

# Microfluidic-assisted analysis of replicating DNA molecules

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**Single molecule-based protocols have been gaining popularity as a way to visualize DNA replication at the global genomic- and locus-specific levels. These protocols take advantage of the ability of many organisms to incorporate nucleoside analogs during DNA replication, together with a method to display stretched DNA on glass for immunostaining and microscopy. We describe here a microfluidic platform that can be used to stretch and to capture labeled DNA molecules for replication analyses. This platform consists of parallel arrays of three-sided, 3- or 4- $\mu\text{m}$  high, variable-width capillary channels fabricated from polydimethylsiloxane by conventional soft lithography, and of silane-modified glass coverslips to reversibly seal the open side of the channels. Capillary tension in these microchannels facilitates DNA loading, stretching and glass coverslip deposition from microliter-scale DNA samples. The simplicity and extensibility of this platform should facilitate DNA replication analyses using small samples from a variety of biological and clinical sources.**

## INTRODUCTION

DNA replication lies at the heart of biology: accurate and complete genome replication ensures genetic continuity and cell viability at each division and species continuity between generations. Replication is well understood in outline<sup>1,2</sup>. Five ‘classical’ DNA polymerases replicate the bulk of genomic DNA and are aided by an additional group of ‘specialized’ polymerases that replicate specific chromosomal regions such as telomeres or synthesize DNA on damaged templates to complete genome replication<sup>3–7</sup>. This central role of replication in biology has made DNA replication a high-value therapeutic target. Drugs that target and disrupt human DNA replication are widely used as cancer chemotherapeutic and immunosuppressive agents. Replication inhibitors that selectively target bacterial or viral replication, such as DNA gyrase inhibitors and nucleoside analog chain terminators, have also gained wide use as antibacterial and antiviral agents<sup>8–10</sup>.

### Assays available to analyze DNA replication

A wide variety of assays have been developed to analyze DNA replication by examining genomic DNA, chromosomes or individual genes. These complementary assays have been used to gain mechanistic insight and to identify genetic, environmental and pharmacological perturbations that alter DNA replication. Replication assays that focus on single replicating DNA molecules have recently received renewed interest<sup>11</sup>. This reflects the ability of these assays to simultaneously reveal both global replication dynamics and altered replication in subsets of replicating molecules.

Current single-molecule replication assays exploit the ability of many organisms to incorporate halogenated pyrimidine nucleoside analogs into replicating DNA. The incorporated bromodeoxyuridine (BrdU), chlorodeoxyuridine (CldU) and iododeoxyuridine (IdU) are then detected by immunostaining DNA. Sequential labeling with pairs of these analogs followed by detection with analog-specific antisera provides a powerful way to reveal replication dynamics<sup>12,13</sup>. These methods, recently reviewed in detail<sup>11</sup>,

represent facile and more readily applicable extensions of earlier <sup>3</sup>H-thymidine incorporation/autoradiography protocols that were used to visualize replicating bacterial and mammalian DNA molecules<sup>14,15</sup>.

Several single-molecule replication protocols have been developed to visualize replication tracks in DNA or in chromatin fibers<sup>13,16–19</sup>. All of these protocols share common labeling and detection protocols, although their solution differs in the problematic step of reproducibly stretching and capturing long DNA fragments for immunostaining and fluorescence microscopy. We recently developed a facile new solution to this problem wherein microfluidic capillary channels are used to stretch and to capture replicating DNA molecules<sup>20</sup>. This protocol adds new functionality to the previously established procedure of microfluidic presentation of genomic DNA molecules for physical mapping of entire plant, animal and microbial genomes—Optical Mapping<sup>21–25</sup>.

### Advantages and disadvantages of our protocol

Our single-molecule replication analysis protocol, termed microfluidic-assisted replication track analysis or ‘maRTA’, provides a simple, inexpensive and extensible platform for single molecule DNA replication analyses. Though rigorous comparison of maRTA with other available single molecule technologies (SMARD, Dynamic combing, DIRVISH) will require side-by-side testing, several suggestions can be made regarding its comparative advantages and disadvantages. maRTA is based on cheap, easily fabricated polydimethyl siloxane (PDMS) micro-capillary channels that require no specialized equipment to use yet achieve orderly deposition of DNA over a fixed grid-like pattern. Thus, subsequent scanning of fibers is easy and automatable. maRTA offers a simple protocol for preparing silanized glass coverslips that are stable and storable; we have not noticed any between-batch differences in the quality of glass generated by this protocol. Our tests show that similar to other single molecule technologies, the maRTA platform

is compatible with fiber FISH. Also of note is that maRTA uses small ( $\mu$ l-scale) sample volumes. These features should allow maRTA to be further developed to permit multiplexed, microfluidic processing and analysis of DNA replication in small biological or clinical samples.

On the other hand, the front-end requirement to manufacture a negative master for making microchannels, and the fact that PDMS requires an additional treatment to be rendered hydrophilic, can be considered disadvantages of maRTA.

### Microfabrication and glass treatment for maRTA experiments

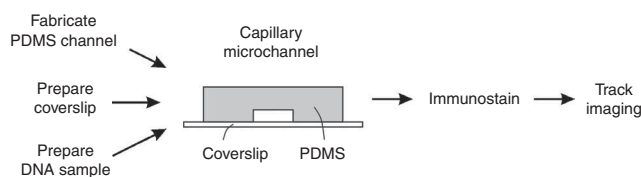
The flow diagram in **Figure 1** depicts the steps needed to set up maRTA. A negative master is fabricated using standard photolithography (**Fig. 2**, Steps 1–10 of the protocol) and it serves as a template for PDMS microchannels, which are cast using a soft lithography procedure (**Fig. 3**, Steps 11–15). Generating a negative master requires access to a clean room with specialized equipment. As microfabrication for biomedical applications becomes more common, our first suggestion for those interested in implementing this protocol is to do a survey of one's colleagues and departments to identify existing facilities and expertise in photolithography.

Fabrication of a master can be also contracted out to a microfabrication foundry. Many of these facilities now exist worldwide and can be found by a simple web search. They will make the SU-8 master and PDMS replicas (see below) for a fee to design specifications.

One master can be used to mold over a hundred PDMS replicas. Replica molding does not require any specialized equipment and can be carried out in a regular lab. However, PDMS microchannels are hydrophobic, as they come off the master and need to be treated with ionized oxygen to render them hydrophilic (Steps 16–18). Hydrophilicity is required for passive capillary filling of PDMS microchannels, which is used in this protocol. Treated microchannels are stable for up to 2 months and they can be used for DNA stretching twice if rinsed and re-treated with oxygen between uses. Oxygen plasma treatment of PDMS microchannels is a second aspect of microfabrication, which is most easily addressed by identifying a local fabrication facility and colleagues. Many researchers have already used such equipment, e.g., Plasma cleaner or UV–ozone cleaner, to generate ultraclean glass surfaces, and this equipment may be used to treat microchannels.

An alternative approach that does not require plasma treatment is to modify the microfluidic platform to include a sample injection port and a feed line that can be used to actively load DNA. For additional background on microfluidics, the properties of micro-scale liquids, and different microfabrication procedures and options including the 'soft lithography' procedure used here, see the following reviews<sup>26–30</sup>.

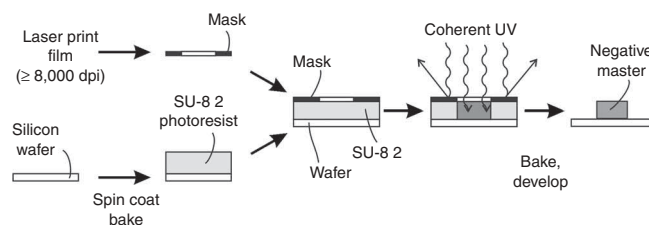
Glass coverslips are used both to seal the open face of PDMS patches to create capillary microchannels and to provide an optical surface for DNA deposition and imaging (**Fig. 4**). These coverslips need to be derivatized with a mixture of two silanes (Steps 19–24). Once silanized, coverslips can be stored for at least 1 month. DNA is stretched by a combination of forces of capillary tension that pulls DNA solution into the channels and of binding to reactive moieties of silanes on glass (Step 26).



