

Chapter 2

Assaying for Radioresistant DNA Synthesis, the Hallmark Feature of the Intra-S-Phase Checkpoint Using a DNA Fiber Technique

Amanda W. Kijas and Martin F. Lavin

Abstract

During S-phase the cell replicates its DNA which is critical to maintaining the integrity of the genome and cell survival amidst damaging events. The cell is equipped with a series of checkpoints to slow progress throughout the cycle and facilitate DNA repair. Ataxia telangiectasia mutated (ATM), defective in the human genetic disorder ataxia-telangiectasia (A-T), is the key to initiating a signaling cascade activating the intra-S-phase checkpoint. This was first identified in A-T cells as radioresistant DNA synthesis using ^{14}C thymidine and ^3H thymidine to pulse label replicating cells before and after damage. This technique has been superseded now by direct labeling that distinguishes DNA replication initiations from ongoing sites of replication which are the target for the intra-S-phase checkpoint. Here, we outline how sites of replication are pulse labeled with two different thymidine analogs before and after damage. The DNA is then stretched out as fibers for immunolabeling to enable visual distinction and counting of ongoing replication forks from new initiations. It is this extent of new initiations that is used to detect the intra-S-phase checkpoint after DNA damage.

Key words Intra-S-phase checkpoint, Radioresistant DNA synthesis, DNA fibers, Replication

1 Introduction

During S-phase cells replicate their genome, creating duplicate sister chromatids in the preparation for cell division. Mammalian cells replicate their genome in a coordinated fashion firing multiple replicons of varying sizes, 20–400 kb, throughout S-phase. These replication forks move bidirectionally at about 2–3 kb per minute, depending on the stage of S-phase [1].

To preserve the integrity of this replication process cells have mechanisms to detect aberrant or unreplicated DNA and respond in a coordinated manner to initiate S-phase checkpoints and repair, or in more extreme cases of damage, initiate cell death. The intra-S-phase checkpoint induced by double strand breaks (DSB), signals

to halt new replicon initiations, distinct from the other S-phase checkpoints that affect actively progressing forks [2].

Defects in this intra-S-phase checkpoint were first discovered in ataxia telangiectasia (A-T) patient cells that were unable to reduce the rate of replication after exposure to ionizing radiation and was termed, radioresistant DNA synthesis, RDS [3, 4]. These original experiments utilized two forms of radiolabeled thymidine ^{14}C and ^3H to consecutively pulse label DNA synthesis pre and post DSB-induced damage. This yielded a measure of total synthesis post damage, thus only a crude measure for new initiations. Since these initial observations linking mutant ATM to defects in the cell cycle, two members of the MRE11-RAD50-NBN (MRN) complex, MRE11 and NBN, were subsequently identified as key in the activation of the intra-S-phase checkpoint [5, 6]. It was demonstrated later that the key activation events involve a signaling cascade through which cyclin-dependent kinases and cyclins become activated to inhibit progress through the cell cycle [7]. Since these early reports, a RAD50-deficient patient was identified and the patient's fibroblasts also displayed RDS [8, 9]. However, in that case the methodology used to determine the effect of radiation-induced DSB on the level of inhibition on new origin firing compared to control cells was a DNA fiber assay that directly visualizes new initiations after damage. This same fiber assay-based methodology has since been applied to A-T lymphoblastoid cells that reported the effects of multiple autophosphorylation sites of ATM in the activation of the intra-S-phase checkpoint [10], and more recently has also been applied in the characterization of A-T stem cells [11, 12].

DNA fibers have been widely used for investigations of replication, with pioneering work by Cairns [13] and Huberman and Riggs [14] who used autoradiography to visualize [^3H]thymidine-labeled DNA, showing that it can be stretched to a resolution comparable to native B-DNA at $0.34\text{ }\mu\text{M}/\text{kb}$ [15]. These techniques have since been used to utilize thymidine analogs in combination with fluorescently labeled antibodies where they have also been applied to high-resolution gene mapping [16].

