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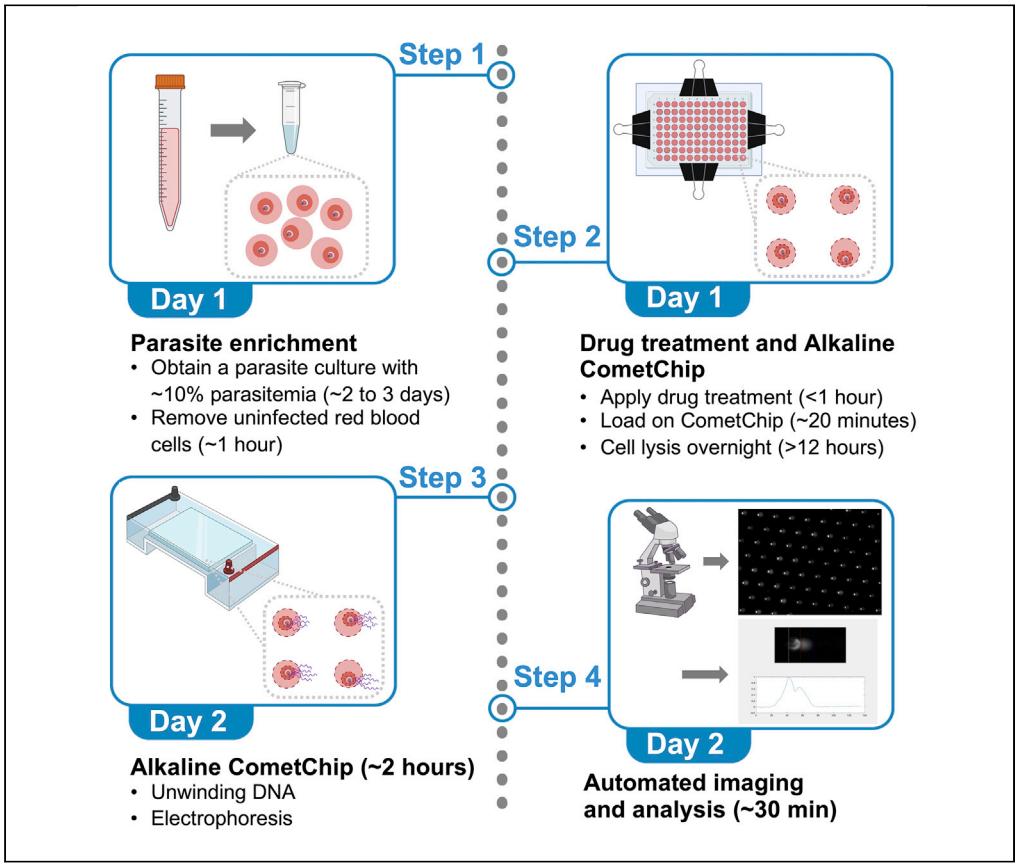
MalariaCometChip for high-throughput quantification of DNA damage in *Plasmodium falciparum*



Comet assay is a standard approach for studying DNA damage in malaria, but high-throughput options are not available. The CometChip was previously developed using mammalian cells as a high-throughput version of the comet assay. It is based on the same principle as the comet assay but provides greater efficacy, automated data processing, and improved consistency between experiments. In this protocol, we present MalariaCometChip to quantitatively assess drug-induced DNA damage in *Plasmodium falciparum*.

Aoli Xiong, Simran Kaushal, Ian Junjie Tay, Bevin P. Engelward, Jongyoon Han, Peter R. Preiser

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Highlights

Concentrating malaria parasites allow quantification of DNA damage despite small genome

High-throughput platform to simultaneously assess DNA damage under different conditions

Automated imaging and analysis allow rapid data processing

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MalariaCometChip for high-throughput quantification of DNA damage in *Plasmodium falciparum*

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SUMMARY

Comet assay is a standard approach for studying DNA damage in malaria, but high-throughput options are not available. The CometChip was previously developed using mammalian cells as a high-throughput version of the comet assay. It is based on the same principle as the comet assay but provides greater efficacy, automated data processing, and improved consistency between experiments. In this protocol, we present MalariaCometChip to quantitatively assess drug-induced DNA damage in *Plasmodium falciparum*.

For complete details on the use and execution of this protocol, please refer to Xiong et al. (2020).

BEFORE YOU BEGIN

Malaria cell culture

Timing: ~1 week

1. Obtain synchronized *P. falciparum* cell culture with high parasitemia (~10%).
   a. Maintain parasite culture at 2% hematocrit in an incubator under these conditions: 37°C, humid atmosphere, 5% CO_2, 3% O_2, and 92% N_2.
   b. Synchronize regularly at early- to mid-ring stage (approximately 0–18 h post infection, h.p.i) using 5% D-sorbitol prepared in materials and equipment.
      i. Spin down the parasite culture at 1000 RCF, 3 min, brake set to 2 and remove supernatant.
      ii. Slowly add 5% D-sorbitol drop by drop while mixing by shaking the tube, with a 4:1 ratio of D-sorbitol volume to parasite pellet (i.e., for a 1 mL pellet add 4 mL 5% D-sorbitol).
      iii. Incubate in water bath at 37°C for 8 min, and shake every minute.
      iv. Add iRPMI to reach final volume of 45 mL.
      v. Spin down at 1000 RCF, 3 min, brake set to 2, and wash it twice with 40 mL iRPMI, remove the supernatant.
   c. Check parasite stage and parasitemia using thin blood smear after synchronization.

i. Use 2.5 μL of the pellet from step b-v and make a thin blood smear, air dry.
ii. Fix the smear in 100% methanol for 5 s, air dry.
iii. Stain the smear using Giemsa for 20 min, rinse and air dry.
iv. Check smear for parasite stage and calculate parasitemia (percentage of parasitized red blood cells to total red blood cells) under 100× light microscope.
v. If the parasitemia is high and parasites haven't been synchronized to the desired time window, adjust the parasitemia to below 1% by adding fresh red blood cells, (i.e., resuspend 0.5 mL parasite pellet with 2% parasitemia and 0.5 mL fresh red blood cells up to 40 mL with cRPMI).
vi. Adjust the hematocrit to 2% by adding additional cRPMI to the flask (i.e., 1 mL pellet with 49 mL cRPMI in the flask).