**Figure 1** | Overview of a setup for microchannel-assisted replication track analysis (maRTA). PDMS microchannels are fabricated and glass coverslips are silanized before the procedure. High-molecular-weight DNA is isolated out of nucleoside analog-labeled cells. A microchannel patch is reversibly sealed onto a coverslip and a microliter-scale droplet of DNA solution is placed at one opening of microchannels. Capillary action draws DNA solution into microchannels and a combination of flow force and attachment to silanized glass stretches DNA. The PDMS patch is then removed and DNA on a glass can be stained for nucleoside incorporation.

### Experimental design for maRTA experiments

Although the specific design of a maRTA experiment will depend on the question(s) being addressed, it must always include pulse labeling of cells in culture with CldU and IdU or BrdU. Labeling intervals in mammalian cells typically range from 10 to 40 min, or up to 1 h if a replication-slowing drug is used. The optimal labeling duration should be determined experimentally and optimized for the question being addressed and the organism being examined (see **Table 1**). The DNA isolation procedure and experimental outline described below are tailored to mammalian cells. When working with yeast, note that genetically engineered strains are required to enable incorporation of halogenated nucleoside analogs (reviewed in ref. 11). Consecutive labeling with two analogs has not yet been reproducibly successful for yeast, particularly in an unperturbed S-phase. Isolation of chromosome length yeast DNA suitable for maRTA analyses can be carried out as described for pulsed-field gel electrophoresis (see ref. 31). In this case, it is important to incubate cells for 30–60 min after labeling and before harvesting in order to ensure that all chromosomes are fully replicated, and therefore will be able to enter a pulse-field gel. Cell-cycle synchronization is not generally required but can be used to increase the number of S-phase cells before pulse labeling. A sample experimental design to address the effects of DNA damage on DNA replication in human fibroblasts is given in **Box 1**.

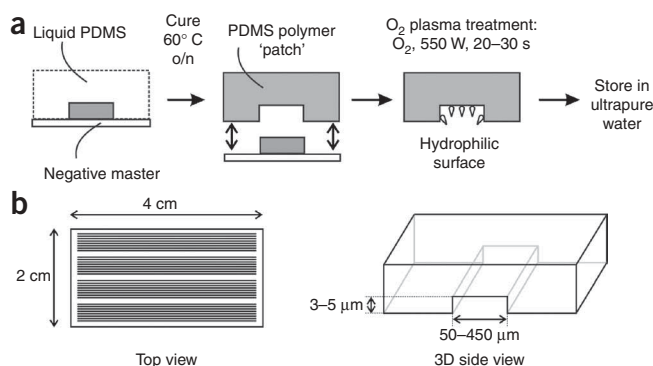


**Figure 2** | Fabrication of a 'negative master' for casting PDMS microchannels using standard photolithography procedures. A pattern for microchannels is printed on a photomask. A layer of SU-8 2 photoresist is spun onto a silicon wafer, and then the photomask is layered on top of SU-8 2 and the sandwich is exposed to UV. SU-8 2 polymerizes and hardens only in areas that are not covered with the photomask. After development, unpolymerized SU-8 2 is washed away, leaving a negative master pattern of raised lines on a wafer.

**MATERIALS**

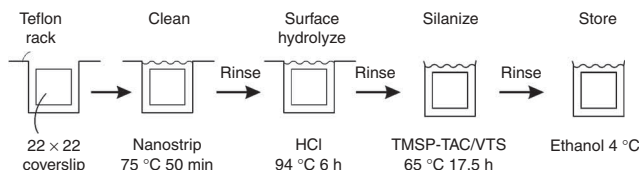
**REAGENTS**

- Silicon wafers (3-inch diameter, P/Boron; Silicon Senses Inc.)
- SU-8 2 photoresist (MicroChem Corporation) used to fabricate a ‘negative master’ mold for casting PDMS microcapillary channels. SU-8 (formulated in  $\gamma$ -butyrolactone) and SU-8 2000 (formulated in cyclopentanone) are epoxy-based, negative photoresists that have high functionality and high optical transparency. Negative photoresists of this type become polymerized and developer-resistant upon exposure to coherent UV light. Standard formulations of these photoresists allow a wide range of film thicknesses to be generated (from  $<1$  to  $>200$   $\mu\text{m}$ ). The thickness of the finished photoresist is determined primarily by the photoresist formula and the spin speed used to coat silicon wafers. SU-8 2 is used in this protocol to give finished feature heights of 3 to 5  $\mu\text{m}$ . SU-8 5, SU-8 2002 and SU-8 2005 can also be used for negative mastering by modifying process parameters (see [http://www.microchem.com/products/su\\_eight.htm](http://www.microchem.com/products/su_eight.htm) for additional details) **▲ CRITICAL** See manufacturer’s data sheets for process guidelines **▲ CRITICAL** SU-8 is highly sensitive to UV light/ambient room light. Store and process in a room equipped with UV filters.
- SU-8 developer (1-methoxy-2-propyl acetate; MicroChem Corporation) used for removing unexposed SU-8 **! CAUTION** 1-methoxy-2-propyl acetate is toxic. Wear protective gear and dispose according to EHS (Environmental Health and Safety) regulations.
- Tridecafluoro-1,1,2,2-tetrahydrooctyl-1-trichlorosilane (TFOCS, United Chemical Technology) used for treating the SU-8 masters to facilitate PDMS replica release **! CAUTION** TFOCS is corrosive and toxic. Wear protective gear and dispose according to EHS regulations.
- Isopropyl alcohol
- Sylgard 184 PDMS polymer components (Dow Corning) **▲ CRITICAL** PDMS consists of two components: a silicone elastomer base and a Sylgard 184 elastomer curing agent. PDMS is a biocompatible transparent elastomeric polymer that can be inexpensively replica molded and is widely used in soft lithographic applications. PDMS surfaces reversibly self-seal against smooth surfaces and can be rendered hydrophilic by a brief exposure to oxygen plasma.
- Nano-Strip solution (Cyantek Corporation, cat. no. 539200) **! CAUTION** Nano-Strip solution is highly caustic. Wear protective gear and dispose according to EHS regulations.
- *N*-trimethoxysilylpropyl-*N,N,N*-trimethylammonium chloride (TCMP-TAC, Gelest, cat. no. SIT8415.0)
- Vinyltrimethoxysilane (VTS, Gelest, cat. no. SIV9220.0)
- CldU (Sigma, cat. no. c6891)
- IdU (Sigma, cat. no. i7125)
- Mimosine (Sigma, cat. no. m0253)
- Methyl methane sulfonate (MMS, Sigma, cat. no. m4016) **! CAUTION** MMS is toxic and mutagenic. Wear gloves and handle in a ventilated hood. Solutions of MMS in water are inactivated over time and can be disposed.
- $\beta$ -Agarase (New England Biolabs, cat. no. m0392S)
- Proteinase K powder (Invitrogen, cat. no. 25530-015)
- YOYO-1 dye (Invitrogen, cat. no. Y3601)
- Rat anti-CldU/BrdU antibody (Serotec, cat. no. MA2060 or Accurate Chemical, cat. no. OBT0030G)
- Normal goat serum (NGS; Accurate Chemical, cat. no. ACL1200-100)
- Bovine serum albumin (BSA; Sigma, cat. no. a3294)
- Goat anti-rat Alexa 594-conjugated antibody (Invitrogen Molecular Dynamics, cat. no. A1100)
- Mouse anti-IdU/BrdU antibody (Becton Dickinson, cat. no. 347580)
- Goat anti-mouse Alexa 488-conjugated antibody (Invitrogen Molecular Dynamics, cat. no. A11001)
- Mouse anti-DNA antibody (anti-deoxythymidine) (Chemicon, cat. no. MAB3034)
- Low gelling temperature (LGT) agarose (any brand, but must be DNase-free)
- Ethylenediaminetetraacetic acid (EDTA), disodium salt (any high-quality supplier can be used for this and the chemicals below)
- Deoxycholic acid, sodium salt
- *N*-Lauroylsarcosine, sodium salt
- NaCl



**Figure 3 |** Fabrication of PDMS capillary microchannels by soft lithography. (a) A liquid mixture of a monomer and a curing agent is poured onto the negative master. Baking at 65 °C speeds up PDMS polymerization. Once polymerized, a PDMS patch is lifted off the master. Oxygen plasma treatment is used to render it hydrophilic. (b) Size parameters for PDMS patches that were commonly used. One patch contains several clusters of microchannels.