Thymidine nucleotide analogs such as 5-Chloro-2'-deoxyuridine and 5-Iodo-2'-deoxyuridine are transported into the cell and then incorporated in an uninhibited manner into newly synthesized DNA, enabling direct pulse labeling of actively replicating sites in the genome. The subsequent ability to differentially detect these analogs by immunofluorescence in DNA fiber spreads allowing high-resolution analysis of replication fork speed, termination, as well as new replicon initiations. These techniques have been applied to investigate replication dynamics after DNA damage in cells where the proportion of new initiations (shown as red-only tracks, Fig. 1a) to ongoing tracks (shown as green and red) before and after damage was determined. The two early studies used two

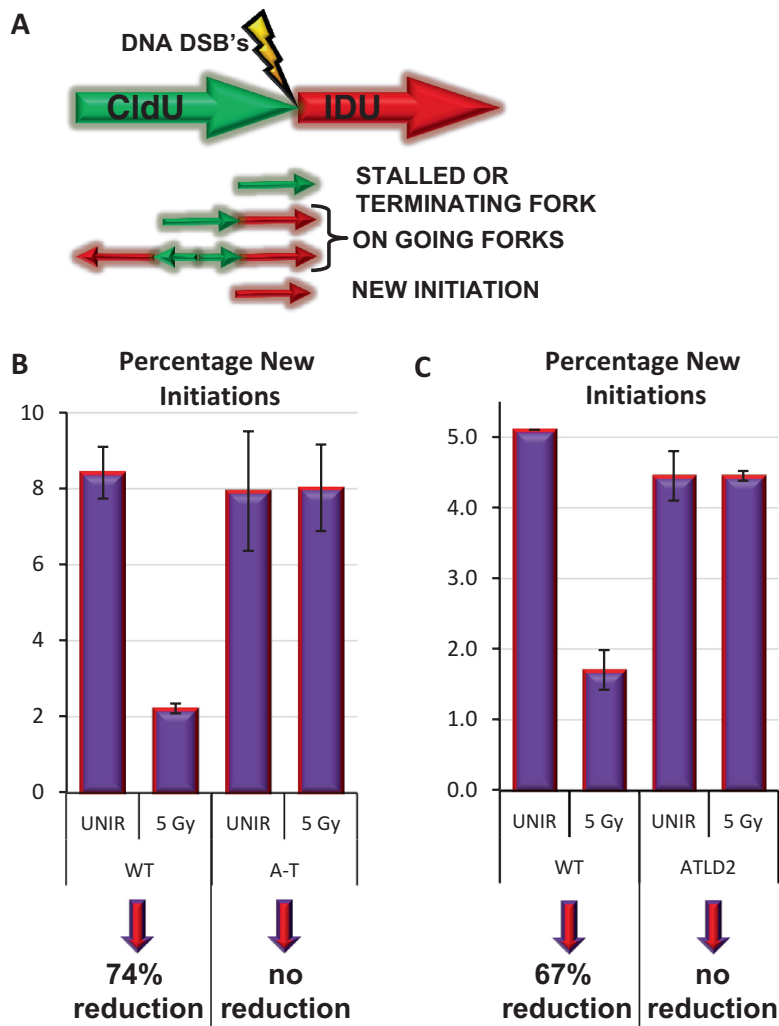


Fig. 1 (a) Replication forks are consecutively pulse labeled with 5-Chloro-2'-deoxyuridine (CldU) and 5-Iodo-2'-deoxyuridine (IDU). Double strand break (DSB) inducing agent introduced at the end of CldU labeling enables the subsequent scoring of ongoing forks (*green* and *red tracks*) from new initiations (*red-only tracks*). (b) A-T lymphoblastoid cells (A-T) and corrected lymphoblastoid cells (WT) expressing a wild-type ATM cDNA were consecutively labeled with CldU and IDU. Cells were either left as unirradiated (UNIR) or irradiated with 5 Gy at the end of the CldU labeling (*see* Fig. 1a). Cells were then treated as outlined in the lymphoblastoid method below to spread the DNA fibers and immunolabel for the thymidine analogs. The proportion of ongoing (*green* and *red tracks*) and new initiations (*red-only tracks*) was scored. The percentage of new initiations was calculated as the number of new initiations/(ongoing + new initiations) \times 100. A total of over 600 forks were scored for each treatment for each cell line. The standard deviation of the mean is shown, $n = 3$. (c) ATLD2 fibroblasts (ATLD2) and corrected fibroblasts (WT) expressing a wild-type MRE11 cDNA were consecutively labeled with CldU and IDU. Cells were either left as unirradiated (UNIR) or irradiated with 5 Gy at the end of the CldU labeling (*see* Fig. 1a). Cells were then treated as outlined in the fibroblast method below to spread the DNA fibers and immunolabel for the thymidine analogs. The proportion of ongoing (*green* and *red tracks*) and new initiations (*red-only tracks*) was scored. The percentage of new initiations was calculated as the number of new initiations/(ongoing + new initiations) \times 100. A total of over 460 forks were scored for each treatment for each cell line. The standard deviation of the mean is shown, $n = 2$

different damaging agents, but both observed inhibition of new initiations in cells after ultraviolet light C exposure [17] and camptothecin treatments [18].