CometChip fabrication

Timing: 30 min

This step describes the preparation of the MalariaCometChip in detail, with modifications to the previous CometChip protocol (Wood et al., 2010). For demonstration of steps, we encourage users to watch the detailed NextGenProtocols video at: https://nextgen-protocols.org/protocol/nextgen-protocol-on-fabricating-the-cometchip/ and the JoVE video on the CometChip by Ge et al. (Ge et al., 2014).

2. Prepare a CometChip fabrication apparatus.
   a. Obtain a rectangular petri plate with the dimensions of a 96-well plate.
   b. Affix a PDMS stamp to a glass plate that is bigger than uniwell plate dimensions using double-sided tape (i.e., the dimensions 180 mm × 110 mm with 3 mm thick glass work well).
      i. The PDMS stamp can be fabricated using photolithography, as previously described. (Wood et al., 2010)
   c. Place the glass stamp-side-down on the rectangular petri plate; there should be a gap between the stamp and the bottom of the plate.
      i. If necessary, adjust the gap to 2–3 mm by adding tape as spacers on the PDMS stamp side of the glass plate; this will result in consistent thickness of the CometChip between experiments.

3. Fabricate the CometChip.

Note: Premade 1% CometChip gel (consisting of an agarose microwell array on glass) can be purchased from BioTechne. However, it has not been tested for MalariaCometChip, which we have found to be effective at 3% agarose.

   a. Cut a piece GelBond® film to roughly the dimensions 80 mm × 110 mm.
   b. Make a 3% (w/v) normal melting point agarose solution in 1× PBS and heat until fully dissolved (i.e., 6 g agarose powder in 20 mL 1× PBS solution).

Note: Make at least 20 mL normal melting point agarose per chip.

   c. Place 2 mL molten normal melting point agarose on a rectangular petri plate lid and use molten agarose to adhere the GelBond® film facing hydrophilic side up to the lid.

Note: Water will bead on the hydrophobic side of the GelBond® film.

   d. Pour ~13 mL of the 3% molten normal melting point agarose over the hydrophilic side of the GelBond® film.
   e. Immediately, gently overlay the PDMS stamp with an array of micropillars on top of the molten agarose gel.

Note: The PDMS stamp will generate an array of microwells with around 40–50 μm in both diameter and depth in a grid with 240 μm between microwells.

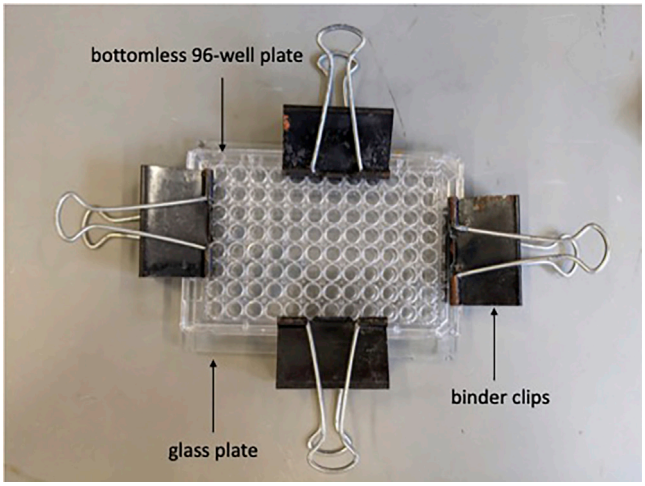

Figure 1. Depiction of the CometChip apparatus

The CometChip apparatus consists of a glass plate onto which a GelBond® film with micropatterned agarose is placed. The agarose gel is then overlaid with a bottomless 96-well plate to create 96 macrowells, each of which contains ~300 microwells. The bottomless 96-well plate is affixed to the glass plate using 1.5" binder clips.

⚠ CRITICAL: Overlay the PDMS stamp down at an angle to avoid any bubbles between the agarose gel and the stamp.

f. Allow the agarose to gelate at room temperature (23°C–26°C) for 15 min.

Pause point: Steps 3-a to 3-f can be performed in advance and the CometChip gel can be stored at 4°C for at least up to a week.

g. Add ~5 mL 1× PBS around the edges of the stamp to facilitate the removal of the stamp.

h. Gently remove the stamp; avoid horizontal movement during removal to prevent collapse of the microwells.

i. Carefully remove the CometChip from the petri plate lid using forceps; use a scalpel or Kim wipe to clean the agarose underneath the CometChip that was used to adhere it to the petri plate lid.

Note: Forceps can be used to manipulate the CometChip during this step and all following steps.

Note: To make the CometChip gel orientation clear, the GelBond® film can be labeled on the hydrophobic side using a permanent chemical resistant marker; alternatively, the corners of the gel can be notched using scissors.

j. Place the CometChip on a glass plate slightly larger than a 96-well plate; the dimensions 180 mm × 110 mm with 3 mm thick glass work well.

k. Inspect the CometChip under a bright field microscope for quality control; make sure that the array of microwells is well formed.

⚠ CRITICAL: If microwells are not in a uniform circular shape, re-pour the CometChip.

l. Gently press a bottomless 96-well plate upside-down onto the CometChip on the glass plate.

Note: This step forms 96 macrowells, each with an array of ~300 microwells.

m. Secure the bottomless 96-well plate onto the CometChip using four 1.5" binder clips, one on each side pressing against the first row of wells (Figure 1).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
RPMI Medium 1640	Gibco	Cat#31800089
Sodium bicarbonate	Sigma-Aldrich	Cat#S5761
Hypoxanthine	Sigma-Aldrich	Cat#H9636
Gentamicin	Gibco	Cat#15750060
AlbuMAX™ II Lipid-Rich BSA	Gibco	Cat#11021037
D-Sorbitol	Sigma-Aldrich	Cat#S6021
Low melting point agarose	ThermoFisher Scientific	Cat#16520050
Normal melting point agarose	Bio-Rad	Cat#1613101
Rectangular petri plates	VWR	Cat#73521-420
Phosphate Buffered Saline	Lonza	Cat#17–516F (38210090)
Sodium chloride	Sigma-Aldrich	Cat#S3014
Disodium EDTA	Sigma-Aldrich	Cat#E5134
Trizma® base (Tris base)	Sigma-Aldrich	Cat#T1503
Triton X-100	Sigma-Aldrich	Cat#T8787
Trizma® hydrochloride	Sigma-Aldrich	Cat#T3523
SYBR™ Gold	Invitrogen™	Cat#S11494
Streptolysin O	Sigma-Aldrich	Cat#S5265-25KU
Dithiothreitol	Sigma-Aldrich	Cat#10197777001
Percoll	Sigma-Aldrich	Cat#P1644
Sodium hydroxide	Sigma-Aldrich	Cat#S5881-500G
Artesunate	Sigma-Aldrich	Cat#A3731
Experimental models: organisms/strains		
Plasmodium falciparum 3D7	MR4	N/A
Software and algorithms		
ImageJ	Schneider et al., 2012	https://imagej.nih.gov/ij/
MATLAB	The MathWorks Inc.	2018a
Guicometanalyzer	Wood et al., 2010	https://github.com/audreyx0206/MalariaCometChip
Other		
PDMS stamp	Wood et al., 2010	N/A
GelBond® film	Lonza	Cat#53748; 110 mm × 205 mm
Bio-One™ 96-Well No Bottom Microplates	Greiner	Cat#07-000-626