- Tris base
  - Tween 20 non-ionic detergent
  - Phosphate-buffered saline (PBS)
  - HCl, concentrated
  - Nitric acid (optional, see Step 19).
  - ‘Hard-as-Nails’ clear nail polish (Sally Hansen, cat. no. 2103, from your local drugstore)
  - Methanol
  - Acetic acid, glacial
- EQUIPMENT**
- Design software to generate a negative master (e.g., CorelDraw, Corel Corporation or AutoCAD, Autodesk Inc.)
  - Photoresist spinner (Headway Research Inc., cat. no. PWM32)
  - UV mask aligner (Quintel Model 2001, 3 inch, Neutronix-Quintel)
  - Two hot plates (preferably programmable)
  - Surface profilometer (KLA-Tencor P-15, KLA-Tencor)
  - Fume hood
  - Balance
  - Rocking platform with variable tilt and rotation speed controls
  - Two desiccators (one for PDMS only and one for TFOCS only) **! CAUTION** Owing to the toxicity and corrosiveness of TFOCS, a desiccator for TFOCS use only should be set up in a fume hood for silicon wafer silanization.
  - Plasma processing reactor center (Plasma Preen II-973, Plasmatic Systems Inc.), also referred to as a ‘plasma etcher’, a vacuum chamber, in which oxygen radicals are generated **▲ CRITICAL** We use a microwave-based plasma etcher (Plasma Preen II) to increase the hydrophilicity of PDMS surfaces. Other types of plasma etcher can also be used.
  - A small oven that can maintain 65 °C setting to cure PDMS
  - A water bath that can maintain 65 °C for silanization of coverslips



**Figure 4 |** Derivatizing glass coverslips for DNA capture, immunostaining and microscopy. Glass surface is cleaned and enriched for hydroxyl groups either by sequential incubation with Nano-Strip and HCl, as shown, or by incubation with freshly mixed  $\text{HNO}_3$  and HCl. After extensive rinsing in water and then in ethanol, glass is incubated in a silane-water solution and then rinsed again and stored in ethanol.

**TABLE 1** | Quality controls

What to do	What it checks	What to expect
Pulse-labeling conditions: when labeling replicating cells add the second label for different times, e.g., for 10 and 20 min	Whether track lengths of ongoing forks accurately reflect the speed of elongation and are not limited by replicon boundaries Whether stretched DNA length is at least replicon-sized	For ongoing forks, the rate of growth of second label segments should be similar when derived using length measurements for two different periods of time
Add the 1st and 2nd labels consecutively for the same periods of time	Whether lengths of tracks laid by ongoing forks are not limited by replicon boundaries Whether stretched DNA is sheared to lengths no smaller than replicon size Whether replication is not under stress	Under normal conditions, the ratio of lengths of labeled segments in ongoing forks should be distributed around 1–1.5
Reverse the order in which CldU and IdU are added	Whether antibody staining is influencing the results	Results should not depend on the order of label addition
Stain a sample of DNA with anti-total DNA antibody and determine DNA fragment lengths	Whether DNA is too sheared	The lengths of molecules should be longer than the lengths of replication tracks
Collect two subsets of data: viewing the fields through a filter to one fluorescent label, then to another, e.g., first in green then in red	Whether scanning process is biasing data collection	Calculated fractions of track types may depend on the way the images are collected

- A sand bath or a heating block with removable rack units. The receptacle of a heating block can be filled with a dry, heat conducting medium such as sand to accommodate polypropylene containers (see below)
- Agarose gel insert ('plug') molds (Bio-Rad, cat. no. 1703706)
- Glass coverslips (Finest, Fisher, 12-548-B)
- Glass microscope slides (any brand)
- Teflon racks (Invitrogen, cat. no. C14784)
- Kimwipes
- Parafilm
- Polypropylene containers (All-Pak, cat. no. PLC03696)
- Fluorescent microscope (we use a Zeiss Axiovert confocal microscope equipped with  $\times 40$ ,  $\times 63$  or  $\times 100$  objectives, filter sets to red and green fluorescence, and a digital camera; note that the ability to do confocal imaging is not required for this protocol)

**REAGENT SETUP**

**CldU** Prepare a 10-mM solution in water. Store at  $-20\text{ }^{\circ}\text{C}$  in the dark for up to several months.

**IdU** Prepare a 2-mM solution in water. Store at  $-20\text{ }^{\circ}\text{C}$  in the dark for up to several months. Both CldU and IdU can also be prepared as 50 mM solutions in dimethyl sulfoxide and stored as above.

**Mimosine** Prepare a 10-mM solution in growth medium (typically Dulbecco modified Eagle's medium). Filter-sterilize and store at  $+4\text{ }^{\circ}\text{C}$  for up to several months.

**LGT agarose** Prepare a 2% (w/v) solution in water. Store at room temperature (defined here and elsewhere as  $20\text{ }^{\circ}\text{C}$ ) for up to several months. Reheat as needed **▲ CRITICAL** Any brand of LGT agarose can be used as long as it is DNase-free, i.e., certified for recovery of DNA fragments.

**Sodium deoxycholate** Prepare a 10% (w/v) solution in water. Store at room temperature, indefinitely.

**Sodium lauryl sarcosine** Prepare a 10% (w/v) solution in water. Store at room temperature, indefinitely.

**Agarose insert buffer** Prepare a 10 mM Tris 7.5, 20 mM NaCl, 50 mM EDTA solution in water. Store at room temperature, indefinitely.

**Lysis buffer** Prepare a fresh 100 mM EDTA pH 8.0, 0.2% (wt/vol) sodium deoxycholate, 1% (wt/vol) sodium lauryl sarcosine, 1 mg ml<sup>-1</sup> proteinase K solution in water, out of stock solutions (see above) and dry proteinase K powder.

**Block buffer** Prepare a 5% (wt/vol) BSA, 0.5% (vol/vol) Tween 20 solution in PBS. Filter-sterilize and store at  $+4\text{ }^{\circ}\text{C}$  for up to several months.

**Wash buffer** Prepare a 1% (wt/vol) BSA, 0.1% (vol/vol) Tween 20 solution in PBS by diluting the Block buffer (1:5) with PBS. Store at  $+4\text{ }^{\circ}\text{C}$  for up to several months.

**TE buffer** Prepare a 10 mM Tris, 1 mM EDTA solution in water. Adjust pH to 8.0 with HCl. Sterilize and store at room temperature indefinitely.

**BOX 1** | **SAMPLE EXPERIMENTAL DESIGN**

A sample experimental design is outlined below to address the effects of MMS on DNA replication elongation.

1. Plate SV40-transformed human fibroblasts at a density of 200,000 cells per 60-mm plate.
2. The next day, incubate cells for 12 h with 0.5 mM mimosine to arrest in late G1.
3. Release from mimosine by changing media, incubate for 8–10 h. Cells are now in the mid-S-phase.
4. Pulse-label with 50  $\mu\text{M}$  of 1st label (IdU or CldU) for 20 min.
5. Wash away the 1st label, add the 2nd label (CldU or IdU) at 50  $\mu\text{M}$  for 20 min (untreated control) or add 0.015–0.02% MMS for 20 min. Wash away MMS and add the 2nd label for 20 or 40 min.
6. Harvest cells or perform an optional step: wash away the 2nd label and incubate untreated and MMS-treated cells without label for additional 30–60 min. This step allows replication forks to move away from labeled tracks. Junctions between replicated and unreplicated DNA can be more susceptible to breakage during DNA isolation and stretching.
7. Harvest cells as described in Preparation of high-molecular-weight DNA.

**Anticipated results:** Inhibition of replication elongation by MMS will cause the tracks labeled by the 2nd label to become shorter than in untreated controls. In particular, in ongoing forks, the 1st label to 2nd label segment ratios will be higher after MMS treatment (**Fig. 6d**).





