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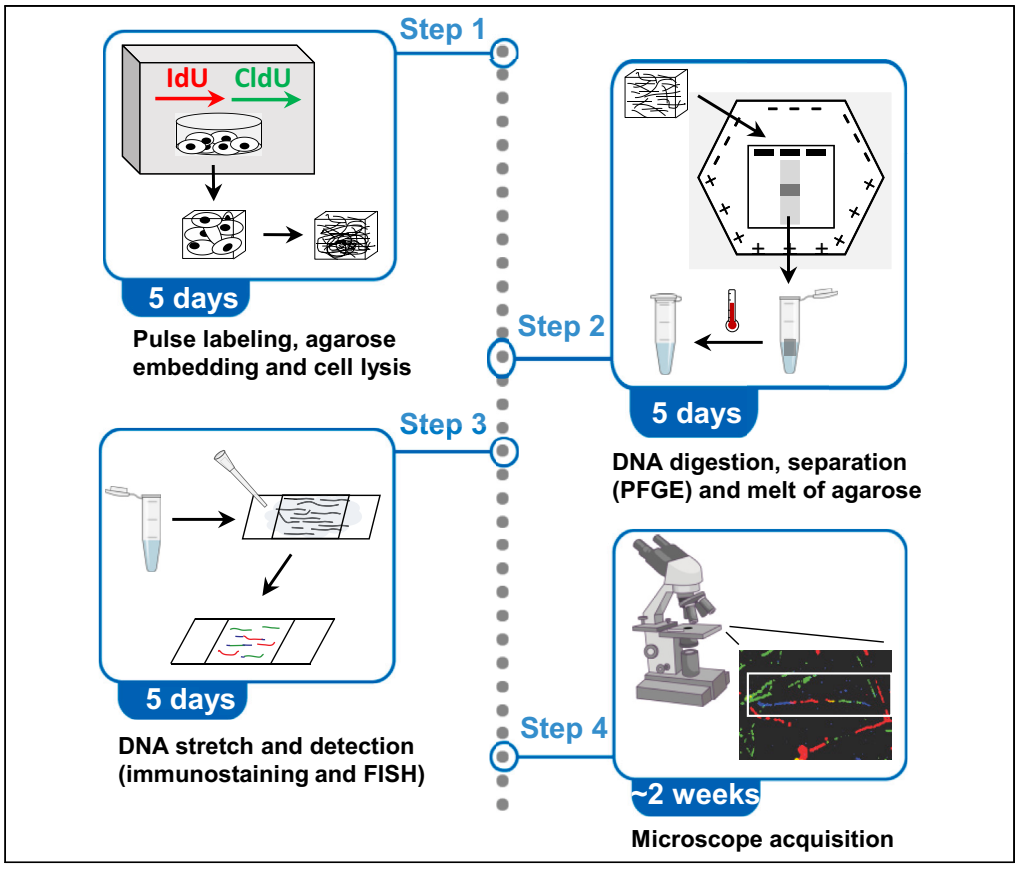
Visualizing DNA replication by single-molecule analysis of replicated DNA



Single-molecule analysis of replicated DNA (SMARD) is a unique technique that enables visualization of DNA replication at specific genomic regions at single-molecule resolution. Here, we present a protocol for visualizing DNA replication by SMARD. We describe steps for pulse labeling DNA, followed by isolating and stretching of genomic DNA. We then detail the detection of the replication at chromosomal regions through immunostaining and fluorescence *in situ* hybridization. Using SMARD, we can visualize replication initiation, progression, termination, and fork stalling.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

SMARD reveals how specific genomic loci are replicated

This unique technique combines DNA fiber analysis with FISH

SMARD is able to detect replication fork direction and replication fork stalling

Using SMARD, initiation and termination sites are detected simultaneously

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Visualizing DNA replication by single-molecule analysis of replicated DNA

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SUMMARY

Single-molecule analysis of replicated DNA (SMARD) is a unique technique that enables visualization of DNA replication at specific genomic regions at single-molecule resolution. Here, we present a protocol for visualizing DNA replication by SMARD. We describe steps for pulse labeling DNA, followed by isolating and stretching of genomic DNA. We then detail the detection of the replication at chromosomal regions through immunostaining and fluorescence *in situ* hybridization. Using SMARD, we can visualize replication initiation, progression, termination, and fork stalling.

For complete details on the use and execution of this protocol, please refer to Norio et al. (2001) and Gerhardt et al. (2014).[1,2]

BEFORE YOU BEGIN

SMARD is a specialized experimental approach to visualize the replication fork progression, replication initiation and termination at a defined genomic region in mammalian cells. From the SMARD profiles the sites of replication fork stalling can be mapped, and the replication fork speed can be calculated. SMARD is a high-resolution DNA fiber assay that utilizes halogenated thymidine analogs[3] and locus-specific DNA probes that allow visualization of DNA replication at large well-defined chromosomal regions. SMARD was developed by Norio & Schildkraut.[1] The protocol below describes in detail the steps to analyze the DNA replication by SMARD at specific loci in the human genome. We have performed SMARD at various genomic loci and in different types of mouse and human cell lines, including embryonic stem cells.[2,4–12]

