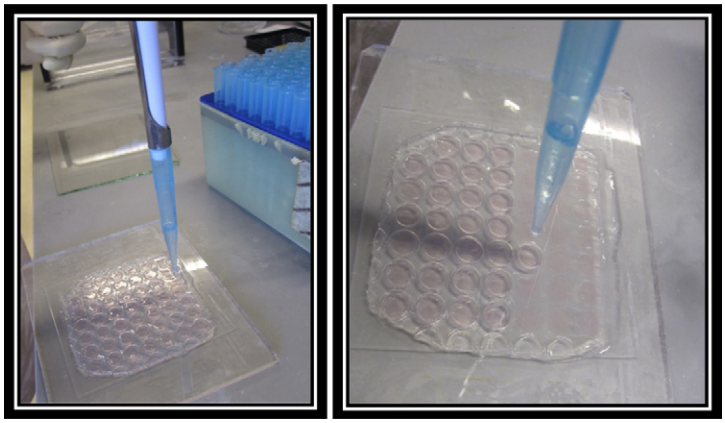

FIGURE 12

Application of LMP agarose overlay. For color version of this figure, the reader is referred to the online version of this book.

Procedure	
2.1	Add 100 μL of each single-cell suspension (>100,000 cells mL^{-1}; as low as 10,000 cells mL^{-1} has been shown to be effective) to each well of the 96-well plate (Fig. 10).
2.2	Cover the plate with GelBond to prevent evaporation of media during incubation (Fig. 10).
2.3	Allow microwells to load for at least 30 min in a 37 °C incubator (Fig. 10).
2.4	Remove the plate from the incubator and aspirate the media from each well (Fig. 11(1,2)).
2.5	Remove 96-well plate (Fig. 11(3,4)) and gently rinse the agarose with PBS to remove excess cells (Fig. 11(5)).
Tips	Look under the microscope to ensure that >70% of wells contain cells. Reload if loading is insufficient.
2.6	Overlay the gel with 37 °C 1% LMP agarose, and allow to gel in refrigerator for 5 min (Fig. 12).
Tips	Place the chip on an even surface and apply approximately one drop of molten LMP agarose per well. Allow to gel at room temperature for 3 min before transporting to refrigerator for further solidification.

5.3 Step 3—Dosing and Repair

Overview	With cells arrayed and encapsulated in the CometChip, experiments can now be performed on chip by reattaching the bottomless 96-well plate onto gel. Each well can be dosed with a different chemical agent of interest, a different dose, or after dosing, placed in media for studies of DNA repair.

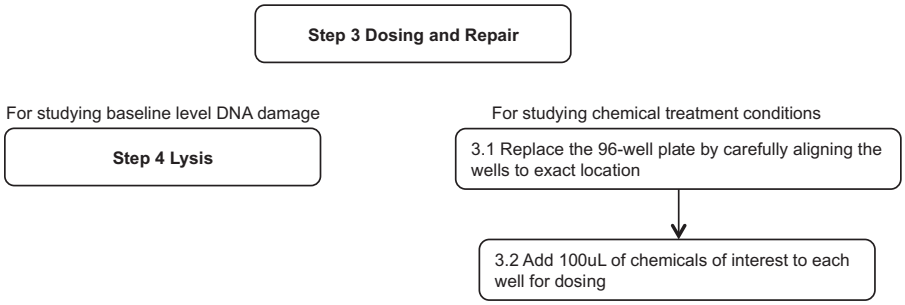

FIGURE 13

Overview of Step 3: dosing and repair.