PROCEDURE

Design of the photomask for the SU-8-negative master ● **TIMING variable**

1| Generate a pattern for a negative master using design software. Send the resulting file to a commercial printing service (e.g., CAD/Art services) to print the pattern at high ( $\geq 8,000$  dpi) resolution on a transparency photomask. Our pattern has clusters of 35-mm long lines ranging from 50- to 450- $\mu\text{m}$  wide and spaced 450  $\mu\text{m}$  apart.

Fabrication of the SU-8-negative master ● **TIMING 4–5 h**

2| Clean silicon wafers by immersing new wafers in Nano-Strip for 20 min, followed by a de-ionized water rinse. Dehydrate the surface by baking at 200 °C for 10 min on a contact hot plate or for 30 min in an oven.

**! CAUTION** Nano-Strip is highly caustic.

**▲ CRITICAL STEP** Perform Steps 1–8 in a dust-free ‘clean room’. A dust-free environment is critical because dust particles can be larger than the desired microstructure feature size and will interfere with photoresist coating. To obtain maximum process reliability and to promote photoresist adhesion, silicon wafers should be clean and dry before applying the SU-8 photoresist. Use new wafers only.

3| Coat wafers with photoresist by spin coating clean silicon wafers on a photoresist spinner. This ensures that the SU-8 photoresist will be of uniform thickness. Use the manufacturer’s guide for setting up coating spins (for instructions, see [http://www.microchem.com/products/su\\_eight.htm](http://www.microchem.com/products/su_eight.htm)). For example, for the SU-8 2 photoresist, a ‘spread’ spin speed at 500 rpm for 10 s (set acceleration ramp rate to 100 rpm  $\text{s}^{-1}$  and hold for 5 s) will allow to cover the wafer and spread from the center. A second spin at 1,000–2,000 rpm for 30 s (holding time) is then used to generate the desired finish height. Acceleration and deceleration rates for this second spin should be set to 500 and 1,000 rpm  $\text{s}^{-1}$ , respectively. Quickly place and center the wafer on a photoresist spinner vacuum holder. Turn on the vacuum, dispense  $\sim 1$  ml photoresist (SU-8 2) onto the center of the wafer and start the ‘spread’ spin. Finish with a second spin to generate a uniform, thin coating.

4| Preset two hot plates at 65 and 95 °C, respectively. Transfer the coated wafer to the 65 °C hot plate and bake for 1 min and then immediately transfer the wafer to the 95 °C hot plate and bake for an additional 3 min.

**▲ CRITICAL STEP** After coating, the photoresist must be soft baked to evaporate the solvent and solidify the film. Two steps of contact hot plate baking are typically used for thin ( $< 100\text{-}\mu\text{m}$  thick) SU-8 photoresists.

5| Place the SU-8-coated wafer onto the vacuum chuck of a UV aligner and bring the photomask (emulsion side facing the wafer) into contact with the photoresist. Use the exposure dose recommendations from MicroChem on the basis of photoresist film thickness and UV power output. A radiation of 200  $\text{mJ cm}^{-2}$  is used in this protocol to overexpose the photoresist film. This is done to ensure that the negative master pattern we are using, with 35-mm long lines ranging from 50- to 450- $\mu\text{m}$  wide, adheres to the wafer substrate and is not damaged or lost during post-baking and developing.

**▲ CRITICAL STEP** SU-8 is optimized to polymerize and harden when exposed to near-UV (350–400 nm) light. Excessive UV doses at wavelengths below 350 nm may lead to overpolymerization of the top portion of the photoresist, resulting in exaggerated negative sidewall profiles or ‘T-topping’ upon development. When using a broad spectral output source, filter out excessive energy below 350 nm to ensure the straightest channel sidewalls. Underexposure, in contrast, often results in catastrophic adhesion failure and excessive cracking. Thus, if feature size is not absolutely critical, overexposure is preferred.

6| Place the wafer onto a 65 °C hot plate for 1 min and then onto a 95 °C hot plate for 1 min. Afterward, ramp down the 95 °C hot plate temperature to 25 °C (or the preferred room temperature) at 4 °C per min for better adhesion.

**▲ CRITICAL STEP** After UV polymerization, a second bake must be performed to selectively crosslink the exposed portions of the film.

7| Develop the negative master by immersing the wafer in the SU-8 developer for 5 min. Avoid strong agitation. Although the recommended time is 1 min, the actual developing time depends on pattern features, agitation speed, temperature, etc. The cross-linked SU-8 is very stable in the developer, thus prolonged immersion causes little harm to the SU-8 mold. We use a 5-min immersion with no agitation to ensure development and to minimize the possibility of wafer adhesion failure.

**? TROUBLESHOOTING**

8| After development, rinse the wafer briefly with isopropyl alcohol and then air dry. When completely dry, a raised SU-8 master pattern of long lines should be visible on the wafer surface. All other unexposed area of the wafer should be completely free of photoresist and uniformly shiny (no streaks or shadows). Examine the patterns under a microscope.

**▲ CRITICAL STEP** A 5-min development time should be adequate to remove all unhardened photoresist. If a white film forms on the wafer after isopropyl alcohol rinsing, return the wafer to the developer (Step 7) for an additional 5 min or until fully developed.

9| Measure the height of the SU-8 patterns using a surface profilometer. This step is recommended though not obligatory because, according to the MicroChem guidelines, using the aforementioned spin speed will produce a negative master with the



## PROTOCOL

desired features, e.g., 2- to 5- $\mu\text{m}$  tall. We are using a master with 3- to 4- $\mu\text{m}$  tall features (i.e., it will cast microchannels that are 3- to 4- $\mu\text{m}$  deep). Deeper microchannels (e.g., 10  $\mu\text{m}$ ) lead to higher flow rates and inefficient DNA deposition.

■ **PAUSE POINT** Store the finished SU-8 master mold in a dust-free environment for up to several years.

**10|** Surface silanize by vapor coating with TFOCS in a vacuum chamber. A convenient way to do this is to attach a desiccator jar (containing a small piece of absorbent paper towel in place of the drying pellets) to a vacuum source. Then add a drop of TFOCS to the paper and evacuate air from the chamber. Apply vacuum for 1 min, close off the vacuum line and allow 30 min for deposition.

▲ **CRITICAL STEP** To prevent bonding of oxidized silicon to PDMS and to facilitate release, the SU-8 master needs to be silanized before PDMS replica molding.

! **CAUTION** The desiccator chamber must be located inside a chemical fume hood because of the toxicity and corrosiveness of TFOCS vapor.

■ **PAUSE POINT** The negative master for PDMS molding is complete at this point. A single silanized master can be used for many cycles (>100) of PDMS replica molding. Store the master for up to several years in a closed container to protect it from dust.

### Replica molding of PDMS devices from the SU-8 master ● **TIMING** $\geq 4$ h

**11|** Weigh PDMS prepolymer out in a weigh boat. Next, add curing agent at a 10:1 polymer/curing agent weight ratio. An approximately 20–30 g mixture is needed to cover the surface of each 3-inch silicon wafer. Mix the PDMS prepolymer and the curing agent thoroughly to ensure uniform cross-linking. Thorough mixing can be accomplished by stirring the PDMS mixture with a wooden tongue depressor for 5 min.

**12|** Use a vacuum chamber (a desiccator without drying pellets) to de-gas the PDMS mixture for 10–15 min until bubbles clear.

**13|** Slowly pour PDMS onto the SU-8 master (from Step 10) placed in a weigh boat or a P-100 Petri dish to a height of approximately 2–3 mm. A weigh boat is advantageous for preventing wafer breakage if the wafer needs to be released or transferred from the container at the end of molding. De-gas again for 10–15 min or until bubbles clear.

**14|** After de-gassing is complete, place PDMS into a 60–65 °C oven (> 2 h) for curing.

**15|** Remove the cured PDMS-covered master from the oven. Using a clean razor blade or a sharp scalpel, cut a 5 cm  $\times$  3 cm rectangle through the PDMS around the line pattern and then carefully peel the PDMS center piece off. A replica pattern of lines should be visible on the PDMS patch surface. The harvested patch then needs to be cleanly trimmed so that the pattern runs end-to-end and the channels are open. These PDMS microchannel patches form three of the four walls of the microcapillary channels that will be used for DNA stretching. PDMS replica molding is now complete. Additional replicas can be made from the same master by repeating Steps 11–14. Cured PDMS outside the pattern region can be left on wafer; thus, for subsequent use liquid PDMS mixture needs only to be poured into the center region.