Before beginning, choose a rare-cutting restriction enzyme that yields a genomic region of 100–400 kb and contains your genomic region of interest. The Gene Construction Kit (GCK) program can be used to determine the availability of rare-cutting restriction enzymes. Next, to detect the region of interest choose FISH probes that bind within this DNA segment. We usually pick two FISH probes that bind on both ends of the regions, but with different sizes (one FISH probe binding to a ~40 kb region and the other binding to a ~10–15 kb region), to determine the orientation of the DNA segments. For larger DNA segments (>300 kb) we usually choose three FISH probes that bind in a recognizable nonsymmetrical pattern. The FISH probes are selected from the UCSC genome browser website and ordered from BACPAC resources. We have used this protocol to determine the replication fork progression through endogenous fragile sites, such as the *FMR1* gene locus in Fragile X

embryonic stem cells[2] and CFS in FANCD2-deficient cells,[7] Pol Eta-deficient cells,[11] PHF6-deficient cells[13], and in response to environmental carcinogenic exposures.[9] We have also utilized SMARD to visualize replication fork stalling at expanded GAA repeats in stem cells derived from Friedreich ataxia's patients[5] and recently at the *BRCA* genes in cells carrying a BRCA germline mutation.[4]

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse monoclonal anti-bromodeoxyuridine (dilution 1:15)	Becton Dickinson	Cat #347580
Rat monoclonal anti-bromodeoxyuridine (dilution 1:15)	Abcam	Cat #ab6326
Goat anti-rat IgG (H + L) cross-adsorbed secondary antibody, Alexa Fluor 488 (dilution 1:15)	Invitrogen	Cat #A-11006
Goat anti-mouse IgG (H + L) highly cross-adsorbed secondary antibody, Alexa Fluor 568 (dilution 1:15)	Invitrogen	Cat #A-11031
Anti-avidin antibody, biotinylated (0.5 g/mL) (dilution 1:15)	Vector Laboratories	Cat #M0392S
Chemicals, peptides, and recombinant proteins		
3-Aminopropyltriethoxysilane	Sigma-Aldrich	Cat #440140
5-Chloro-2-deoxyuridine (CldU)	MP Biomedicals	Cat #ICN10547883
5-Iodo-2-deoxyuridine (IdU)	MP Biomedicals	Cat #ICN10035701
Agarose	SeaKem	Cat #50070
Avidin, NeutrAvidin, Alexa Fluor 350 conjugate	Thermo Fisher Scientific	Cat #A11236
Bovine serum albumin	Sigma-Aldrich	Cat #1703605
β-Agarase	New England Biolabs	Cat #C-8052
CHEF DNA size marker, 0.2–2.2 Mb, *S. cerevisiae* ladder	Bio-Rad	Cat #1705061,
EDTA (pH 8) (0.5 M)	Thermo Fisher Scientific	Cat #AF-100-15
Ethidium bromide	Bio-Rad	Cat #G6257
Glutaraldehyde	Sigma-Aldrich	Cat #A144-212
IGEPAL CA-630 (NP40)	Sigma-Aldrich	Cat #I-1882
InCert agarose	Lonza	Cat #1708840
Lambda DNA ladder (48.5 kB - 1 MB)	Lonza	Cat #354277)
Low melting agarose	Sigma-Aldrich	Cat #A412-4
Methanol	Fisher Chemical	Cat #85850
$MgCl_2$ (1 M)	Sigma-Aldrich	Cat #L5777
N-lauroylsarcosine sodium salt	Sigma-Aldrich	Cat #BP359-212
NaCl	Fisher BioReagents	Cat #1004960700
NaOH	Fisher BioReagents	Cat #D8537
Paraformaldehyde	Merck	Cat #15070063
Phosphate-buffered saline	Sigma	Cat #P7626
Phenylmethanesulfonyl fluoride	Sigma-Aldrich	Cat #J60300
Restriction enzyme (*PmeI*, *SbfI*, *SfiI*, *NruI*, *PacI*, *SwaI*)	New England Biolabs	Cat # varies
Proteinase K	Invitrogen	Cat #P10144
ProLong Gold antifade mountant	Thermo Fisher Scientific	Cat #SML2496
SeaPlaque GTG agarose gel	Lonza	Cat #15567-027
SSC buffer (20X)	Thermo Fisher Scientific	Cat #15568-025
Tris-HCl pH 7.5 (TE 7.5)	Invitrogen	Cat #X100
Tris-HCl pH 8.0 (TE 8)	Invitrogen	Cat #12604013
Triton X-100	Sigma-Aldrich	Cat #T8154
Trypan blue	Sigma-Aldrich	Cat #T8154
YOYO-1 iodide (491/509)	Thermo Fisher Scientific	Cat #Y3601
Critical commercial assays		
CHEF disposable plug molds	Bio-Rad	Cat #1703713
Biotin-nick translation mix	Sigma	Cat #11745824910
Biotin-16-dUTP	Sigma	Cat #11093070910
PCR mix kit (GoTaq green)	Promega	Cat #PAM7123
Maxi prep kit	QIAGEN	Cat #12162

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Oligonucleotides		
FISH probes	BACPAC	https://bacpacresources.org/
Primers	IDT	https://www.idtdna.com/
Software and algorithms		
Photoshop	Adobe	Version 2022
GCK	http://www.textco.com/gene-construction-kit.php	
Others		
CHEF Mapper XA system	Bio-Rad	Cat #1703670
Hybridization oven	Fisher Scientific	Cat #UV95003001
Benchmark tube rocker	Benchmark Scientific	Cat #M2100

Alternatives: Instead of using a hybridization oven, you can just incubate the slides in a 37°C warm room.

STEP-BY-STEP METHOD DETAILS

Part 1: Pulse labeling cells and cell lysis

Timing: 5 days

Before the experiment, ensure that cells (for example human embryonic stem cell lines, fibroblasts or breast epithelial cells) are in their exponential growth phase. For maximum 5-iodo-2-deoxyuridine (IdU) and 5-chloro-2-deoxyuridine (CldU) incorporation, the cells have to actively proliferate and replicate.

Day 1.

1. Pulse labeling cells with IdU and CldU.
   a. Pulse the cells with 30 μM (final concentration in the media) IdU (10 mM stock concentration), growing for 4 h.
   b. Wash cells with PBS to remove IdU.
   c. Pulse the cells with 30 μM (final concentration in the media) CldU (10 mM stock concentration), growing for 4 h.