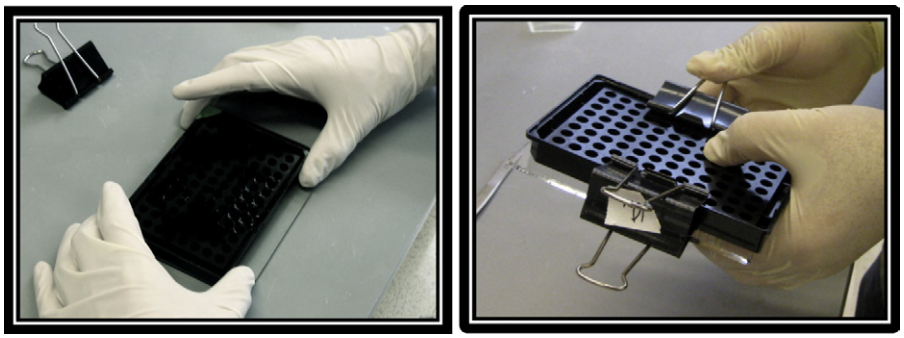

FIGURE 14

Replacement of 96-well plate on chip for dosing and repair. For color version of this figure, the reader is referred to the online version of this book.

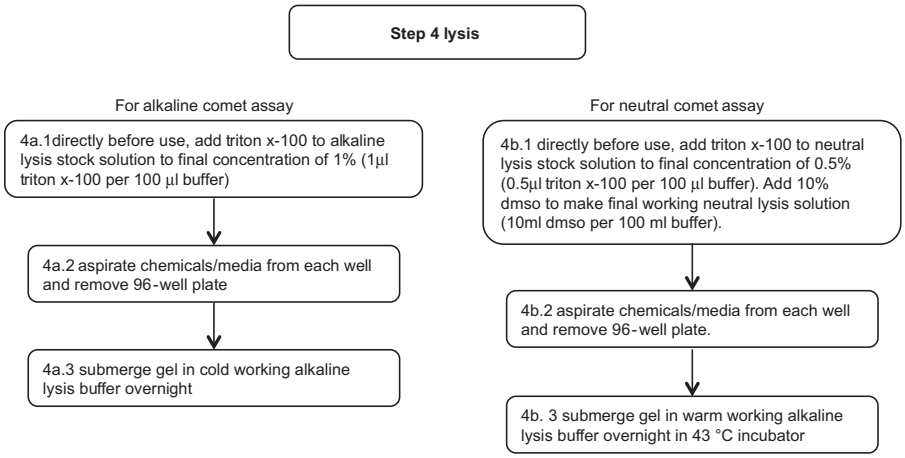

FIGURE 15

Overview of Step 4: lysis.

Duration	Varied by experiment.

See Fig. 13 for the flowchart of Step 3.

Procedure	
3.1	If multiple chemical conditions or repair time points are to be conducted, replace the 96-well plate by carefully aligning the wells to exact location (Fig. 14). If baseline level DNA damage is to be detected, proceed to Step 4.
Tips	(1) Use a marker to circle the backside of the 2–3 wells to help with realignment.
	(2) 96-well plate should slip back into position with minimal force.
3.2	Add 100 μL of chemicals of interest to each well for dosing.

5.4 Step 4—Lysis

Overview	Placing cells in lysis buffer stops all cellular activities, and exposes and prepares the DNA for next step of the assay.
Duration	Overnight.

See Fig. 15 for the flowchart of Step 4.

5.4.1 Step 4A—Alkaline Lysis

Alkaline Lysis Stock Solution Preparation

1)	Dissolve crystalline substances into half of the desired final volume.
2)	Adjust the pH to 10: add NaOH until cloudy solution becomes clearer (~pH 9.8).
3)	Fill with distilled water to the final volume.
4)	Store at 4 °C.
4A.1	Directly before use, add Triton X-100 to the alkaline lysis stock solution to a final concentration of 1% (1 mL Triton X-100 per 100 mL buffer).
4A.2	Aspirate chemicals/media from each well and remove 96-well plate.
4A.3	Submerge the gel in cold working alkaline lysis buffer overnight.