▲ **CRITICAL STEP** When cutting out a PDMS patch, avoid cutting across the lines of a negative master, as this will destroy the master.

■ **PAUSE POINT** Keep cured PDMS patches and the SU-8 master in a dust-free environment between casting.

### Surface treatment of PDMS microchannels ● **TIMING** 30 min

**16|** If dust particles are visible on the PDMS microchannel (from Step 15) surface, clean it with a piece of Scotch tape. Gently apply the tape to the surface of the PDMS patch and lift to remove dust particles.

▲ **CRITICAL STEP** A clean, dust-free PDMS surface is critical for good sealing to glass substrates.

**17|** Place the PDMS microchannels inside a Plasma Preen II oxygen plasma etcher with the microchannel side up. Set the power to 550 W, oxygen pressure at 30 psi and flow rate 3–5 SCFH (standard cubic feet per hour). Treat for 20–30 s. Optimal system parameters such as oxygen pressure, flow rate, plasma power and treatment time should be established empirically using the test described in Step 18. Generally, the settings should be the lowest that produce the desired effect, e.g., a completely hydrophilic PDMS surface.

▲ **CRITICAL STEP** To facilitate DNA stretching, hydrophobic PDMS microchannels need to be rendered hydrophilic using oxygen plasma.

**18|** Test PDMS surface. A simple test of PDMS surface treatment efficacy is to see how well the treated surface can be wetted by water. After oxygen plasma treatment, place the PDMS microchannels in a Petri dish channel side up and leaning against the edge of the dish. Drop  $\sim 0.5$  ml de-ionized water onto the PDMS surface. Water should easily wet the whole surface, leaving a uniform film if the plasma treatment was successful. After the test, immediately immerse PDMS devices in de-ionized water channel side down. If left dry, PDMS surfaces will revert to a hydrophobic state within 15 min and will not be useable.

■ **PAUSE POINT** Treated PDMS microchannel patches can be stored submerged in sterile de-ionized water at 4 °C without losing surface properties for up to 2 months. The PDMS surface can be re-treated with oxygen plasma if it is not sufficiently hydrophilic or is inadvertently dried.

**Preparation of silanized glass coverslips** ● **TIMING 24 h**

19| Clean and surface hydrolyze coverslips using either option A (Nano-Strip and hydrochloric acid) or option B (nitric and hydrochloric acid).

**(A) Nano-Strip and hydrochloric acid method**

(i) Place coverslips in Teflon racks in a polypropylene container and immerse in the Nano-Strip for 50 min at 75 °C in a sand bath to maintain a uniform temperature.

! **CAUTION** Nano-Strip is a toxic and caustic mixture of sulfuric acid and hydrogen peroxide. Wear protective gear and handle in a fume hood; dispose of properly.

(ii) Remove Nano-Strip and rinse coverslips 4–5 times with ultrapure water.

(iii) Add concentrated hydrochloric acid to coverslips in a polypropylene container and incubate at 95 °C for 6 h in a sand bath to maintain a uniform temperature.

**(B) Nitric and hydrochloric acid method**

(i) Incubate coverslips for 8 h to overnight in a freshly made 2:1 (v/v) mixture of nitric and hydrochloric acid.

! **CAUTION** This alternative reagent is extremely corrosive and emits chlorine gas for several hours upon mixing. Incubation should be carried out only in a well-ventilated fume hood. Dispose by neutralizing with baking soda.

20| Remove surface treatment and rinse coverslips 7–8 times with ultrapure water.

21| Wash coverslips in pure ethanol twice, then air-dry in clean, dust-free air.

22| Make up a solution of 12.4 μl of *N*-trimethoxysilylpropyl-*N,N,N*-trimethylammonium chloride and 6 μl of vinyltrimethoxysilane in 50 mL of sterile, de-ionized water. Shake the solution vigorously by hand or on a shaker for 5 min to start silane hydrolysis. Vortex can be used as long as the mixture is moved briskly.

▲ **CRITICAL STEP** The solution must be made fresh immediately prior to use and thorough mixing is important to initiate silane hydrolysis.

23| Incubate coverslips with a silane mixture from Step 22 at 65 °C for 17.5 h. Carry out incubation in glass containers (standard histology staining jars) with the coverslips laid flat on the bottom with no overlaps. Use a water bath with a lid to minimize evaporation of silane solution.

▲ **CRITICAL STEP** After silanization, it is important to keep track of which side of the coverslip was facing up, as this is the side to use for capturing DNA.

24| Rinse the silanized coverslips five times in ultrapure water and twice in ethanol.

■ **PAUSE POINT** The finished coverslips can be stored in Teflon racks in ethanol at 4 °C for at least 1 month.

**Preparation of high-molecular-weight DNA** ● **TIMING 24–48 h**

25| Harvest 200–400,000 nucleoside analog-labeled cells by trypsinization and wash once with 0.5 ml of Agarose insert buffer.

26| Resuspend cells in 50 μl of Agarose insert buffer and add an equal volume of melted 2% LGT, DNase-free agarose in water. Pipet to mix and immediately place in a well of an agarose insert mold with one end taped closed.

▲ **CRITICAL STEP** Use a wide-bore tip for mixing and transfer. The resulting 100 μ volume of the mixture is sufficient for one insert of specified size (see EQUIPMENT). Other insert sizes and fewer cells can be used, but adjust volumes accordingly. Some cell types may require adjusting cell concentration in plugs.

27| Let the gel inserts solidify at 4 °C for 10 min or longer. Then remove tape from the bottom of the mold and push inserts out using a ‘tooth’ that comes with the mold into flat-bottomed microtubes containing 1 ml of Agarose insert buffer.

■ **PAUSE POINT** At this stage, gel inserts can be stored for up to several months at 4 °C in the dark.

28| Aspirate off Agarose insert buffer and add 0.5 ml of Lysis buffer per 100 μl of gel insert. Incubate in a 50–55 °C water bath overnight. This lyses embedded cells to free DNA.

▲ **CRITICAL STEP** For MMS-treated DNAs, incubate at 30 °C for 36 h to avoid breakdown of methylated DNA (see ref. 32).

29| Wash agarose inserts at room temperature with 4× 1–2 ml changes of TE buffer for 30 min each. Rock or rotate gently to facilitate buffer exchange.



## PROTOCOL

**30|** Add 0.3–0.5 ml 1× agarase buffer per 100 µl gel insert. Heat at 75 °C for 5 min and then inspect the tubes to ensure that the agarose inserts have melted. If needed, heat for another 2–5 min.

▲ **CRITICAL STEP** From this point on, minimize agitation to avoid shearing the high-molecular-weight DNA that is now in solution.

**31|** Transfer tubes to 42 °C water bath, and let the temperature equilibrate for 5 min. Add 2–4 µl β-agarase to each tube containing melted agarose and mix by gently inverting once, and then incubate for 3–4 h at 42 °C. Alternatively, resuspend agarase in 50 µl of buffer and add to melted agarose solution without further inversion.

▲ **CRITICAL STEP** For MMS-treated DNAs, incubate at 37 °C for 4–5 h.

**32|** Adjust pH of the DNA solution by adding 1:10 volume of 10× TE pH 8.0 to neutralize the β-agarase buffer (pH 6.5).

▲ **CRITICAL STEP** Proper stretching of DNA on glass is pH-dependent.

■ **PAUSE POINT** After β-agarase digestion, DNAs can be stored at 4 °C in the dark for at least a month.

### Assembly and loading of capillary microchannels ● **TIMING** ≥ 1.5 h

**33|** Remove PDMS microchannel patch stored in water from Step 18 and blot dry against a clean glass surface. One patch of the size specified in **Figure 3** should be enough for two 22 x 22 mm coverslips if cut crosswise.

▲ **CRITICAL STEP** Microchannels must be absolutely dry for proper use. Residual liquid between channels will result in a failed seal and the entry of DNA into space between channels. ‘Plugs’ of liquid inside channels will impede capillary flow. Once dry, however, microchannels should be used within 15 min (see Step 18).