   Note: SMARD can be performed on both suspension and adherent cell lines. Spin down suspension cells after first pulse and wash with 1x PBS before second pulse.

   Note: IdU and CldU are dissolved in ddH_2O according to manufacturer's instruction and both are light sensitive, thus we recommend that labeling is performed without light under the hood and the steps after labeling under yellow light.

2. Near the end of the second pulse (about 1 h before), prepare agarose.
   a. Make 1% low melting agarose (InCert agarose) gel in 1x PBS and boil the agarose solution for 10 min and then equilibrate to 50°C in water bath.
   b. Pre-cool 1x PBS buffer and plug mold (for making the plugs) for 30 min on ice.
3. After the second pulse, detach, spin cells down and wash cells with 1x PBS.
4. Count the cells (use yellow light from now on for the rest of the procedure if possible).
5. Resuspend the cells in ice-cold 1x PBS to make the final concentration of 3×10^7 cells/mL (that will lead to a final concentration of 1×10^6/plug).
6. Equilibrate the cells to 42°C in water bath for 5 min. Then add equal volume of agarose solution, mix by pipetting up and down and leave the suspension in 42°C water bath for 1 min.

Note: Before mixing the agarose solution transfer the agarose solution from 50°C to 42°C about 2 min before mixing with the cells.

7. Pipette the mixture into the plug molds (~80 μL per well for Bio-Rad molds). Leave on ice for 20 min to let the plugs solidify.
8. Take the plugs out of the plug cast and put them into the lysis buffer (0.5 M EDTA + 1% sarcosine). Add 0.5 mL proteinase K stock solution (20 mg/mL) per 50 mL lysis buffer. Incubate in a 50°C water bath for 14–18 h.

Day 2–5.

9. Change the lysis buffer (by carefully pouring off the liquid so that the plugs stay at the bottom) the next day and add fresh proteinase K (20 mg/mL) in a 50 mL corning tube. Incubate in a 50°C water bath for 24 h.
10. Repeat step 9.
11. After proteinase K digestion, wash the plugs with TE (pH 8) for 1 h at room temperature (RT: 23°C), rotating (we use rocker with 24 rpm, 30-degree tilt angle).
12. Wash the plugs with TE 8 and incubate the plugs for 1 h at 50°C.
13. Discard buffer and incubate the plugs in TE 8 buffer + PMSF (100 μL stock PMSF/50 mL TE 8, PMSF stock concentration is 100 mM) at 50°C for 30 min.
14. Repeat step 13.
15. Wash the plugs in TE 8 buffer twice at RT for 1 h, rotating.
16. Add fresh TE 8 buffer, leave at 4°C for 14–18 h.
17. Transfer plugs to storage buffer (50 mM EDTA + 10 mM Tris-HCl 8.0) the next day, leave at 4°C for 14–18 h.
18. Transfer plugs into fresh storage buffer and continue storing the cell plugs at 4°C.

⚠ CRITICAL: The cell plugs should be solid before removal and handed very careful. At this point in the process, since the cell plugs are in long-term storage buffer, you can pause and continue to use the cell plugs for SMARD for up to 2 years.

Part 2: DNA digestion and separation

Timing: 4–5 days

Day 1.

19. Take out the plugs needed for digestion from the storage buffer, wash with 50 mL TE 8 (for 3–6 plugs), twice for 1 h at RT, rotating.
20. Quickly rinse the plugs with water; and then with Pre-digestion buffer (10 mM $MgCl_2$ + 10 mM Tris-HCl 8.0).
21. Wash with 50 mL Pre-digestion bufferat RT for 1 h, rotating.
22. Repeat step 21 twice.
23. Transfer plugs to 1.5 mL tubes (one plug/tube). Equilibrate each plug in 1 mL digestion buffer of the enzyme you choose to cut the genomic DNA (NEB buffer with 2X BSA and 4 μL/mL of 100 mM spermidine) .
    a. We usually use the rare-cutting enzymes PmeI, SfiI, SbfI, PacI or SwaI to cut the genomic DNA.
    b. Use GCK or any other program to choose the enzyme and predict DNA fragment size (between 100 to 400 kb) that contains the region of interest.
24. Wash with digestion buffer a second time for 30 min at RT, rotating.
25. Add 150 μL digestion buffer per plug with 30 unit of enzyme.
    a. Pipette up and down to mix solution (be careful not to touch the cell plug).

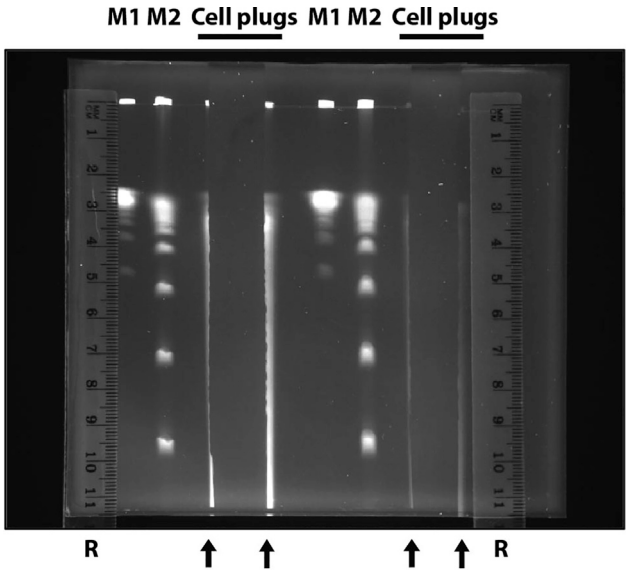

Figure 1. PFGE gel after ethidium bromide staining

Two Markers (M2: CHEF DNA Size Marker (0.2–2.2 Mb) *S. cerevisiae* and M1: Lambda DNA Ladder (48.5 kB - 1 MB)) are placed flanking the DNA inthe cell plugs. Black arrows on the bottom indicate the cut positions. Use a ruler (R) to determine the position of the DNA fragments according to the size of the DNA segment of interest.

b. Leave the plug on ice for 30 min to let the enzyme diffuse into the plug.