MATERIALS AND EQUIPMENT

- Malaria culture stock solutions
  - Complete RPMI (cRPMI, used as culture medium)

Reagents	Final concentration	Amount
RPMI 1640	-	Up to 1 L
Sodium bicarbonate	2.3 g/L	2.3 g
AlbuMAX® II Lipid-Rich BSA	2.5 g/L	2.5 g
Hypoxanthine	0.05 g/L	0.05 g
Gentamicin	10 mg/L	1 mL
Total	**n/a**	**1 L**

Note: Filter sterilize the media and store at 4°C for up to 2 weeks. Warm up to 37°C before use.

  - Incomplete RPMI (iRPMI, used as washing buffer)

Reagents	Final concentration	Amount
RPMI 1640	-	Up to 1 L
Sodium bicarbonate	2.3 g/L	2.3 g
Hypoxanthine	0.05 g/L	0.05 g
Gentamicin	10 mg/L	1 mL
Total	**n/a**	**1 L**

Note: Filter sterilize the solution and store at 4°C for up to 2 weeks. Warm up to 37°C before use.

- 5% D-sorbitol

Reagents	Final concentration	Amount
D-sorbitol powder	5%	25 g
1× PBS	-	500 mL
Total	**n/a**	**500 mL**

Note: Filter sterilize the solution and store at 4°C for up to 1 year. Warm up to 37°C before use.

- Percoll gradient

Reagents	Final concentration	Amount
Percoll solution	63%	63 mL
10× PBS	10%	10 mL
iRPMI	27%	27 mL
Total	**n/a**	**100 mL**

Note: Filter sterilize the solution and store at 4°C for up to 2 months. Warm up to 37°C before use.

- Streptolysin O (SLO) Lysis
  - SLO

Reagents	Final concentration	Amount
SLO	-	25,000 units
1× PBS	-	2.25 mL
Total	**n/a**	**2.25 mL**

Note: Make 20 μL aliquots and store at −80°C for up to 6 months.

- Dithiothreitol (DTT)

Reagents	Final concentration	Amount
DTT	1 M	154 mg
Deionized water	-	1 mL
Total	**n/a**	**1 mL**

Note: Make 50 μL aliquots and store at −20°C for up to 6 months.

Note: Determine the hemolytic unit (HU) of the current batch of SLO as described by Kulzer et al (Kulzer et al., 2015). As SLO loses activity over time, HU may have to be re-assessed every month.

- CometChip stock solutions
  - Alkaline lysis buffer (pH 10)

Reagents	Final concentration	Amount
NaCl	2.5 M	146.1 g
Disodium EDTA	100 mM	37.22
Tris base	10 mM	1.211 g
Sodium hydroxide	-	~1 g
Distilled water	-	Up to 1 L
Total	**n/a**	**1 L**

Note: Adjust to pH 10 using hydrochloric acid. Store at 4°C for up to 6 months.

Note: Sodium hydroxide added is to fully dissolve reagents.

  - Alkaline electrophoresis buffer (0.3 M NaOH and 1 mM disodium EDTA, pH 13.5)

Reagents	Stock concentration	Amount	Final concentration
NaOH	5 M	60 mL	0.3 M
Disodium EDTA	0.2 M	5 mL	1 mM
Distilled water	-	935 mL	-
Total	**-**	**1 L**	**n/a**

Note: Store the electrophoresis buffer at 4°C for up to 1 month.

Note: make a stock of at least 100 mL of 5 M NaOH stock solution and at least 10 mL of 0.2 M disodium EDTA stock solution.

Note: 700–900 mL of alkaline electrophoresis buffer should be sufficient to fill standard gel boxes.

  - Neutralization buffer (0.4 M Tris, pH 7.5)

Reagents	Stock concentration	Amount	Final concentration
Tris	1 M	400 mL	0.4 M
Distilled water	-	600 mL	-
Total	**-**	**1 L**	**n/a**

Note: Store at room temperature (RT) for up to 1 year.

Note: Make a 1 L stock solution of 1 M Tris at pH 7.5.

  - 1% w/v low melting point agarose

Reagents	Final concentration	Amount
Low melting point agarose powder	1%	0.5 g
1× PBS	-	50 mL
Total	**-**	**50 mL**

Note: Store the molten low melting point agarose at 43°C and use within two weeks of preparation.

Note: For each CometChip, ~5 mL low melting point agarose is needed.

Note: Low melting point agarose does not solidify at temperatures above 37°C.

- 3% w/v normal melting point agarose

Reagents	Final concentration	Amount
Normal melting point agarose powder	3%	0.6 g
1× PBS	-	20 mL
Total	-	20 mL

Note: Heat up until fully dissolved. Use the molten agarose immediately after preparation.

STEP-BY-STEP METHOD DETAILS

Concentrate parasites

Timing: 2–3 days

This step describes how to collect highly concentrated parasites at the schizont stage [Part A] or ring stage [Part B] for MalariaCometChip experiments. Depending on which stage of parasites will be used to assess DNA damage, one could choose from either Part A or Part B.