5.4.2 Step 4B—Neutral Lysis

Neutral Lysis Stock Solution Preparation

1)	Dissolve crystalline substances into half of the desired final volume.
2)	Adjust the pH to 9.5.
3)	Fill with distilled water to final volume.
4)	Store at 4 °C.
Tip	*Heat up the stock solution to completely dissolve all components in solution, and then adjust the pH accordingly after the temperature comes down to room temperature.*
4B.1	Directly before use, add Triton X-100 to the neutral lysis stock solution to a final concentration of 0.5% (0.5 mL Triton X-100 per 100 mL buffer). Add 10% DMSO to make final working neutral lysis solution (10 mL DMSO per 100 mL buffer).

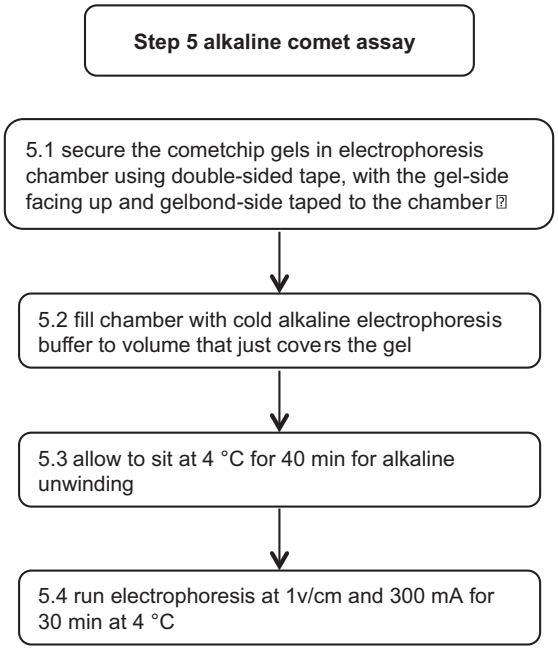

FIGURE 16

Overview of Step 5: alkaline Comet assay.

Tip	*Preheat neutral lysis stock solution in 43 °C incubator before adding Triton and DMSO.*
4B.2	Aspirate chemicals/media from each well and remove the 96-well plate.
4B.3	Submerge the gel in warm working alkaline lysis buffer for an overnight in 43 °C incubator.

5.5 Step 5—Alkaline Comet Assay

Overview	In this version of comet assay, DNA is unwound and electrophoresed under alkaline conditions. Relaxed loops and low-molecular-weight fragments migrate out of the packed chromatin, forming a comet tail.
Duration	1–2 h.

See Fig. 16 for the flowchart of Step 5.

Procedure	
5.1	Secure the CometChip gels in an electrophoresis chamber using double-sided tape, with the gel-side facing up and GelBond-side taped to the chamber (Fig. 17).