26. Leave the digestion for 14–18 h at the temperature required for each enzyme.

Day 2.

27. Next day, tremove (by carefully pipetting) the digestion buffer and rinse the plugs with TE 8 first and then wash the plugs with TE 8 for 1 h at RT, rotating. You can also wash plugs for several hours with TE 8 on ice or leave it for 14–18 h at 4°C.
28. Pulsed Field Gel Electrophoresis (PFGE system:CHEF mapper or CHEF DRIII system, Bio-Rad):
    a. Cast low melting agarose gel (usually 0.7% agarose gel is used GTG SeaPlaque). The low melting agarose is dissolved in 150 mL of 0.5x TBE in the microwave. Then, let the solution cool at RT before casting the gel.
    b. Keep the rest of the melted agarose in the 65°C water bath.
    c. Let the gel solidify for at least 1.5 h.
    d. Pre-cool 3 L of 0.5x TBE in the PFGE apparatus for 1 h before the run. Circulation is set to 60–70. During the run, the temperature inside the apparatus is around 12°C.
    e. After the gel has solidified, remove a 1–3 cm strip from top of this gel and place plugs and markers on the edge in horizontal position. Pour remaining agarose to fill the gap and to solidify the plugs to gel.
    f. Pre-cool the gel inside the tank for 10–30 min before starting the run.
29. Set up the PFGE program.

Note: The parameters depend on the size of the DNA fragment you need to isolate after the run. For example, 145 kb PmeI fragment: 0.7% agarose gel, 0.5x TBE, 200 volt with an Initial time: 5, Final time: 16, Run time: 36 h.

Day 4.

30. After the PFGE run, remove the gel and cut the gel region with the DNA as pictured in Figure 1.
    a. Set aside the gel with the DNA.

b. Fill the rest with 1% agarose (SeaKem) and stain gel with ethidium bromide (about 0.5 μg/mL) for about 2 h, shaking gently.

31. Determine the position of your DNA fragment based on the size of the PFGE marker and ruler (Figure 1).
32. Cut 0.5 cm DNA fragment containing your region of interest out of the gel strip you set aside using a ruler.
33. To confirm that this gel piece contains your region of interest cut ~1–2 mm slivers from both ends of this gel piece for PCR, keep the rest of the gel piece in storage buffer.
34. Melt the agarose and perform PCR to confirm the presence of your regions of interest:
    a. Wash the gel slices in agarose digestion buffer (TE 8 + 100 mM NaCl + 0.1% β-mercaptoethanol), equilibrate for at least 2 h.
    b. Transfer gel slices to a 1.5 mL tube and add 150 μL agarase digestion buffer.
    c. Melt at 72°C for 20 min and then equilibrate solution at 45°C for 1 min.
    d. Add 1.5 μL agarase slowly in circular motion on the surface, mix by rotating the tube and digest the agarose at 45°C for 1.5 h.

⚠ CRITICAL: When adding the agarase (do not mix by vortexing or pipetting!) simply mix by rotating the tube gently with your hands for 1-2 min.

35. Perform PCR to confirm the presence of your region of interest:
    a. The separation of the genomic DNA may vary between different PFGE runs. To identify the exact 0.5 cm DNA slice that contains the DNA of interest, use PCR, set up PCR reactions for 6–7 DNA samples surrounding the 0.5 cm gel slice likely to contain the DNA of interest based on the DNA marker.
    b. Use 1–2 μL of the melted DNA solution for PCR reaction.
    c. Using NCBI-BLAST design primers specific to your region of interest.
    d. PCR reaction conditions vary according to the annealing temperature of the PCR primers chosen. Set up a reaction with at least 30–35 cycles.
    e. To confirm amplification, run the PCR products on a 1% agarose gel.

Optional: For confirmation that the gel piece contains the region of interest Southern blot analysis can also be performed. The slices can be stored in storage buffer at 4°C for up to 2 years.

Pause point: At this point in the process, since the slices are in long-term storage buffer, you can pause and continue to use the gel slices for SMARD for up to 2 years.

Part 3: Hybridization and immunodetection

Timing: 5 days

STEP 1: Pre-hybridization steps - Slide preparation, Biotin-Nick translation and Agarose Melting to release DNA of interest.

Day 1–2.

36. Slide Preparation (Timing: 2 days + 1–2 days of desiccation).
    a. Soak slides in 1% SDS for at least 1 h.
    b. Wash the slides with 1% SDS solution followed by rinsing with hot water and a final wash with distilled water.
    c. Immerse the slides in HNO_3: HCl (2:1) solution for 14–18 h in a chemical hood.
    d. Next day, remove the slides from the acid and transfer to a trough of distilled water.
    e. Wash the slides in distilled water 3 times, 3 min each time.

f. Drain excess water from the slides on a paper towel.
g. Rinse the slides in a trough of Methanol, moving up and down.
h. Wash the slides in Methanol containing Amino Silane (2 mL stock of Amino Silane in 500 mL Methanol) for 1 h, shaking constantly at 70 rpm.
i. Rinse off the excess Amino Silane using the first trough of Methanol from step 36g.
j. Wash the slides in distilled water 3 times, 3 min each time.
k. Rinse slides in 95% Ethanol twice, air dry with air from a hose with a filter at the end.
l. Place the slides in a desiccator for a day.