#### ? **TROUBLESHOOTING**

**34|** Remove silanized coverslip from ethanol and air-dry. Position a PDMS microchannel patch on a silane-coated glass substrate from Step 24 (channel side down). Microchannels will seal onto the surface spontaneously or after light tapping with forceps. Do not press hard on the microchannel patch, as this will result in flattening of the channels. A good seal should be visible as a striped pattern because of a difference in light reflection between sealed areas (between channels) and open areas (inside channels). A seal is more easily observed against a dark background. It is acceptable to break the seal to reposition microchannels on a coverslip if needed.

#### ? **TROUBLESHOOTING**

**35|** Using a wide-bore tip, load no more than 2 µl of DNA solution from Step 32 at one end of the microchannels. The DNA solution should enter and progress only along the channels at a rate of 1 cm/3–5 s. Individual speeds may vary between channels of different widths. Exceedingly uniform and fast filling of all channels usually means the seal is imperfect. See Steps 33 and 34.

#### ? **TROUBLESHOOTING**

**36|** Insert a razor blade at a shallow angle between a microchannel patch and a coverslip at the front end of the patch (i.e., the end from which DNA entered the channels). Slowly push the razor blade forward, separating the patch from the glass, taking care that the seal breaks evenly and its front recedes perpendicular to the axis of channels. Use forceps to pick up the patch after it is separated from the glass. Note that PDMS patches can be recycled once. Rinse and store used PDMS patches in ultrapure water at room temperature for up to several months. Dry completely and re-treat with oxygen plasma as described for new patches before reuse. Reusing more than once is not recommended because PDMS deteriorates after the third oxygen plasma treatment. This will be apparent as PDMS becomes increasingly sticky: it will begin adhering to glass.

▲ **CRITICAL STEP** Do not drop or slide the PDMS patch onto the coverslip or allow it to reseal, as this will result in the disorganized deposition of DNA.

**37|** Lay coverslips flat with the DNA side up in a dust-free place and allow water to evaporate until no liquid remains.

#### ? **TROUBLESHOOTING**

**38|** Flood the dried coverslips with a freshly prepared mixture of methanol–acetic acid (3:1) for 3 min. Drain gently and air-dry coverslips for 1 h to overnight. Stretched DNA can be stained with YOYO-1 (1 µM in TE with 20% β-mercaptoethanol) at this point to check the quality of stretching. If staining with YOYO-1, proceed to Step 39 after microscopy. DNA can also be stained with YOYO-1 in gel inserts, before agarase digestion. Alternatively, one can use an antibody against total DNA to examine the quality of stretching (see REAGENTS and Step 51 note below).

▲ **CRITICAL STEP** Minimize exposure of YOYO-1-stained DNA to the light beam of the microscope, as excessive illumination will break DNA.

### Immunostaining of stretched DNA on coverslips ● **TIMING** 7 h

**39|** Incubate fixed coverslips in 1–2 mls of 2.5 M HCl for 40 min to denature the DNA.

▲ **CRITICAL STEP** This and subsequent steps are performed at room temperature unless otherwise noted. For this and other steps that involve washing, coverslips can be kept DNA-side up in a six-well tissue culture plate.



- 40| Wash coverslips twice with 0.1 M Na Borate pH 8.5, once with PBS and once with Wash buffer to remove HCL.
- 41| Incubate coverslips in 1 ml Block buffer for 30 min.
- 42| Drain excess blocking buffer by touching the edge of a coverslip against a Kimwipe. Incubate blocked coverslips with rat anti-CldU/BrdU (primary antibody) freshly diluted 1:6 in Block buffer supplemented with 10% NGS for 1 h in the dark. Incubation with antibodies can be carried out with coverslips laid DNA side down into 10–20  $\mu$ l of antibody solution spotted onto a parafilm. Use forceps to transfer coverslips between parafilm and a six-well plate.
- 43| Remove primary antibody by washing three times for 5 min each time with 1 ml Wash buffer.
- 44| Incubate with a goat anti-rat Alexa 594-conjugated antibody (secondary antibody) diluted 1:1,000 in Block buffer supplemented with 10% NGS for 1 h in the dark. Note that the diluted secondary antibody in Block buffer can be kept at 4  $^{\circ}$ C for up to 1 week.
- 45| Remove the secondary antibody by washing three times for 5 min each time with 1 ml Wash buffer.
- 46| Incubate coverslips for 20 min in 1 ml Block buffer supplemented with 10% NGS before staining with a second anti-analog antibody.
- 47| Incubate blocked coverslips with a mouse anti-IdU/BrdU antibody (second primary antibody) diluted 1:6 in Block buffer supplemented with 10% NGS for 1 h in the dark.
- 48| Remove second primary antibody by washing three times for 5 min each time with 1 ml Wash buffer.
- 49| Incubate coverslips with a goat anti-mouse Alexa 488 conjugated antibody (2nd secondary antibody) diluted 1:1,000 in Block buffer supplemented with 10% NGS for 1 h in the dark.
- 50| Remove secondary antibody by washing three times for 5 min each time with 1 ml Wash buffer. Rinse once with PBS.
- 51| Place in a clean, dust-free place and dry until all liquid evaporates (usually 5–7 min). Mount coverslips face down on microscope slides, affixing them with ‘Hard-as-Nails’ clear nail polish at the corners.
- ▲ **CRITICAL STEP** We do not recommended the use of mounting medium because some brands, such as Vectashield, can cause deterioration of staining within hours after preparation. However, other brands, such as Prolong Gold antifade (Invitrogen), are reported to be compatible with staining. If using Prolong Gold, store coverslips at –20  $^{\circ}$ C in the dark for up to several months.
- **PAUSE POINT** Doubly-stained slides can be stored for weeks at 4  $^{\circ}$ C in the dark.
- 52| If desired, also stain stretched DNA with a mouse anti-deoxythymidine antibody. To do this, perform Steps 47–51, but using a mouse anti-deoxythymidine antibody in place of the primary antibody in Step 47 at a 1:2,000 dilution in Block buffer supplemented with 10% NGS, followed by a goat anti-mouse Alexa-conjugated secondary antibody in Step 49. We recommend that beginners perform this staining to verify that the key elements of DNA isolation and stretching protocol are working.

**Fluorescence microscopy of labeled and immunostained DNA on coverslips** ● **TIMING** variable, depending on nature of track analysis and number analyzed

53| Observe stretched and immunostained DNA using either a confocal or a regular fluorescent microscope. We use a confocal Zeiss Axiovert microscope equipped with a  $\times$ 63 and  $\times$ 100 objective and a digital camera. Briefly inspect the whole coverslip for DNA abundance, the quality of stretching and staining. Locate the areas with optimal DNA density and alignment (the density of DNA molecules decreases the farther they are from the loading end). See ANTICIPATED RESULTS for further guidance on locating these areas.

? **TROUBLESHOOTING**

**Data acquisition, analysis and quality control** ● **TIMING** 2–4 h per coverslip for image collection and track measurement

54| Collect images from the areas with optimal DNA density and alignment for subsequent analysis. We typically acquire 50–80 digital images from three to four microchannels for each sample, with the goal of collecting data on 300–400 replication tracks. This total number may have to be higher if a specific small subset of all tracks is sought after, e.g., tracks containing three segments (e.g., red–green–red). The only criteria for image acquisition should be the proper density and alignment of tracks. Photograph fields consecutively in two fluorescent colors to generate merged two-color images of tracks. See ANTICIPATED RESULTS for further guidance regarding data analysis.

? **TROUBLESHOOTING**

Troubleshooting advice can be found in **Table 2**.