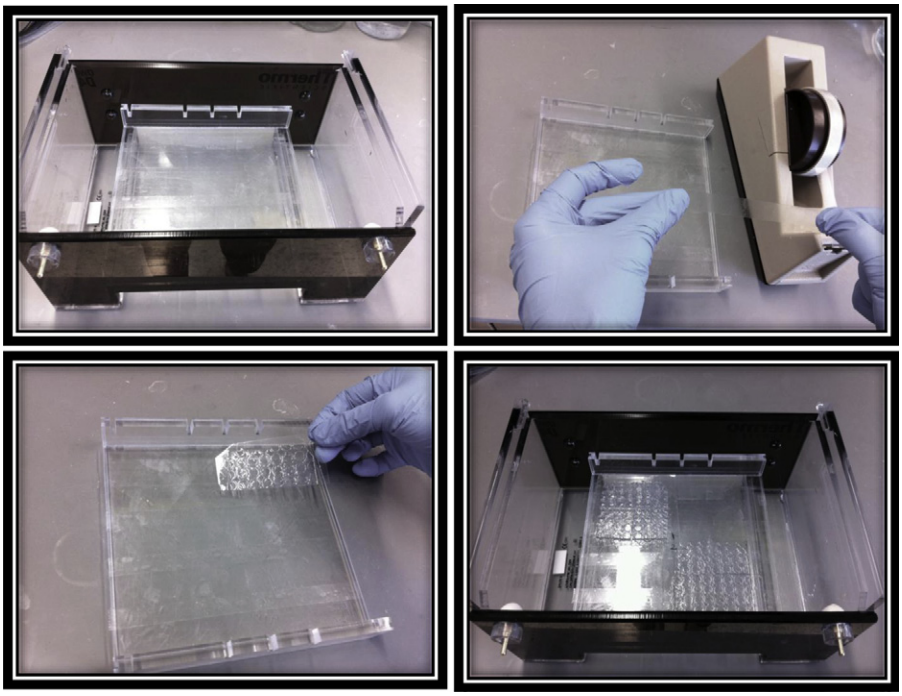

FIGURE 17

Securing of CometChip gels in electrophoresis chamber. For color version of this figure, the reader is referred to the online version of this book.

5.2	Fill the chamber with cold alkaline electrophoresis buffer to a volume that just covers the gel.
5.3	Allow to sit at 4 °C for 40 min for alkaline unwinding.
5.4	Run electrophoresis at 1 V cm^{-1} and 300 mA for 30 min at 4 °C.
Tip	Adjust the level of electrophoresis buffer in the chamber to achieve 300 mA current.

5.6 Step 6—Neutral Comet Assay

Overview	In this version of comet assay, DNA is electrophoresed under neutral conditions to reveal double-strand breaks.
Duration	2–3 h.

See Fig. 18 for the flowchart of Step 6.

Procedure

6.1	Remove the lysis buffer and rinse with neutral electrophoresis buffer (TBE) for 2 × 15 min at 4 °C.

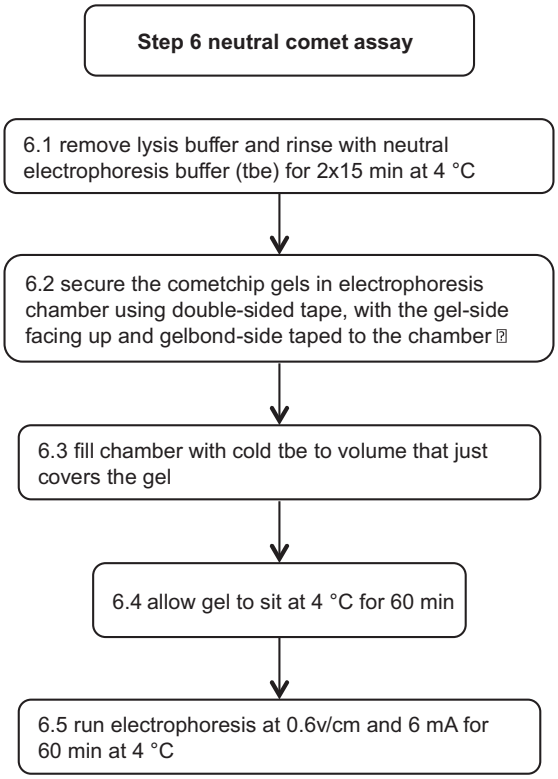

FIGURE 18

Overview of Step 6: neutral Comet assay.

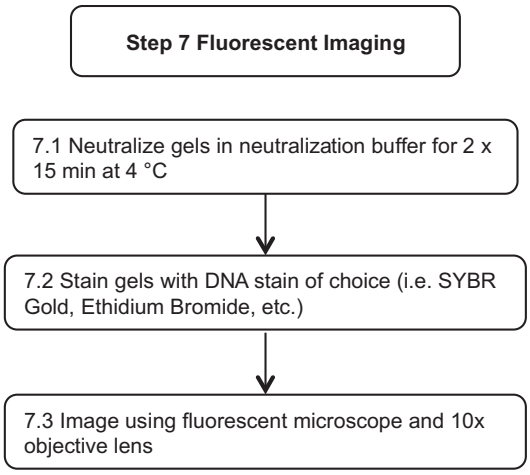

FIGURE 19

Overview of Step 7: fluorescent imaging.