Troubleshooting

To obtain well stretched DNA in the following steps, optimal desiccation of the silane coated slides is essential (1–2 days).[14] This can vary depending on the desiccator used, age of the amino silane and atmospheric humidity. The desiccation time might need to be altered to accommodate these variations. The slides can be used within a week if stored within the desiccator.

37. Biotin-Nick translation (Timing: 3 h).
    a. DNA probes specific to the region of interest are prepared for hybridization by biotinylation using nick translation.
    b. Fosmids containing DNA complimentary to the region of interest are obtained from BACPAC resources.
    c. A standard midi or maxi prep (QIAGEN) is carried out to obtain enough DNA for multiple experiments.
    d. Biotin-Nick translation of the fosmid DNA is carried out using the Invitrogen kit, following the manufacturer's protocol.

Optional: Appropriate size of the biotinylated probe (100–300 bp) can be ascertained by resolving a sample of the nick translated product on a 1% agarose gel. Radio isotopes can be included in the nick translation reaction to ascertain sufficient biotinylation of probes by Southern blotting.

38. Agarose Melting to release DNA of interest (Timing: 7 h).
    a. From the gel slice that contains your region of interest, cut ~1–2 mm slivers from both ends for melting.
    b. Wash the gel slices in 50 mL agarase digestion buffer (TE8 + 100 mM NaCl + 0.1% β-mercaptoethanol) andequilibrate for at least 2 h, rotating.
    c. Transfer gel slices into a 1.5 mL tube and add 150 µL agarase digestion buffer.
    d. Melt at 72°C for 20 min and then equilibrate the tube at 45°C for 1 min.
    e. Add 1.5 µL agarase slowly in circular motion on the surface, mix by rotating the tube and digest the agarose at 45°C for 4 h. After digestion, add 0.2 µL YOYO to the DNA slowly in a circular motion and incubate for 30 min.

⚠ CRITICAL: When adding the agarase (do not mix by vortexing or pipetting!) simply mix by rotating the tube gently with your hands for 1–2 min.

Optional: To save time, this can be done in parallel on Days 1–3. Alternatively, it can be done after PCR detection of the slice containing the region of interest.

STEP 2: DNA Stretching and Hybridization (Timing: 2 days under yellow light or in the dark).

Day 4.

39. Prepare the humid chamber by placing two moistened kimwipes inside a black slide box and place box in the 37°C room.

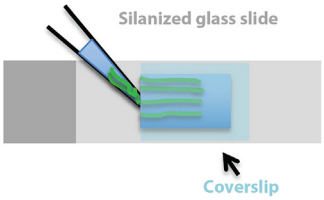

Figure 2. Stretching DNA on a slide

Using a 200 μL large orifice pipet tip, extract 3.5–4 μL of YOYO-stained DNA gently. Pipet out DNA solution slowly from one side of the coverslip, moving up and down along the edge of the coverslip to allow liquid movement by capillary action under the coverslip. Mark the position of the coverslip with a diamond glass marker.

40. Prepare the following buffers (5 slides per jar).
    a. Methanol + 0.1% β-mercaptoethanol.
    b. Denaturation Buffer: 0.1 N NaOH in 70% ethanol + 0.1% β-mercaptoethanol.
    c. Fixation buffer: 0.5% glutaraldehyde in denaturation buffer. Glutaraldehyde must be added to the denaturation buffer from previous step just before use.

Note: Methanol and glutaraldehyde are toxic if inhaled. Prepare and store in the chemical hood.

    d. Dehydration buffers: Two 70% ethanol washes followed by one 95% ethanol wash and a final 100% ethanol wash.
41. Stretch the DNA.
    a. To begin, mark denaturation times and cell line names on slides.
    b. Place a 22 × 22 mm coverslip at the center of the slide. The coverslips must be cleaned with ethanol or methanol just before use.
    c. Stretch the DNA underneath the coverslip (Figure 2).

Hint: The stretch of the DNA fibers can be checked under the microscope. For more information regarding good versus badly stretched DNA, please refer to the troubleshooting section below.

42. Remove the coverslip gently with a razor blade. Dip the slides into the Methanol + 0.1% β-mercaptoethanol buffer. Keep the slides in Methanol buffer for at least 10 min before denaturation.
43. Denature the slides with **Denaturation buffer** for a series of times: 8 min, 12 min, and 16 min.

Note: longer denaturation times can cause DNA breakage

44. Next, add glutaraldehyde to **Fixation buffer** and fix the slides with **Fixation buffer** for 5 min.
45. Dehydrate the slides by washing them with a series of ethanol washes of increasing concentrations: first 70% (# of 70% jars = # of fixation jars), second 70%, one 95% and one 100% ethanol wash. Wash the slides for 1–2 min each time.
46. Place the slides in the original Methanol + 0.1% β-mercaptoethanol jars for 60 min before proceeding with the hybridization step.
47. Turn on the dry heating block and adjust the temperature to 95°C to denature the probes for hybridization.
48. Prepare the hybridization master mix in a 15 mL falcon tube and mix in a rotator for at least 30 min (hybridization master mixture contains 40% formamide, 5 M NaCl, 10% SDS, 50% Dextran Sulfate, 1 M Tris pH 7.4, 1 μg/μL COT1 DNA and 10 μg/μL Salmon Sperm DNA).

Hint: Prepare approximately 3 mL for 16 slides.