**TABLE 2** | Troubleshooting table.

Step	Problem	Likely reasons	Solution
7	Patterns lift off in the developer during photolithography	Photoresist adhesion problem	Reduce surface moisture on the silicon wafer surface by drying the wafer on a 200 °C hotplate for 10 min before coating Increase exposure time and post-exposure baking time Ramp down temperature slowly post-UV exposure bake Avoid agitation during developing
33 and 34	DNA stretching is disorderly: DNA is not confined to channels	Residual moisture on PDMS surface has resulted in a poor seal and leaking of DNA into space between channels PDMS was not peeled off the coverslip properly PDMS patch surface is buckled, resulting in a poor seal and leaking of DNA into space between channels	Make sure PDMS channels are completely dry by blotting several times against a glass surface before applying them onto coverslips Remove PDMS from glass as described in Step 35 PDMS patches are too thick and thus rigid. Prepare thinner, more flexible patches for a better seal
35	DNA solution is not flowing into microchannels when loading	Blocked channels Hydrophobic PDMS surface	Check if both ends of the microchannels are cut open Do not allow channels to remain dry for over 15 min before DNA stretching Re-treat PDMS with oxygen plasma
37	DNA is not fully attached to glass: 'flapping' molecules can be seen	Suboptimal glass properties: vinyl silane concentration may be too low	Remake coverslips according to the recipe; if needed, increase the vinyl silane concentration Increase coverslip drying time after DNA capture
	DNA is stretched poorly: unevenly stretched, curled and clumped molecules are abundant	Suboptimal pH of DNA solution	Make sure DNA solution has a pH 8.0
53	DNA staining is too beady, impossible to identify tracks, beady background in channels	DNA concentration may be too high Proteinase K and/or agarase digests may be incomplete	Dilute DNA solution used for loading 1:10 in TE Treat DNA samples with more agarase; reduce cell concentration in agarose gel inserts
	Staining against CldU generates bright granular background throughout the cover slip	Some batches of rat anti-CldU antibody may be prone to generating aggregate background	Change the antibody batch, centrifuge antibody preparation to eliminate aggregates Preincubate antibody dilution for 15 min with a silanized coverslip that has no DNA but has been treated with methanol-acetic acid, dried and subjected to HCL-neutralization-blocking steps

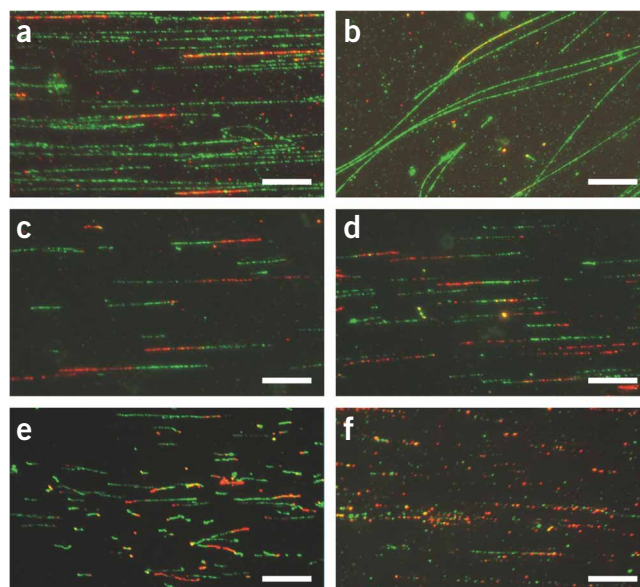
## ANTICIPATED RESULTS

### Visual appearance of DNA

DNA stretched inside the microchannels should appear as bands composed of molecules oriented parallel to the microchannel axis. **Figure 5a** shows a field within a microchannel, which displays a desired quality of stretching and reasonable density and length of DNA molecules. DNA is pulse-labeled with CldU and stained with antibodies against total DNA (anti-thymidine, green) and anti-CldU/BrdU (red). Note that some of the CldU tracks are located at the ends of molecules. It is expected that DNA at a replication fork may be more fragile to breakage during stretching than regular duplex DNA.

**Figure 5** | Examples of stretched DNA. Scale bars represent 10  $\mu\text{m}$ .

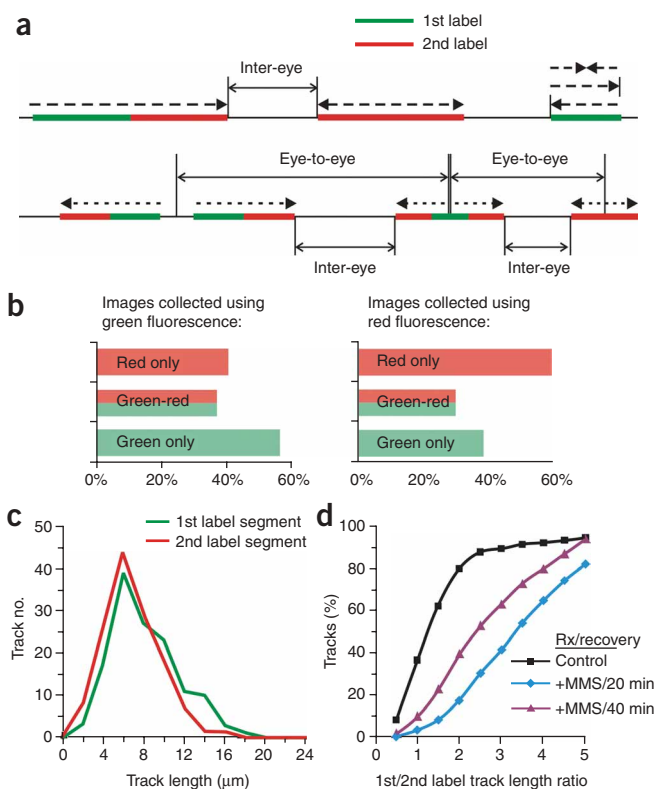
(a) A fragment of area that was inside a microchannel when DNA was loaded. Flow inside microchannels results in deposition of highly aligned DNA molecules. Here, human fibroblasts were labeled for 30 min with CldU and harvested after another 30 min. DNA was prepped, stretched and stained with antibody against total DNA (anti-thymidine, green) and against CldU (red). Note that a subset of tracks is located at the ends of DNA molecules. (b) A fragment of area outside a microchannel. Staining is as in a. (c–f) Areas inside microchannels. (c,d) Examples of good quality staining and stretching. Human fibroblasts were labeled with IdU and then with CldU for 30 min each, and DNA was stained with antibodies against CldU (red) and IdU (green). (e) An example of a less than optimal stretching resulting in ‘curling’ of a subset of tracks. Human fibroblasts were labeled with IdU and then with CldU for 40 min each, and DNA was stained with antibodies against CldU (red) and IdU (green). (f) An example of suboptimal staining resulting in ‘beady’ appearance of tracks. Human fibroblasts were labeled with CldU and then with IdU for 20 min each, and DNA was stained with antibodies against CldU (red) and IdU (green).



Typically, DNA is also deposited and stretched outside the channels (Fig. 5b). These molecules are usually unaligned or oriented at an angle to microchannel axes. Collecting data on this DNA should be avoided, as it is often more sheared than the DNA inside channels.

When DNA is labeled consecutively with CldU and IdU (or BrdU and any one of the above nucleoside analogs) and stained with antibodies to these analogs, the anticipated patterns should be as in Figure 5c and d, in which densities of DNA molecules and staining quality are acceptable for data collection. The staining is optimal when tracks appear largely continuous. However, some discontinuity is unavoidable. At close examination, tracks appear as chains of beads of about 1–1.5  $\mu\text{m}$  in diameter, and it is common that some beads in this chain are missing or are dimmer than others. As a general rule of thumb, when discontinuities are larger than the width of two beads, the interpretation of track lengths and boundaries can be problematic. Typically, double-labeled replication tracks comprise a fraction of all tracks labeled with the first label. Note that double-labeled tracks in Figure 5 have red and green segments of similar length. This should be expected if labels were added for the same periods of time (see Table 1 and Fig. 6, panel c).

Figure 5e and f show examples of the most frequent problems encountered. In Figure 5e, immunostaining against CldU and IdU is good, but DNA is not well stretched, as revealed by the variable thickness and buckling of some replication tracks. In Figure 5f, stretching is fair but immunostaining has not been successful: a majority of tracks are extremely discontinuous.