We describe below the application of this fiber-based methodology to assess new initiations after 5 Gy irradiation that induces approximately 180 double strand breaks in DNA. This is described for both fibroblast and lymphoblastoid cell lines. The results generated when applied to A-T and corrected A-T cells as a control are shown in Fig. 1b. The data very clearly demonstrate the radioresistant-based nature of the intra-S-phase check point failure in A-T lymphoblastoid cells. No change in new initiations is observed after damage in an A-T cell line, but corrected A-T cells expressing a full-length ATM cDNA (WT) show a 70% reduction, demonstrating activation of the intra-S-phase checkpoint (Fig. 1b). Figure 1c demonstrates the technique applied to ataxia telangiectasia like disorder (ATLD) cells that have no functional MRE11 protein. Here, an ATLD2ht fibroblast cell line was compared to a corrected ATLD2ht cell line expressing a full-length MRE11 cDNA (WT). As observed for the A-T cell line, a comparable level of radioresistant DNA synthesis is observed where there is no change in new initiations after damage in the ATLD cells. The corrected ATLD cells, on the other hand, expressing a full-length MRE11 cDNA (WT), show activation of the intra-S-phase checkpoint with a 67% decrease in new initiations after damage (Fig. 1c).

2 Materials

2.1 Thymidine Nucleotide Analog Incorporation

1. Media: Dilute two parts of cell growth media (usually 12% fetal calf serum supplemented DMEM) with preconditioned media (media that is harvested from an actively growing culture). This is to be used for all media containing steps.
2. Prepare aliquots of 2 mM 5-Iodo-2'-deoxyuridine (IDU), MW = 354.1. Preheat 40 mL sterile high-purity water to 60 °C. Add 28.34 mg IDU, vortex vigorously, then wrap in foil, and place on a rotating platform to dissolve. Make 1 mL aliquots once dissolved, store at -20 °C.
3. Prepare aliquots of 2 mM 5-Chloro-2'-deoxyuridine (CldU), MW = 262.6. Preheat 40 mL sterile, high-purity water to 60 °C. Add 21.01 mg CldU, vortex to dissolve, then make 0.5 mL aliquots and store at -20 °C.
4. Optional: Prepare 10 mM Thymidine aliquots.
5. Versene.
6. Trypsin-EDTA.
7. PBS.

8. TBST.
9. Trypan blue (for cell counting).
10. Plasticware for tissue culture (6-well plates, 1.5 mL tubes, 5 and 10 mL pipettes, 1 mL pipette tips).

2.2 Preparing DNA Fibers

1. Menzel–Glaser Superfrost plus or ULTRA plus, positively charged slides.
2. Lysis solution: 0.5% sodium dodecyl sulfate, 200 mM Tris–HCL pH 7.4 and 50 mM EDTA.
3. 15–20° ramp and a large glass plate (capable of holding multiple slides). We made one by marking of 20° with protractor on a small styrofoam box. Then cut with razor blade and used an old gel casting glass plate about 20 × 20 cm.
4. Vertical glass staining jars/glass coplin jars. Vertical eight slide jars are good size.
5. Ice-cold methanol/acetic acid (3:1).
6. Lint-free paper towel.

2.3 Immunolabeling of Incorporated CldU and IDU at Sites of DNA Synthesis

1. Vertical glass staining jars/glass coplin jars. (Vertical eight slide jars are good size).
2. 50 × 22 mm cover slips for mounting slides.
3. 2.5 M HCL.
4. PBS.
5. PBS containing 0.01% Tween 20 (0.01% PBST).
6. PBS containing 0.05% Tween 20 (0.05% PBST).
7. Blocking buffer (10% BSA in 0.01% PBST).
8. Antibody dilution buffer, 1% BSA in 0.01% PBST.
9. High stringency wash buffer (1 L: 29.2 g sodium chloride, 4.44 g Tris–HCL adjust to pH 8 with NaOH and then add Tween20 to 0.5% final concentration).
10. Flat plastic tray for washing and staining glass slides.
11. Parafilm cut to the size of the slide (excluding super frosted region).
12. Rat monoclonal anti-bromodeoxyuridine antibody (Rat monoclonal [BU1/75] catalog number Ab6326, Abcam).
13. Goat anti-rat Alexa488 conjugated secondary antibody.
14. Mouse monoclonal anti-bromodeoxyuridine antibody (Anti-BrdU (B44), catalog number 347580, Becton Dickinson).
15. Goat anti-mouse Alexa594 conjugated secondary antibody.
16. Mounting media.