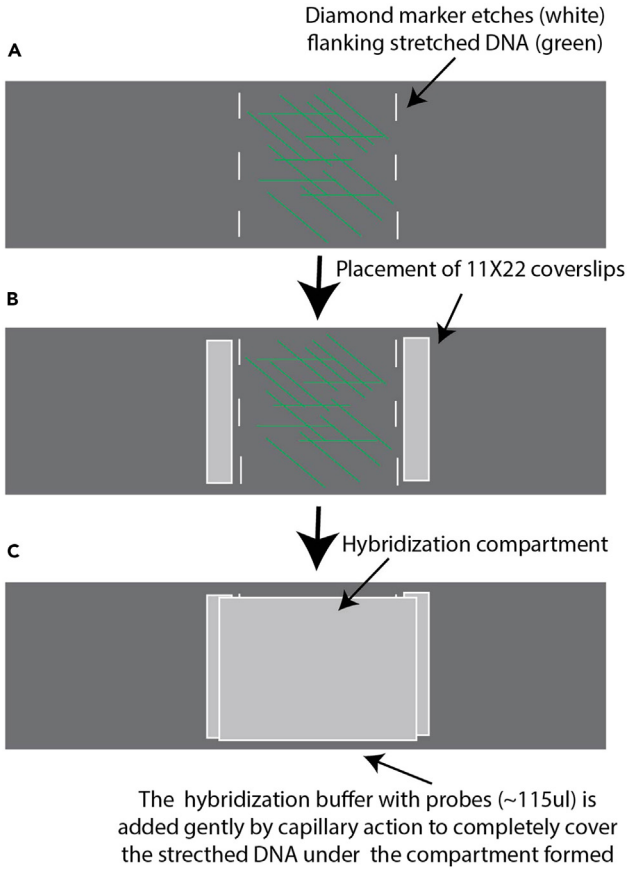

Figure 3. Assembly of the hybridization compartment

(A) Add hybridization buffer (~2 μL) onto the positions outside of the stenciled area containing the stretched DNA (green area)(B) place one 11 × 22 mm coverslip onto the buffer at each side. The 11 × 22 mm coverslips should be placed over the diamond glass marker stenciled regions to provide enough space for the hybridization compartment to be formed.

(C) Add hybridization buffer onto the two 11 × 22 coverslips, lay one 22 × 40 mm coverslip onto the 11 × 22 coverslips to form a compartmentfor hybridization with the probe.

49. After the slides have been in Methanol + 0.1% β-mercaptoethanol for 30 min, start preparing the hybridization-probe mixture in individual Eppendorf tubes by adding 2.5 μL biotinylated probe to 110 μL hybridization mixture.
50. Dry the slides a little by wiping with kimwipes gently on the back and the side of the slides.
51. Assemble the hybridization compartment by placing one 22 × 40 mm coverslip over two 11 × 22 mm coverslips as follows (see Figure 3A–C). Hybridize no more than 5 slides at once.
52. Denature the probe at 95°C for 5 min.
53. Pipette the probe into the compartment and place the slides in the humid chamber.
54. Incubate the slides at 37°C (using warm room or hybridization oven) in the humid chamber for 14–18 h.

Hint: During the denaturation step, you can assemble the hybridization compartment for the next 5 slides. At the same time, you can pipette the probe mixture onto the previously denatured 5 slides.

STEP 3: Immunodetection.

Day 5.

55. Prepare the post hybridization wash buffers in ddH_2O: 4x SSC + 40% formamide (2 Coplin Jars: 125 mL), 2x SSC + 1% SDS (2 Coplin Jars: 125 mL), 2x SSC + 0.1% IGEPAL (1 Coplin Jar: 60 mL), 4x SSC + 0.1% IGEPAL (4 Coplin Jars: 250 mL) and 1x PBS + 0.03% IGEPAL (2 Coplin Jars: 125 mL).
56. Prewarm the 4x SSC + 40% formamide buffer in the 45°C water bath for 1 h.
57. Remove coverslip from slide using a razor. Rinse slides sequentially in two jars of 2x SSC + 1% SDS. Transfer 5 slides at a time to the 4x SSC + 40% formamide buffer in the 45°C water bath.
58. Incubate slides in 4x SSC + 40% formamide for 5 min.
59. Next rinse the slides with 2x SSC + 0.1% IGEPAL at RT.
60. Wash sequentially in 4 jars of 4x SSC + 0.1% IGEPAL for 1–10 min each time at RT.
61. Next, rinse in 1x PBS + 0.03% IGEPAL at RT.
62. Wipe solution off with kimwipes on the back, sides, and front outside of the stenciled area, making sure that the DNA containing area does not dry.
63. Add 20 μL blocking buffer delivered on a 22 × 30 coverslip.
64. Incubate slides in the humid chamber at RT for 1 h or longer.

Note: Slides can be incubated in blocking buffer for longer than 1 h.

65. During this incubation, prepare the antibodies for immunodetection.
    a. Detection Mixture 1 (will be used for steps 66 and 69 so prepare 2X) - Dilute Avidin Alexa Fluor 350 conjugate antibody (1:15) with blocking buffer.
    b. Detection Mixture 2 (will be used for step 68) - Dilute Biotinylated Anti-Avidin (BAA) antibody (1:15) with blocking buffer.
    c. Detection Mixture 3 - Dilute Biotinylated Anti-Avidin (1:15), and mouse-anti-IdU (1:15) and rat-anti-CldU (1:15) with blocking buffer.
    d. Detection Mixture 4 – Dilute Avidin Alexa Fluor 350 conjugate antibody (1:15), Goat anti-Rat IgG Secondary Antibody Alexa Fluor 488 (1:15) and Goat anti-Mouse IgG Secondary Antibody, Alexa Fluor 568 (1:15) with blocking buffer.
66. Razor off the coverslip, add 15 μL of 1st detection mixture for each slide via a 22 × 30 cover slip. Incubate in the humid chamber at RT for 20 min.
67. Rinse off the coverslips in a jar of 1x PBS + 0.03% IGEPAL and wash a second time with 1x PBS + 0.03% IGEPAL CA-630. Drain and wipe off the liquid on the back, sides, and front outside of the stenciled area, making sure that the DNA containing area does not dry (wash step).
68. Add 15 μL of 2nd detection mixture. Incubate in the humid chamber at RT for 20 min.
69. Repeat wash step 67 and add 15 μL of 1strd detection mixture. Incubate in the humid chamber at RT for 20 min.
70. Repeat wash step 67 and add 15 μL of 3rd detection mixture. Incubate in the humid chamber at RT for 1 h.
71. Repeat wash step 67 andt add 15 μL of 4th detection mixture. Incubate in the humid chamber at RT for 1 h.