1. [Part A] If the objective is to quantify DNA damage at schizont stage:
   a. After obtaining synchronized ring stage culture with high parasitemia in Step 2 of the before you begin section, maintain the culture until the majority of the parasites reaches mid- to late-schizont stage (roughly after 24 h for *P. falciparum* 3D7).
   b. Obtain purified schizont stage parasites (~90% parasitemia) using Percoll gradient.
      i. Transfer parasite culture from the flask to a 50 mL tube.
      ii. Spin down at 1000 RCF, 3 min, brake set to 2 and remove supernatant.
      iii. Resuspend 1 mL pellet into 4 mL iRPMI.
      iv. Add 10 mL Percoll solution into a 15 mL tube.
      v. Gently lay the parasite suspension onto the Percoll solution by slowly dripping the parasite suspension along the tube wall using a sterile dropper.

   Note: Try to avoid sudden drops across the interface between Percoll and cell suspension.
      vi. Centrifuge at 1000 RCF, 11 min, brake 0.
      vii. Collect the schizont stage parasites, which are concentrated at the interface of the iRPMI and Percoll solutions, by inserting a dropper to the interface and sucking up the schizont pellet.
      viii. Add 20 mL iRPMI to the schizont pellet, resuspend, spin down at 1000 RCF, 3 min, brake set to 2 and remove the supernatant.
      ix. Repeat previous step once.

2. [Part B] If the objective is to quantify DNA damage at ring stage:
   a. Obtain ring stage culture with high parasitemia.
      i. Inoculate purified late schizont stage parasites, ideally segmented schizonts, into fresh blood to obtain a final parasitemia around 3%.
      ii. Culture for 18 h and synchronize the cell culture using 5% D-sorbitol as described above.
      iii. Check blood smear to make sure the parasitemia is near or exceeds 10%.
      iv. Resuspend the pellet in 1 mL iRPMI and transfer to a 1.5 mL tube.
      v. Spin down at 1000 RCF, 3 min, brake set to 2 and remove the supernatant.
      vi. Measure the volume of the pellet using a pipette.
   b. Enrich live ring stage parasites using SLO.

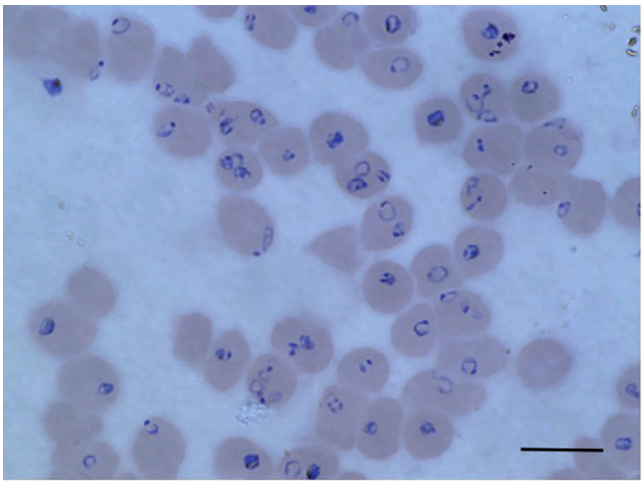

Figure 2. Blood smear of enriched live ring stage parasites after SLO treatment
Scale bar, 10 μm.

Note: As a bacterial toxin, SLO can form pores in cholesterol-containing membranes and lyse cells in a non-osmotic manner. Previous studies found when added in a mixed population of uninfected and infected RBCs, SLO preferentially permeabilizes uninfected RBCs and thus enriches intact ring stage parasitized RBCs. Figure 2 shows the blood smear after SLO enrichment. The steps below are based on studies by Jackson et al. and Kulzer et al.

i. Calculate the volume of SLO needed to lyse the pellet from Step 1c-vi:

$$V_{(activated\ SLO\ needed)} = \frac{V(pellet)}{20\ ul} \times 4\ HUs$$

Note: HU should be determined after the preparation of SLO, and the protocol is described in detail by Kulzer et al (Kulzer et al., 2015).

ii. Activate SLO with 1 M DTT: for every 10 uL of SLO, add 1 uL of 1M DTT; mix gently and incubate at room temperature for 15 min.
iii. Add 4 HUs of activated SLO as calculated above to parasite pellet and gently mix.
iv. Incubate for 6 min at RT and invert the tubes ten times every 2 min during incubation.
v. Spin down at 1000 RCF, 3 min and remove supernatant.
vi. Add 500 μL 1× PBS to the pellet and mix.
vii. Spin down at 1000 RCF, 3 min and remove supernatant.
viii. Repeat step vi-vii until the supernatant turns colorless.
ix. Check blood smear to make sure parasitemia exceeds 90%.

Drug treatment of parasites

Timing: 20 min to 1 h

These steps describe how to treat parasites with agents/drugs on-chip [Part I] or off-chip [Part II]. In [Part I], cells are loaded in the CometChip and then treated with chemical (hydrogen peroxide in this protocol). Alternatively, in [Part II], cells are treated with chemical before being loaded in the CometChip. Figure 3 shows the key steps in [Part I] and [Part II], respectively.

Here we use the alkaline CometChip, which detects single-stranded breaks, abasic sites, and alkali-sensitive moieties. Double strand breaks can be detected using the neutral CometChip as outlined in Ge et al. (Ge et al., 2012).