6.2	Secure the CometChip gels in electrophoresis chamber using double-sided tape, with the gel-side facing up and GelBond-side taped to the chamber (Fig. 17).
6.3	Fill the chamber with cold TBE to a volume that just covers the gel.
6.4	Allow the gel to sit at 4 °C for 60 min.
6.5	Run electrophoresis at 0.6 V cm^{-1} and 6 mA for 60 min at 4 °C.
Tip	Adjust the level of electrophoresis buffer in the chamber to achieve 6 mA current.

5.7 Step 7—Fluorescent Imaging

Overview	Electrophoresed DNA is stained with fluorescent dyes and imaged under fluorescent microscope. Comet images are collected and later analyzed to reveal DNA damage level.
Duration	Varied by experiment.

See Fig. 19 for the flowchart of Step 7.

Procedure

7.1	Neutralize the gels in neutralization buffer for 2 × 15 min at 4 °C.
7.2	Stain the gels with DNA stain of choice (i.e. SYBR Gold, Ethidium Bromide, etc.).
7.3	Image using fluorescent microscope and 10× objective lens.

5.8 Step 8—Data Analysis

Overview	Comet images are analyzed by standard software such as Komet 5.5. These software identify beginning and end of comet as well as head/tail divisions and calculates comet parameters (i.e. percentage of head DNA, percentage of tail DNA, Olive Tail Moment, tail length, and total comet length).
Duration	Varied by selection of analysis software.
Tips	Users can select any standard comet analysis program for data analysis. The output images are the same as traditional comets, but in an arrayed format which displays improved real estate.

VIDEO 1

CometChip: Single cell microarray for high throughput detection of DNA damage.

Keywords

Keyword Class	Keyword	Rank	Snippet
Methods List the methods used to carry out this protocol (i.e. for each step).	1 Comet assay	3	
	2 Electrophoresis	4	
	3 Microarray	5	
	4 DNA damage	1	
	5 DNA repair	2	

Keyword Class	Keyword	Rank	Snippet
Process List the biological process(es) addressed in this protocol.	1 DNA damage	1	
	2 DNA strand breaks	2	
	3 DNA damage repair	3	
	4 Base damage	4	
	5		
Organisms List the primary organism used in this protocol. List any other applicable organisms.	1 Human	1	
	2 Mouse	3	
	3 Mammalian	2	
	4		
	5		
Pathways List any signaling, regulatory, or metabolic pathways addressed in this protocol.	1 DNA repair	1	
	2		
	3		
	4		
	5		
Molecule Roles List any cellular or molecular roles addressed in this protocol.	1		
	2		
	3		
	4		
	5		
Molecule Functions List any cellular or molecular functions or activities addressed in this protocol.	1		
	2		
	3		
	4		
	5		
Phenotype List any developmental or functional phenotypes addressed in this protocol (organismal or cellular level).	1		
	2		
	3		
	4		
	5		
Anatomy List any gross anatomical structures, cellular structures, organelles, or macromolecular complexes pertinent to this protocol.	1		
	2		
	3		
	4		
	5		
Diseases List any diseases or disease processes addressed in this protocol.	1 Cancer	1	
	2 Aging	2	
	3		
	4		
	5		

Keyword Class	Keyword	Rank	Snippet
Other List any other miscellaneous keywords that describe this protocol.	1 Base excision repair	1	
	2 Nucleotide excision repair	3	
	3 Nonhomologous end joining	2	
	4		
	5		

ACKNOWLEDGMENTS

This work was primarily support by 5-UO1-ES016045 with partial support from 1-R21-ES019498 and R43-ES021116-01. DMW was supported by the NIEHS Training Grant in Environmental Toxicology T32-ES007020. Equipment was provided by the Center for Environmental Health Sciences P30-ES002109.

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