3 Methods

3.1 For Fibroblast Cell Lines

3.1.1 Seeding of Cells

Cells are split 1–3 days before the experiment such that they will be sub-confluent on the day of experiment when you will need at least 1×10^5 cells. Seed to generous media volume (5 mL for 6-well plate) as this will be reused during the labeling experiment as preconditioned media.

3.1.2 Thymidine Nucleotide Analog Incorporation

1. On the morning of the experiment collect excess media from plated cells (about 2.5 mL/well). Dilute this preconditioned media (1/3) with fresh media (2/3) and then place in an incubator to warm to 37 °C and equilibrate to 5% CO₂, so lid needs to be loose or use small filter topped flask. This is to be used for all subsequent media containing steps.
2. Defrost aliquots of CldU (2 mM) and IDU (2 mM), thymidine (10 mM). Prepare 2 mL per well labeling media of each 20 μ M CldU and 80 μ M IDU, as well as 1 mL per well of each 100 μ M thymidine and rinse media.
3. Label cells consecutively with 20 μ M CldU media for 20 min including a mock or 5 Gy treatment just prior to the removal of CldU (*see Note 1*).
4. Remove all media and rinse consecutively with 100 μ M thymidine media (1 mL) and then rinse media (1 mL), removing all residual media between each addition. This can be replaced with just two washes with rinse media, the presence of thymidine does not seem essential for the change over between CldU and IDU.
5. Add 80 μ M IDU media for a further 20 min. As the time after CldU removal and IDU addition must be kept to a minimum, the thymidine rinse can be removed prior to performing one 2 mL media rinse, which saves time when processing multiple samples.
6. Remove media, rinse twice with versene, then add trypsin-EDTA in small volume (about 600 μ L per well) until cells detach fully with a gentle tap.
7. Add cold fetal calf serum or fetal bovine serum to neutralize trypsin and pellet cells in 1.5 mL Eppendorf tubes (spinning at $400 \times g$ for 5 min at 4 °C).
8. Remove trypsin-FCS and rinse cells once with ice-cold PBS.
9. Resuspend cell pellet with 50 μ L ice-cold PBS. Leave cells on ice and perform trypan blue cell count using hemocytometer. Adjust volume to approximately $1\text{--}2 \times 10^6$ cells per mL with ice cold PBS.

3.1.3 Preparing DNA Fibers

DNA fibers are made following the approach by Parra and Windle [16] where labeled DNA fibers are stretched out by gravity as the DNA solution slides down a charged slide.

1. Label slides and set onto clean dust-free flat bench top (check with level).
2. Place 2 μL of cells ($1\text{--}2 \times 10^6$ cells/mL) at the top of a Menzel-Glaser Superfrost plus or ULTRA plus (positively charged) slide, as shown in Fig. 2a (*see Note 2*).
3. Add 8 μL of lysis solution to the cell droplet sitting at the top of the slide and gently pipette up and down once to ensure mixing (Fig. 2a). Allow the cells to lyse in the droplet for 8–10 min without disturbing the slides (*see Note 3*).