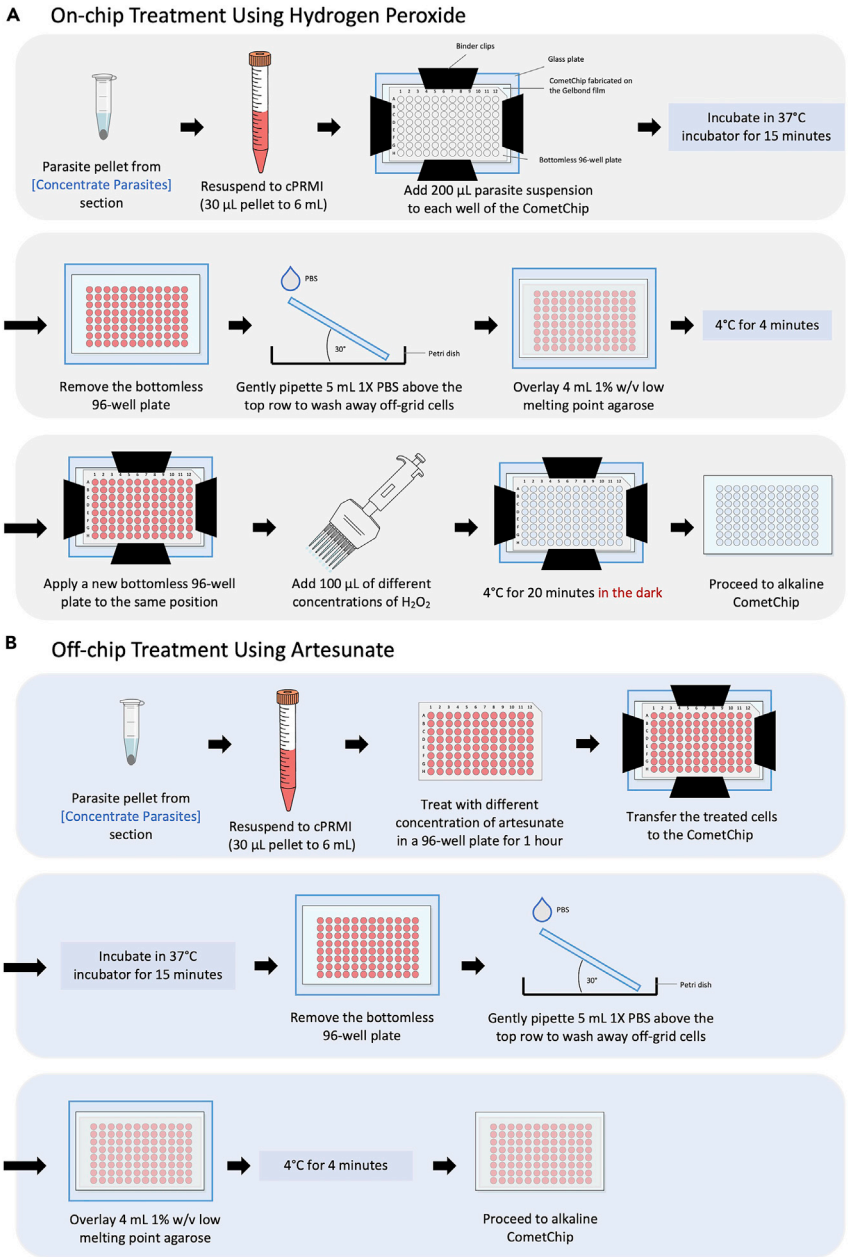

Figure 3. Illustration of key steps for on-chip (A) and off-chip (B) drug treatment

3. [Part I] On-Chip treatment using hydrogen peroxide.

Note: Here we use a dose response to hydrogen peroxide, which causes oxidative damage, to illustrate that the CometChip is working as expected. For data, please refer to Xiong et al. 2020 (Xiong et al., 2020). Hydrogen peroxide was diluted in cold 1× PBS to the following doses: 100, 250, 500, 1000 µM. The negative control was 1× PBS. Each dose of hydrogen peroxide was loaded into 3 macrowells to create 3 technical replicates.

Note: Other chemicals can be used to study different classes of DNA damage.

a. Resuspend 30 μL concentrated parasite pellet from [Concentrate Parasites] section in 6 mL cRPMI to achieve 0.5% hematocrit.

Note: Here, a 2× surplus of parasite suspension is created in case of the need to re-load cells after step 3f.

b. Add 200 μL of parasite suspension into each well of the bottomless 96-well plate clamped to the CometChip gel.
c. Incubate in 37°C incubator supplemented with 5% CO_2, 3% O_2, and 92% N_2 for 15 min to load cells into microwells by gravity.
d. [Optional] If low melting point agarose from Prepare CometChip stock solutions (see "materials and equipment") is being stored at higher temperatures, retrieve it and allow solution to cool to ~43°C.
e. Unclamp binder clips and gently remove the 96-well plate.
f. Place the chip at roughly a 30 degree angle and gently pipette 5 mL 1× PBS above the top row to wash away off-grid cells via shear force.

⚠ CRITICAL: Be careful not to pipette PBS directly on the CometChip wells, as this can wash away cells loaded in microwells. Instead, aim for the area of the CometChip just above the top row of macrowells. Wash by slowly expelling PBS from the pipette in drops - do not wash with too much force, as this can wash away cells loaded in microwells. For a demonstration of this step, we encourage users to watch the detailed JoVE video on the CometChip by Ge et al. (Ge et al., 2014).

g. Check cell loading under a bright field microscope.

Note: When inspecting the loaded CometChip under the bright field microscope, cells loaded in microwells should form a grid pattern; ensure >70% of microwells are loaded before proceeding. If there are a significant number of cells outside the grid, wash once more; if there are too few cells in the wells (e.g., more than 30% of the wells are empty), rinse with 1× PBS and repeat step 3b–3f to re-load cells.

h. Retrieve low melting point agarose gel.

Note: Gel solution should be cooled to ~43°C before proceeding to the next step

i. Overlay the CometChip with 4 mL 1% w/v low melting point agarose by gently dripping the melted agarose onto the chip and keep the CometChip at 4°C for 4 min to allow agarose to solidify.

Note: To ensure the gel is evenly overlaid on the chip, make sure that the chip is completely covered by the low melting point agarose and that the gel is allowed to solidify on a level surface.

j. Apply a bottomless 96-well plate to the same position as in CometChip Fabrication Step 3m by lining up the edges so that the bottomless plate re-forms the same macrowells.
k. Use 1.5" binder clips to clamp bottomless 96-well plate into place on a glass plate.
l. Add 100 μL of different concentrations of hydrogen peroxide to appropriate wells and incubate chip at 4°C for 20 min in the dark.
m. Remove the bottomless 96-well plate and proceed immediately to alkaline lysis (Step 5).

4. [Part II] Off-chip treatment using artesunate.

Note: This is an alternative approach to Part I; here the cells are treated before loading in the CometChip. It uses a completely new set of plates for this purpose.

Note: Artesunate is used in this section. Artesunate is dissolved in 7.5% sodium bicarbonate solution to final concentration of 50 mM (i.e. 19.2 mg in 1 mL 7.5% sodium bicarbonate

solution), and stored in 100 μL aliquots at −20°C for up to 6 months. The aliquots are used one time only (do not re-freeze). The final concentrations of artesunate used in this protocol are: 5, 50, 250, 500, 1000 nM.

a. Prepare 10× solutions by diluting the stock with 7.5% sodium bicarbonate in series, preserve on ice or at 4°C if not used immediately. The same amount of sodium bicarbonate solution is considered as 0 nM control.
   i. Dilute the 50 mM artesunate stock to 100 μM as follows:
      - [50 μL] of 50 mM stock + [450 uL] of sodium bicarbonate = 5 mM artesunate
      - [100 μL] of 5 mM artesunate solution + [900 μL] of sodium bicarbonate = 500 μM artesunate
      - [200 μL] of 500 μM artesunate solution + [800 μL] of sodium bicarbonate = 100 μM artesunate
   ii. Label five 1.5 mL reaction tubes and add the amount of 7.5% sodium bicarbonate solution as shown in the table below.