Note: Longer than 1 h incubation with the 4th detection mixture containing the secondary antibodies can cause high background.

72. Repeat step 67.
73. Seal the slides with a drop of ProLong Gold Molecular Probe on a 22 × 30 mm coverslip. Keep the slides at RT for 14–18 h to dry completely before imaging.

Optional: Slides can be checked on the same day after allowing ProLong Gold to set for at least 1 h.

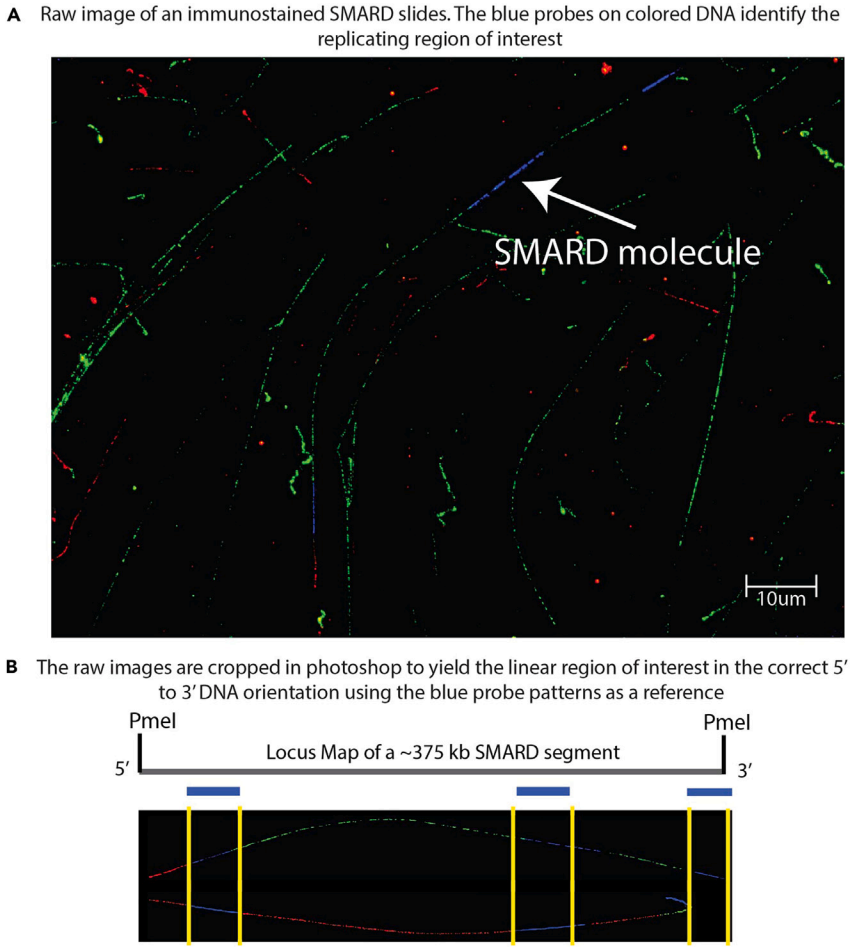

Figure 4. Raw and processed images after immunostaining

(A) Raw image of a completed SMARD slide.

(B) Processed DNA molecules that are aligned using a locus map to form a SMARD profile. Scale bar: 10 μm.

EXPECTED OUTCOMES

Immunodetection concludes the experimental steps of SMARD. The next stage involves data collection using an upright fluorescent microscope. The image depicted in Figure 4A is an example of a raw image from a completed SMARD slide. Each colored line in the image is a DNA molecule that is ~375 kb. The molecule present in Figures 4A and 4B contains three blue probes (two 40 kb and one 20 kb). Images are acquired from fields that contain molecules that are colored (red or green or red/green) and are bound by the blue probes. Image acquisition concludes when 40–60 tri-colored (blue/red/green) molecules are identified. These molecules represent temporal replication across the region of interest. The SMARD molecule imaging step is perhaps the most time-consuming step in SMARD and can take 2–6 weeks depending on the size of the segment being analyzed, amongst other factors mentioned below in the troubleshooting section. The longer the region of interest, the easier the molecules are to find since there is less non-specific DNA at that region of the pulse-field gel after enrichment. However, as the region size decreases, there is an increase in non-specific DNA of similar size leading to longer times for molecule identification. The images are then processed in Photoshop to orient the molecule in the same direction using the blue probes as a reference (Figure 4B). The reoriented molecules are arranged below the locus map, as demonstrated in Figure 4B, to form the final composite SMARD profile.