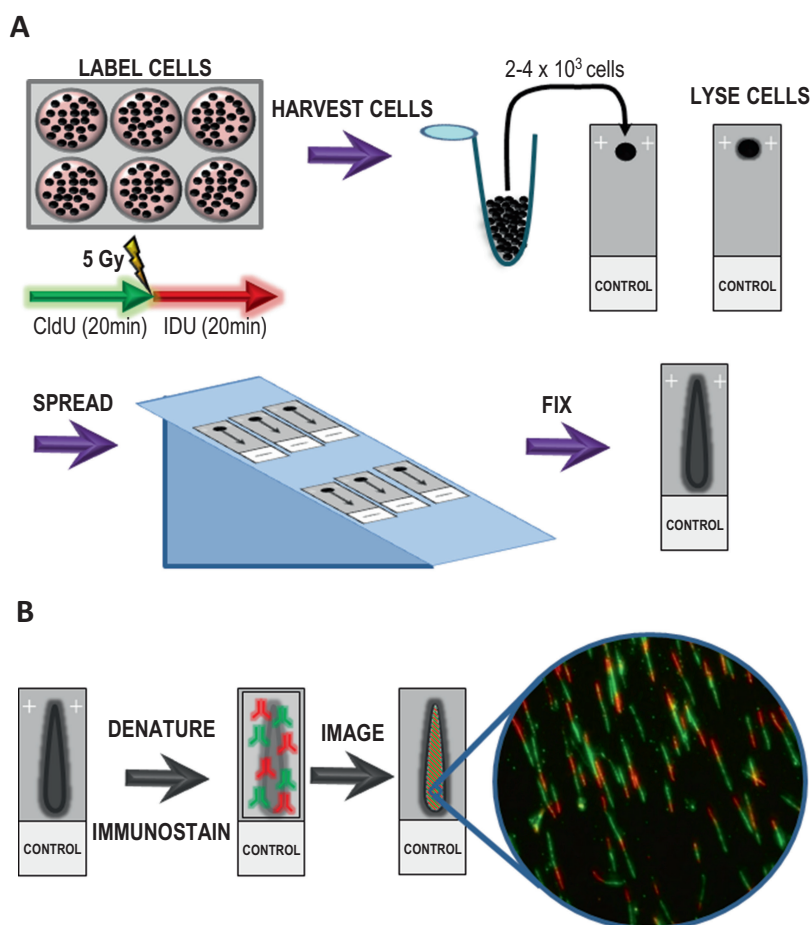


Fig. 2 (a) Cells are seeded to subconfluent density and replication forks consecutively pulse labeled with CldU and IDU. Cells were then harvested and lysed using SDS on positively charged slides before spreading by gravity at 15–20°. Stretched DNA fibers were then fixed in methanol acetic acid before storage. **(b)** DNA fibers were denatured in HCL, before sequential immunolabeling with antibodies recognizing the two thymidine analogs, CldU and IDU. Ongoing forks and new initiations were imaged using fluorescence microscopy

4. Pick up slides carefully so as not to disturb the droplet and place onto a large glass plate (capable of holding multiple slides), tilted to 15–20°. This will allow gravity to slowly stretch out the labeled DNA into fibers down the length of the slide (Fig. 2a). Keep the droplets running roughly down the center of the slide to make subsequent immunolabeling and imaging more straightforward, knowing where to expect the DNA fibers to be.
5. Leave slides to air dry and then fully immerse into glass coplin jar containing ice-cold methanol/acetic acid (3:1) for 10 min (*see Note 4*).
6. Remove slides, draining well before placing DNA side up onto lint-free paper towel in fume hood. Store the slides at 4 °C for at least 1 day (to age the DNA) before immunolabeling.

3.1.4 Immunolabeling of Incorporated CldU and IDU at Sites of DNA Synthesis

The immunolabeling follows the approach of Aten et al. [19] where the duplex DNA fibers are first denatured, exposing the CldU and IDU epitopes before immunolabeling with antibodies recognizing the individual analogs.

1. Immerse the slides into a glass coplin jar containing 2.5 M HCl for 1 h to denature the DNA duplex.
2. Transfer slides to a fresh coplin jar containing PBS, drain, then rinse a further three times.
3. Perform a final rinse with PBS containing 0.01% Tween 20 (PBST). Place the slides in a flat plastic tray.
4. Add 200 µL of blocking buffer to slides, pipetting it down the center of the slide where the DNA has run. Spread this evenly by placing a piece of parafilm cut to the size of the slide, (excluding super frosted region) setting it down first at the top of the slide and then laying it progressively down the length of the slide trying to avoid air bubbles. This will spread the blocking buffer evenly over the full slide surface.
5. Dilute all antibodies in Antibody dilution buffer (1% BSA, 0.01% PBST) and use sequentially as follows:
 - First primary: rat monoclonal anti-bromodeoxyuridine antibody (Abcam), 1/70 to label CldU for 1.5 h.
 - First secondary: goat anti-rat Alexa488 conjugated secondary antibody, 1/300 for 1 h.
 - Second primary: mouse monoclonal anti-bromodeoxyuridine antibody (Becton Dickinson), 1/8 to label IDU for 1 h.
 - Second secondary: goat anti-mouse Alexa594 conjugated secondary highly cross absorbed antibody, 1/300 for 1 h.
6. Prepare 90 µL of each antibody dilution for each slide you are labeling (*see Note 5*). Pipette antibody dilution down the center of the slide where the DNA has run. Spread this evenly

over the slide surface by placing a piece of parafilm cut to the size of the slide, (excluding super frosted region) setting it down first at the top of the slide and then laying it progressively down the length of the slide trying to avoid air bubbles. This will spread the antibody evenly over the full slide surface.