Final concentration	1000 nM	500 nM	250 nM	50 nM	5 nM
10× concentration	10 μM	5 μM	2.5 μM	500 nM	50 nM
Volume of [artesunate solution]	100 μL of [100 μM artesunate solution]	500 μL of [10 μM artesunate solution]	500 μL of [5 μM artesunate solution]	200 μL of [2.5 μM artesunate solution]	100 μL of [500 nM artesunate solution]
Volume of 7.5% sodium bicarbonate solution	900 μL	500 μL	500 μL	800 μL	900 vL

b. Resuspend 30 μL of the concentrated parasite pellet from [Concentrate Parasites] section in 6 mL cRPMI to achieve 0.5% hematocrit.
c. In a 96-well plate, add 20 μL of 10× artesunate solution (or negative control solution) and 180 μL cell suspension to each well, mix gently and incubate at 37°C for 1 h.

Note: Malaria parasites are usually maintained at low parasitemia (i.e. 1%). High parasitemia may lead to death of the parasites. In this protocol, the parasites are concentrated to extremely high parasitemia (>90%) before drug treatment. Hence, it cannot be maintained for long time. Longer treatment times (>1 hour) are not recommended.

d. After incubation, transfer 180 μL cell suspension from last step (Step 4 [Part II] c) to the CometChip for loading.
e. Perform Steps 3 [Part I] -e to -i.

Alkaline MalariaCometChip

Timing: 2 days

This section describes how to perform alkaline MalariaCometChip and collect data images. The alkaline CometChip steps are similar between MalariaCometChip and the CometChip performed using mammalian cells, we thus encourage users to watch the [Alkaline Comet Assay] section of the detailed JoVE video on the CometChip by Ge et al. (Ge et al., 2014)

5. Alkaline lysis
   a. Directly before use, make working alkaline lysis buffer by adding Triton X-100 to alkaline lysis stock solution at 1% v/v (i.e., 1 mL Triton X-100 per 100 mL alkaline lysis stock solution).

Note: A small dish, such as a pipette tip box lid, can be used for the alkaline lysis incubation; a roughly 100 mL volume of working alkaline lysis buffer is usually sufficient to submerge the CometChip in a pipette tip box lid.

Note: Cellular and nuclear membranes are lysed during this step.

b. Submerge the CometChip from Step 3 or 4 (either Part I or Part II, depending on experiment design) in roughly 100 mL cold alkaline lysis buffer; Make sure the chip is fully submerged in the buffer.
c. Check the CometChip after 5 min of incubation in lysis buffer; if the chip has floated to the top of the chamber, forceps can be used to push the chip back down into buffer so that it is submerged; alternatively, double-sided tape may be used to affix the chip to the bottom of the pipette tip box lid before covering in lysis buffer.
d. Keep at 4°C overnight (usually more than 12 h).

Pause point: Chips can stay in alkaline lysis buffer for up to two consecutive nights so that chips from different days can undergo electrophoresis together.

6. Unwinding and Electrophoresis

Note: Standard electrophoresis chambers (i.e. Owl D-Series Horizontal Gel Systems from ThermoFisher) can be used to run the CometChip gel.

Note: The CometChip is lighter than traditional agarose gels and thus double-sided tape should be used to affix the GelBond® side of the chip to the electrophoresis chamber to avoid floating or any movement of the chip during electrophoresis.

a. Affix double-sided tape to the electrophoresis chamber.
b. Using forceps, remove the CometChip from the lysis buffer in the last step and gently dry the bottom of the GelBond® film using Kim wipes.
c. Transfer the CometChip to the electrophoresis chamber; use forceps to gently press the CometChip gel onto the double-sided tape to ensure it is firmly in place.
d. Add cold electrophoresis buffer to the electrophoresis chamber to a volume that just covers the gel; there should be ~5 mm of buffer above the gel.
e. Unwind the DNA by incubating at 4°C for 40 min.

⚠ CRITICAL: To ensure reproducibility, strictly keep the unwinding time consistent for experiments.

f. Electrophorese at 1 V/cm and ~300 mA in the same buffer at 4°C for 30 min [troubleshooting].

⚠ CRITICAL: Adjust the buffer volume to reach the expected voltage and amperage; it is important to keep the voltage and amperage consistent between experiments.

Note: Electrophoresis buffer can be re-used one time.

7. Neutralization

a. Immediately after electrophoresis, transfer the CometChip to neutralization buffer and gently shake at RT for 15 min.

Note: A small dish, such as a pipette tip box lid, can be used for the neutralization buffer incubation; a roughly 100 mL volume of neutralization buffer is usually sufficient to submerge the CometChip in a pipette tip box lid.

b. Wash the CometChip a second time in neutralization buffer: pour out the neutralization buffer and submerge the CometChip in another 100 mL fresh neutralization buffer, gently shake at RT for 15 min.

Pause point: Chips can stay in neutralization buffer for up to two days. This enables chips from different days to be stained and imaged together, reducing potential inter-sample variability.

8. Stain
   a. Submerge the chip in roughly 50 mL 1× SYBR Gold solution in 1× PBS in the dark using a small dish, such as a pipette tip box lid.

   Note: Other DNA staining solutions, such as ethidium bromide, can also be used.

9. Fluorescence microscopy
   a. Perform fluorescence microscopy at 40× magnification with a 480-nm excitation filter.

   Note: It is optimal to image 100–400 comets per condition (Ge et al., 2015), thus it is customary to load 1 to 3 macrowells per condition.

   Note: The number of wells needed per condition will also depend on loading efficiency; i.e. if 70% of microwells are loaded with cells, then an image of 1 macrowell should generate sufficient data for accurate analysis.

   Note: The number of images needed to capture all of the comets in a macrowell will depend on your specific microscope set up.