QUANTIFICATION AND STATISTICAL ANALYSIS

Using Excel, the percentage of origins, termination sites, and replication forks progressing in either direction independently can be quantified from the SMARD profiles. Frequently used replication origins can be detected in replication initiation zones as described before, such as at the *Oct4* gene *(POU5F1* gene).[15] However, replication origins that fire less frequently, as at the *FMR1* gene[2] and dormant origins, such as at the *FRA16D* gene locus,[7] can also be visualized by SMARD. In addition, regions of fork stalling can be detected. To determine replication fork stalling at specific genomic sites the number of replication forks for each 5 or 10 kb DNA segment are quantified from the SMARD profiles.[4,5,8] In addition, the average percentage of replication forks in the whole DNA segment can also be calculated by quantifying all replication forks per DNA segment (blue line).[4] The DNA fragments that contain more replication forks above this line show some degree of fork pausing or stalling. A minimum of 25 red/green molecules containing FISH probes are essential to detect a pause site and make conclusions about origin activity. However, an optimal replication profile would contain 40–70 red/green molecules containing FISH probes. Conclusions drawn from such a profile will yield the most reliable information.

LIMITATIONS

SMARD analysis depends on the availability of enzymes. Regions that can be analyzed with SMARD are limited to availability of restriction enzyme cutting sites that will generate a 100–400 kb segment and contains the region of interest. Only few enzymes are rare-cutting enzymes that can be used to generate such large DNA segments. In addition, if the cut site is in the region of interest you may have to analyze two DNA segments. Thus, the success of the SMARD experiments is dependent on multiple factors that include: an optimum number of cells in S-phase (since these constitute the colored molecules we see on the slide), good resolution of DNA by size in the pulse-field to ensure optimum enrichment of the fragment of interest, well stretched DNA (see Figure 5D), optimal probe hybridization and clean immunostaining with minimal background. Since only 15–40% of cells are actually replicating (in S-phase) in culture, a higher density of total DNA that is replicating will ensure a better chance of obtaining a good number of colored molecules on the finished SMARD slides.

TROUBLESHOOTING

Problem 1

If PCR results show that two slices contain your regions of interest, see step 35.

Potential solution

Proceed with the slice that gives you the brightest band to ensure the success of the subsequent steps.

Problem 2

The DNA is not properly stretched, because slides are too sticky (Figure 5A). See step 41.

Potential solution

Try to stretch again after 1–2 days. Keep the slides in the desiccator during that time.

Problem 3

Agarose left in the DNA (Figure 5B). See step 41.

Potential solution

- Melt the DNA again. Therefore, cut new slices from your stored agarose plugs.
- Then to ensure complete removal of storage buffer before melting, wash the slices with TE 8 three times at RT, rotating.

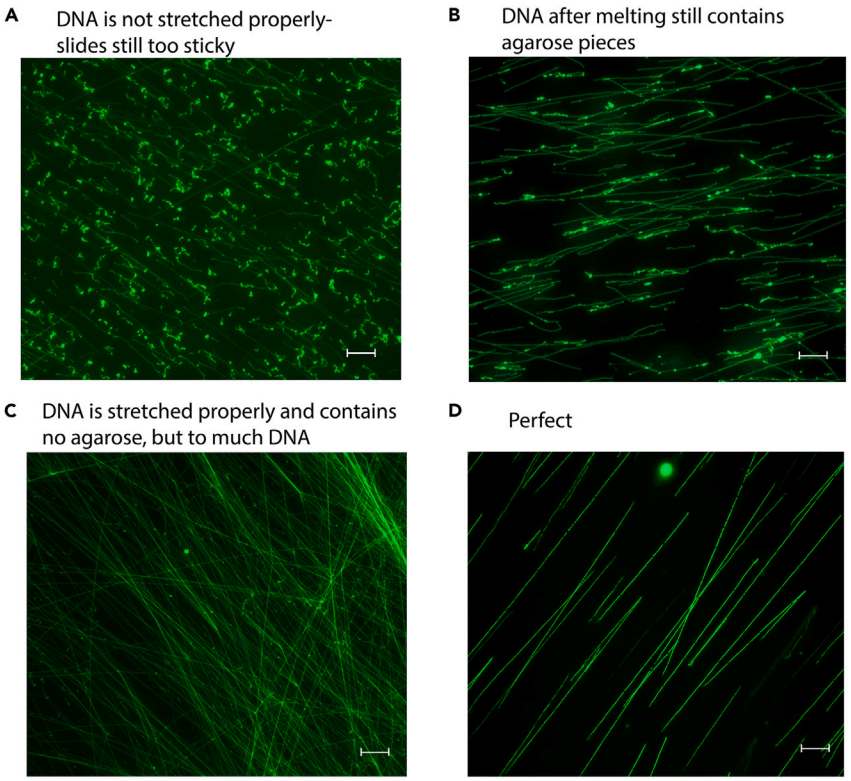

Figure 5. DNA stretched on the glass slides and potential problems

(A) Example of DNA that is not stretched well.

(B) Example of DNA which still contains undigested agarose pieces.

(C) Example of high concentration of DNA and (D) Example of perfectly stretched DNA with the correct DNA concentration. Scale bar: 10 μm.

Problem 4

The DNA is too concentrated (Figure 5C).

Potential solution

For too much DNA, dilute the sample by adding more agarose buffer (10–50 μL) before the melt.

Problem 5

If you have too little DNA in the sample (See step 41) or your cell lines contain less than 20% of S-phase cells, the time needed to collect the SMARD molecules for a full replication profile will be much longer. In average two weeks and preparations of around 20–30 slides are needed to collect 40 to 70 DNA molecules. Time and number of slides needed to obtain a full replication profile depends on the type of cell line, S-phase population, and number of cells in the plugs. However, too many cells in each plug could lead to less optimal cell lysis and enzyme digestion.

Potential solution

Repeat SMARD and increase number of cells per plug. Although only a final concentration of 1.5–2.0 × 10^6 cells/plug is recommended.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Jeannine Gerhardt (jeg2039@med.cornell.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate any datasets and codes.

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AUTHOR CONTRIBUTIONS

A.M. and J.G. contributed equally to this work.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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