**Figure 6** | Replication track types and measures that can be derived from track images. (a) A schematic representation of the types of tracks and intra- and inter-replicon measurements. (b) An example of scoring for the types of tracks in human fibroblasts labeled with IdU (green) and then with CldU (red) for 30 min each. The observed fraction of red-only tracks appears higher if the images were collected looking for red fluorescence than when looking for green fluorescence. Approximately 250 tracks total were scored for each graph. (c) Plotting of the track data as binned distributions of track lengths. Bins are in 2- $\mu\text{m}$  increments, e.g., contain tracks that are 2–4-, 4–6- $\mu\text{m}$  long, etc. Human fibroblasts were labeled consecutively with two labels for 20 min each. Lengths of 1st and 2nd label segments in each two-segment track were measured and plotted. Note that these lengths are similar, as expected. (d) Plotting of the track data as cumulative distributions. Human fibroblasts were labeled consecutively with IdU (green) and then with CldU (red) for 20 min each (control), or treated with 0.015% MMS for 20 min between the two labels (as described in more detail in Box 1). In this case, the second, post-MMS label was added either for 20 or for 40 min. Ratios of 1st to 2nd label segment lengths in two-segment tracks were plotted (control,  $n = 134$ ; two-segment tracks: 20 min,  $n = 118$ ; 40 min,  $n = 83$ ). The slowing of replication elongation post-MMS manifests as an increase in values of these ratios. Also, the longer the post-MMS labeling time, the lower the ratios.

### Determination of track types

This is the simplest kind of information that can be derived from replication track images, and it is useful for evaluating overall replication profiles. There are three basic types of tracks one can observe after consecutive pulse labeling with two labels (**Fig. 6a**). Tracks containing *the first label-only* represent one or two replication forks that terminated before the second label was added. Tracks containing consecutive segments of *the first then second label* represent ongoing forks; their direction can be determined from the temporal order of addition of each label. Tracks containing *the second label-only* are origin firing events that occurred during the second labeling period. Note that these are the simplest interpretations of the track appearances and represent the most likely classes of event that could give rise to the specified track appearances. Finally, additional types of tracks may be observed: three-segment tracks of the 1st–2nd–1st label type are most likely two converged forks, and those of the 2nd–1st–2nd label type are origin firing events that occurred during the first labeling period. All these types can be scored manually, using any image display software such as Microsoft Picture Viewer or Adobe Photoshop.

It is important to realize that the process of image acquisition can and will introduce a bias into the estimated track type distribution (**Fig. 6b**), especially when tracks are not very dense (2–5 per image). Scanning of coverslips for image acquisition is performed in one color (i.e., with a filter set to fluorescence of one of the two labels), and the presence or absence of the second color in the field should not factor into a decision to acquire the image. Using first label color-only, scanning may result in a data set that underestimates the proportion of the second label-only tracks among all tracks (**Fig. 6b**). This bias can be avoided by collecting an additional set of images scanned using the second label color (see **Table 1**). Use a consistent color scanning strategy in all experiments.

### Measurement of replication track lengths

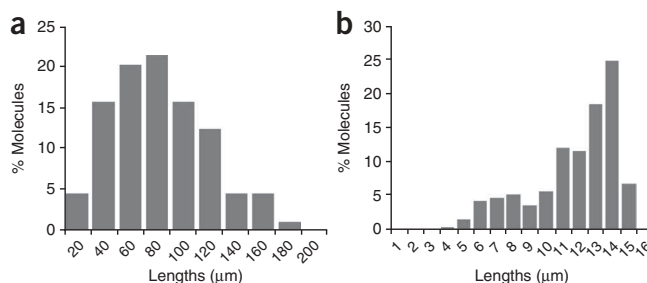
This measure provides information on replication elongation speeds and is particularly powerful when exploring genotype- or genotoxic drug-specific effects on replication. We measure lengths in microns for all types of tracks listed in the earlier section using the AxioVision image analysis software supplied with the Zeiss Axiovert microscope; however, any software that has the capability to measure linear distances in a microscopic image can be used. The resulting sets of values can be processed in Microsoft Excel (or any graphing software). For tracks containing consecutive segments of each color, it is particularly informative to collect data on each segment and to determine mutual ratios of segment lengths (see **Table 1**). Note that DNA stretched as described above will be sufficiently concentrated to detect plenty of IdU and CldU tracks but too dense to measure lengths of individual DNA molecules. To achieve the latter, DNA should be carefully diluted 1:200–500 in TE. **Figure 7a** shows a distribution of DNA molecule lengths in microns obtained from a sample prepared and stretched as described above. Median and maximum lengths observed are 80 and 180  $\mu\text{m}$ , respectively. To convert these lengths into microns, one can use a length standard, such as bacteriophage lambda DNA. **Figure 7b** shows a distribution of molecule lengths measured after stretching a stock solution of phage lambda DNA. The majority of intact molecules in **Figure 7b** measure approximately 13–14  $\mu\text{m}$  in length, with the mean of 12.3  $\mu\text{m}$ . Thus, the conversion factor may be estimated as 48.5 Kb (the length of phage lambda) divided by 12.3, e.g., 1  $\mu\text{m}$  = 3.9 Kb. This estimate is within the range of the original evaluation published for microchannel-assisted stretching on the two-silane platform<sup>21</sup>. According to this estimate, median and maximum lengths of stretched DNA molecules shown in **Figure 7a** are  $\sim$ 300 and 700 Kb.

### Inter-replicon measurements

This is a more demanding task than the *intra-replicon* characterization described above, as it requires identifying more than one adjacent replicon on one DNA molecule. When collecting inter-replicon data, it is helpful to use total DNA staining with an anti-thymidine antibody to identify replication tracks contained in single DNA molecules. If mouse anti-thymidine antibody is used for this purpose, a sheep or a goat antibody against IdU/BrdU should be used.

Absolute length of DNA molecules becomes critical for these types of measures, as the ability to identify more than one replicon on a single molecule is dependent on the presence of intact molecules of  $\geq$  0.5 Mb range.

*Inter-origin or eye-to-eye distance* is a distance between the centers of symmetry of two tracks that represent origin firing events (2nd–1st–2nd label or 2nd label-only) and gives an estimate of the density of active origins and thus replicon size (**Fig. 6a**).



**Figure 7** | Stretching parameters. **(a)** An example of a length distribution of DNA molecules isolated from human fibroblasts and stretched. DNA was stained with YOYO-1. **(b)** A standard preparation of bacteriophage lambda DNA was stretched and stained with YOYO-1, and the lengths of molecules were measured and plotted. The smaller peak corresponds to broken molecules in the DNA sample. The observed lengths of intact molecules can be used to estimate the conversion, or stretching factor (see PROCEDURE).



Pairs of closely spaced double-labeled tracks that represent diverging forks are also included into this measure: in this case, an origin is implied to be at a halfway point between diverging forks. The acceptable spacing between such tracks should be less than the lengths of tracks.

The less stringent but more easily measured *distance between converging forks, or inter-eye distance*, may be affected by the density of origins as well as by fork processivity and speed. *Origin density* can also be calculated as a number of origin firing events per unit of total DNA length, per specified time. Note that all of these track measures may be biased by the position of cells in S-phase at the time of labeling and harvesting.

When performing an experiment to take inter-replicon measurements, include a chase step after pulse labeling with nucleoside analog(s). This will allow replication forks to clear the vicinity of labeled segments of DNA and complete replication. Fully replicated DNA is thought to be more resilient to shear that incurs during stretching.

### Analysis of track length, inter-origin distance or segment ratio data

We use two ways of displaying numerical values obtained from length or segment ratio measurements. Values are grouped into bins (usually at 2- $\mu\text{m}$  resolution, e.g., 0–2, 2–4, 4–6, 6–8  $\mu\text{m}$  and so on), and the number of tracks that fall within each length class or bin is plotted to give a frequency distribution as a function of length (**Fig. 6c**). This type of graph readily visualizes the mode of the distribution; it also shows that the data are not distributed normally. Mean values may as a result be misleading, and non-parametric significance tests should be used to compare data sets. We use a  $\chi^2$  test on the binned distributions described above to assess differences between track length distributions. Cumulative distributions provide another useful and more compact way of plotting track length data (**Fig. 6d**). This type of plot depicts the fraction of track length values that are equal to or smaller than a given value. The Kolmogorov–Smirnov (K–S) test is performed to determine *P*-values of different cumulative distributions.

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