EXPECTED OUTCOMES

To test if the protocol is working, it is easiest to start with on-chip treatment using hydrogen peroxide before testing with artesunate or other agents.

Expected outcomes for hydrogen peroxide treatment from direct observation under fluorescence microscopy: while collecting images using fluorescence microscopy, one should clearly observe increased 'comet tail' as the concentration of hydrogen peroxide is increased as shown in Xiong et al. (2020). Quantitative analysis of images by guicometanalyzer algorithm (covered in the next section on quantification and statistical analysis) should show increased % tail DNA as the concentration of hydrogen peroxide is increased.

QUANTIFICATION AND STATISTICAL ANALYSIS

For demonstration of analysis steps, we encourage users to watch the detailed NextGen Protocols at: https://nextgen-protocols.org/protocol/cometchip-data-analysis-using-the-guicometanalyzer/.

1. Import images into Guicometanalyzer (Figure 4A).

   Note: Other comet assay analysis programs can also be used to analyze the CometChip data.

   a. Click the 'Browse' button and choose the file directory where the images are saved.
   b. The list of images in the file folder will appear on the file list at right.
   c. Select the image(s) to be analyzed and click the 'Select' button.
   d. A new window will appear showing the progress of analysis.

2. Obtain results from Guicometanalyzer [troubleshooting].
   a. .txt files containing the results will be saved in the same folder as the images after analysis (Figure 4C).
   b. Calculate the median for %Tail DNA for each well and input to a table, as illustrated by Table 1.
   c. Mean can be calculated for the technical triplicates A, B and C, as depicted in Table 1.
3. Ensure reproducibility of data.
   a. Repeat the experiment at least two times.
   b. Obtain means from the experimental replicates.

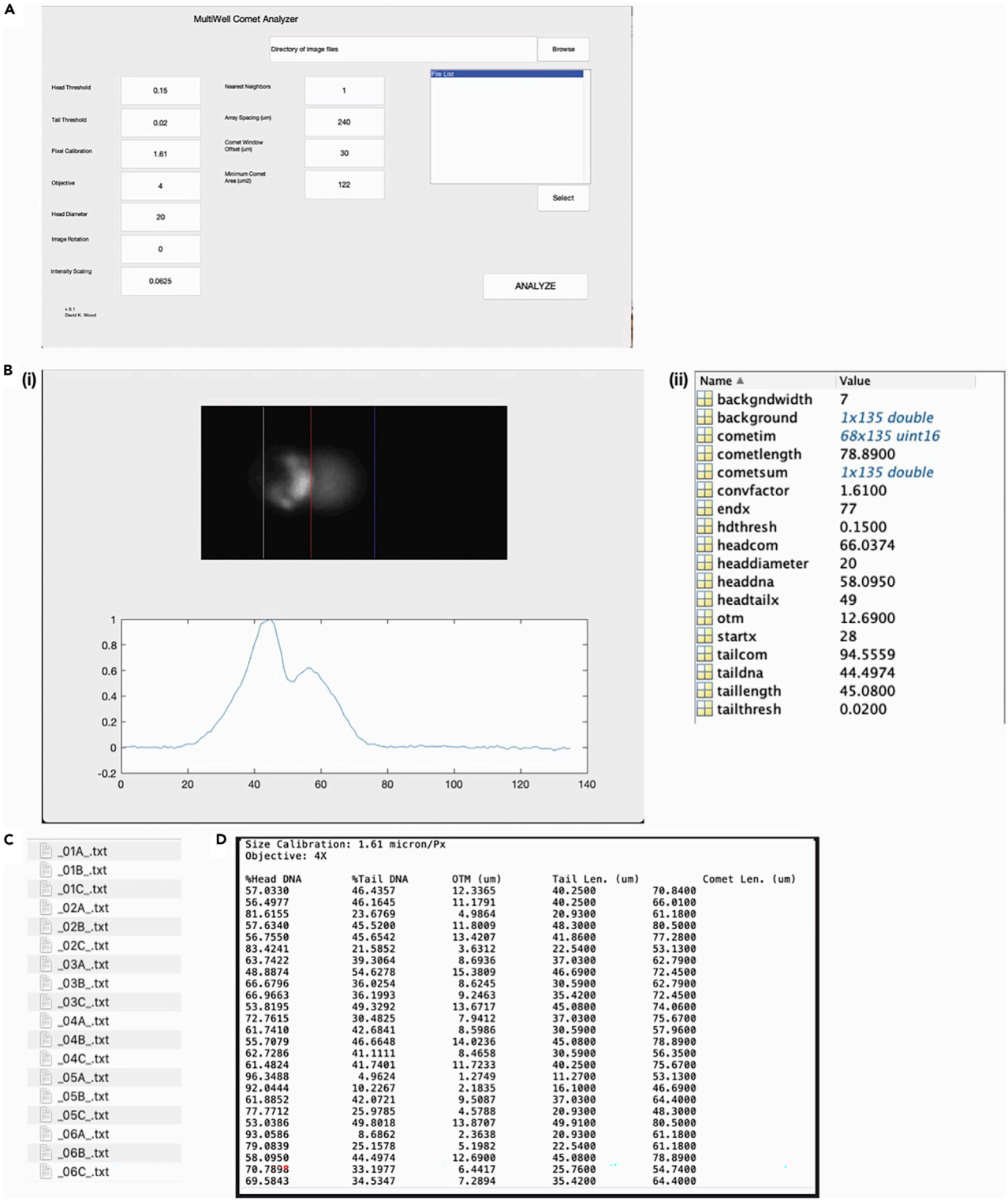

Figure 4. Illustration of image analysis using guicometanalyzer algorithm (Figure S1 from Xiong et al., 2020)

Guicometanalyzer is customized software developed in MATLAB that can be used to analyze images generated from the CometChip experiment. During the analysis, the algorithm can automatically identify comets in the microarray, differentiate the head and tail of each comet, and report values accordingly as shown in the figure.

(A) Dialog window for file selection and parameter settings.

Figure 4. *Continued*
(B) Analysis of one individual comet; (i) the head is defined as the region between the white and red vertical lines and the tail is defined as the region between the red and blue vertical lines. (ii) Parameters reported by the algorithm.
(C) The text file list generated by the algorithm for each macrowell.
(D) Data layout in each text file. For each image file representing one macrowell, a text file containing all the reported value will be generated. Median can then be calculated for each well and exported to an Excel file for further analysis.

c. Mean and standard error of means can be calculated from the experimental triplicates (if repeated three times).
d. Statistical significance can be calculated using one-way or two-way ANOVA, depending on experiment design.