7. Between antibodies rinse once and wash 2×10 min in 0.05% PBST, with the exception after second primary (mouse anti-bromodeoxyuridine antibody) where a high stringency wash is used for 9 min (no longer or may experience loss of signal).

3.1.5 Mounting and Imaging of Labeled DNA Fibers

1. Perform a final rinse in PBS and drain slides well. For each slide dry the back of the slide and then place long edge down on lint-free tissue to drain.
2. Try to dot mounting media down the center of the slide (DNA track). Using forceps and finger slowly lower 50×22 mm cover slip down the length of the slide.
3. Use fluorescent microscope to follow labeled DNA tracks scanning down the slide, using 594 channel as the signal is usually stronger for IDU staining. You will also need a 488-channel filter for CldU detection. Magnification: 63 \times and or 40 \times lenses.
4. Score the proportion of ongoing (green and red tracks) and new initiations (red-only tracks). The percentage of new initiations is calculated as the number of new initiations/(ongoing + new initiations) $\times 100$ for each treatment.

3.2 METHODS for Lymphoblastoid Cultures

3.2.1 Dilution of LCL Cultures

1. Cells are split 1 day before experiment to densities of about $3\text{--}4 \times 10^5$ cells/mL such that they will be at about $6\text{--}8 \times 10^5$ cells/mL on the day of experiment, when you will need 200 μ L of cells per treatment.

3.2.2 Nucleotide Analog Incorporation

1. On the morning of the experiment collect excess media from diluted cells (preconditioned media). Dilute this preconditioned media (1/3) with fresh media (2/3) and then place in an incubator to warm to 37 °C and equilibrate to 5% CO₂, so the lid needs to be loose or use a small filter topped flask. This is to be used for the dilution of the IDU and you will need 5 mL per sample.
2. Defrost aliquots of CldU (2 mM) and IDU (2 mM). Prepare 100 μ M IDU stock (5 mL per sample) in media and put to warm at 37 °C, and equilibrate to 5% CO₂. Also put versene on ice.

3. Label cells consecutively with 20 μM CldU (2 μL of 2 mM stock per 200 μL of cells for each sample) for 20 min at 37 °C and 5% CO_2 , including a mock or 5 Gy treatment during the last minutes of CldU labeling. Then add 5 mL of 100 μM IDU for a further 20 min (at 37 °C, 5% CO_2).
4. Add 5 mL ice-cold versene supplemented with 10 mM EDTA pH 8, pellet cells by centrifugation, and rinse once with ice-cold versene.
5. Resuspend cell pellet in 50 μL of ice-cold PBS. Leave cells on ice while doing cell counts using trypan blue and hemocytometer. Adjust volume to be approx. $1\text{--}2 \times 10^6$ cells per mL using ice cold PBS.

3.2.3 Preparing DNA Fibers

Treat the same as outlined above for fibroblasts.

3.2.4 Immunolabeling of Incorporated CldU and IDU at Sites of DNA Synthesis

Treat the same as outlined above for fibroblasts.

3.2.5 Imaging of Labeled DNA Fibers

Treat the same as outlined above for fibroblasts.

4 Notes

1. Take into account your irradiator location and output for timing of CldU labeling. I routinely start irradiating at 15 min, to give me time to complete irradiation and return to lab within 20 min CldU labeling.
2. The number of cells needs to be optimized depending on the percentage you expect to be in S phase. If you are using rapidly dividing cells, you will need to supplement the labeled cells with unlabeled cells 1:1, you should not need to do this with control and A-T cells.
3. The time varies greatly depending on air conditioning and temperature (affecting droplet drying time), and cell number (affecting the viscosity of the droplet, the more DNA the more viscous). Plan to run several extra slides to get this right. The droplet should migrate slowly, if it migrates too fast, leave the droplet to dry a couple of minutes longer before tilting to 15–20°. If droplet does not reach the bottom of the slide or migrates too slowly (a few minutes), then reduce the droplet cell lysis time by a minute or two, or increase lysis solution to 10 μL .
4. If the DNA track left by the droplet has dried but a droplet at the base of the slide is still sitting there, the corner of a lint-free tissue

can be used to touch into the base of droplet (superfrosted end); this sucks of some of the excess fluid, to enable faster drying and stops the formation of a crusty region at the bottom.

5. If using other primary antibodies raised against these thymidine analogs, you will have to confirm their specificity.

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