LIMITATIONS

The MalariaCometChip used in Xiong et al. (2020) is suitable for rapidly and quantitatively assessing drug-induced DNA damage in *P. falciparum* parasites. However, since the parasites are highly concentrated and thus not suitable for long incubation periods, only short drug pulses (up to 1 h) can be tested under the current protocol. For drug classes that require longer incubation times to act on DNA, other methods may need to be used.

Note that this protocol is not applicable to freshly isolated parasites from Malaria patients. To perform MalariaCometChip, parasites need to be cultured to high parasitemia (>5%) and concentrated to above 90%. The parasitemia of patients is usually low (e.g., <0.1%), and thus it is hard to concentrate to perform MalariaCometChip.

We also recommend inoculating concentrated parasites in fresh blood to test parasite viability after the concentration protocol. After confirming that the viability is not heavily affected, the MalariaCometChip protocol can be performed.

TROUBLESHOOTING

Problem 1

When the voltage is set to 1 V/cm, the amperage is above 300 mA (alkaline MalariaCometChip step 6f).

Potential solution

Reduce the volume of buffer in the electrophoresis chamber, but ensure that the CometChip is still covered in electrophoresis buffer.

Problem 2

When the voltage is set to 1 V/cm, the amperage is below 300 mA (alkaline MalariaCometChip step 6f).

Potential solution

Increase the volume of buffer in the electrophoresis chamber.

Table 1. Example of median values calculated from raw data processed by guicometanalyzer

Concentration of H_2O_2	0 μM	100 μM	250 μM	500 μM	1000 μM
	01	02	03	04	05
A	11.7431	25.4292	38.4646	71.1999	84.9277
B	17.167	37.4377	40.2895	64.3712	74.1999
C	13.9267	35.1313	48.37405	68.3496	81.4094
Mean	14.27893	32.66607	42.37605	67.97357	80.179

Columns 1–5 represent increasing concentrations of drug treatment. Rows A, B, and C represent technical triplicates.

Problem 3

While trying to keep the voltage and amperage at 1 V/cm and ~300 mA, respectively, the chip is not fully covered by the electrophoresis buffer (alkaline MalariaCometChip step 6f).

Potential solution

This is probably because the electrophoresis chamber is not level. To solve this problem, try to adjust the four feet at the base of the chamber to make it level. After the adjustment, the chip should be fully covered by electrophoresis buffer. It is also recommended to affix the CometChip gel to the bottom of the chamber using double-sided tape.

Problem 4

Comet tail in the control group is long (quantification and statistical analysis step 2).

Potential solution

This can be caused by a few conditions. First, the cells may have been highly concentrated for too long, and the parasites may die due to high parasitemia condition. The time gap between concentration and drug treatment should be minimized to less than 10 min. Second, the electrophoresis chamber may not be level, and thus electrophoresis is not even across the chip. Third, the electrophoresis buffer has been reused too many times. Ideally, electrophoresis buffer can be used twice.

Problem 5

Clear increase in comet tails can be seen on the images but is not recognized by the algorithm in the guicometanalyzer (quantification and statistical analysis step 2).

Potential solution

Adjust the settings for image analysis. To adjust and test the settings, search 'debug' in the algorithm code and follow the instructions in the code to comment out the debugging code. Run the code with one representative result image, and a window as shown in Figure 4B (i) should pop out. Try to adjust the intensity scaling or head diameter in the main window Figure 4A and re-run the code so that proper recognition of head and tail can be achieved as shown in Figure 4B (i). A detailed NextGen Protocol is available to demonstrate this step (https://nextgen-protocols.org/protocol/cometchip-data-analysis-using-the-guicometanalyzer/).

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Peter Preiser (prpreiser@ntu.edu.sg).

Materials availability

This study did not generate new unique reagents.

Data and code availability

The Guicometanalyzer is available at https://github.com/audreyx0206/MalariaCometChip (Wood et al., 2010).

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AUTHOR CONTRIBUTIONS

A.X., S.K., B.P.E., J.H., and P.R.P. conceptualized the study. A.X. and I.J.T. performed the CometChip experiments. A.X., S.K., B.P.E., J.H., and P.R.P. drafted the manuscript, which was reviewed by all authors.

DECLARATION OF INTERESTS

B.P.E. is a co-inventor on a patent for the CometChip.

REFERENCES

Ge, J., Chow, D.N., Fessler, J.L., Weingeist, D.M., Wood, D.K., and Engelward, B.P. (2015). Micropatterned comet assay enables high throughput and sensitive DNA damage quantification. Mutagenesis *30*, 11–19.

Ge, J., Prasongtanakij, S., Wood, D.K., Weingeist, D.M., Fessler, J., Navasummrit, P., Ruchirawat, M., and Engelward, B.P. (2014). CometChip: a high-throughput 96-well platform for measuring DNA damage in microarrayed human cells. J. Vis. Exp. *92*, e50607.

Ge, J., Wood, D.K., Weingeist, D.M., Bhatia, S.N., and Engelward, B.P. (2012). CometChip: Single-cell microarray for high-throughput detection of DNA damage. Methods Cell Biol. *112*, 247–268.

Kulzer, S., Bittl, V., and Przyborski, J.M. (2015). Fractionation of Plasmodium-infected human red blood cells to study protein trafficking. Methods Mol. Biol. *1270*, 71–80.

Schneider, C.A., Rasband, W.S., and Eliceiri, K.W. (2012). NIH Image to ImageJ: 25 years of image analysis. Nature Methods *9*, 671–675. https://doi.org/10.1038/nmeth.2089.

Wood, D.K., Weingeist, D.M., Bhatia, S.N., and Engelward, B.P. (2010). Single cell trapping and DNA damage analysis using microwell arrays. Proc. Natl. Acad. Sci. U S A *107*, 10008–10013.

Xiong, A., Prakash, P., Gao, X., Chew, M., Tay, I.J.J., Woodrow, C.J., Engelward, B.P., Han, J., and Preiser, P.R. (2020). K13-mediated reduced susceptibility to artemisinin in plasmodium falciparum is overlaid on a trait of enhanced DNA damage repair. Cell Rep. *32*, 107996.