Chapter 3

High-Resolution Analysis of Mammalian DNA Replication Units

Vadim O. Chagin*, Marius Reinhart*, and M. Cristina Cardoso

Abstract

Genomic DNA of a eukaryotic cell is replicated once during the S-phase of the cell cycle to precisely maintain the complete genetic information. In the course of S-phase, semiconservative DNA synthesis is sequentially initiated and performed at thousands of discrete patches of the DNA helix termed replicons. At any given moment of S-phase, multiple replicons are active in parallel in different parts of the genome. In the last decades, tools and methods to visualize DNA synthesis inside cells have been developed. Pulse labeling with nucleotides as well as detecting components of the replication machinery yielded an overall picture of multiple discrete sites of active DNA synthesis termed replication foci (RFi) and forming spatio-temporal patterns within the cell nucleus. Recent advances in fluorescence microscopy and digital imaging in combination with computational image analysis allow a comprehensive quantitative analysis of RFi and provide valuable insights into the organization of the genomic DNA replication process and also of the genome itself. In this chapter, we describe in detail protocols for the visualization and quantification of RFi at different levels of optical and physical resolution.

Key words 3D-SIM, Confocal microscopy, DNA replication, Fluorescent protein, High-resolution fluorescence microscopy, Immunofluorescence staining, Nucleotide incorporation, Replication foci

1 Introduction

Precise and complete duplication of genome is essential for normal proliferation of cells. Eukaryotic cells duplicate their genome in the course of S-phase of the cell cycle by sequential initiation of DNA synthesis in multiple genomic locations. Stretches of DNA that are replicated from a single initiation event are termed replicons ([1]; reviewed in [2]). At any given moment of S-phase, many replicons are synthesized in multiple locations in parallel.

In addition to labeling and detecting the protein components of the DNA replication machinery, the high processivity of the replicative DNA polymerases allows visualization of the synthesized DNA by incorporation of nucleotide analogs [3–5].

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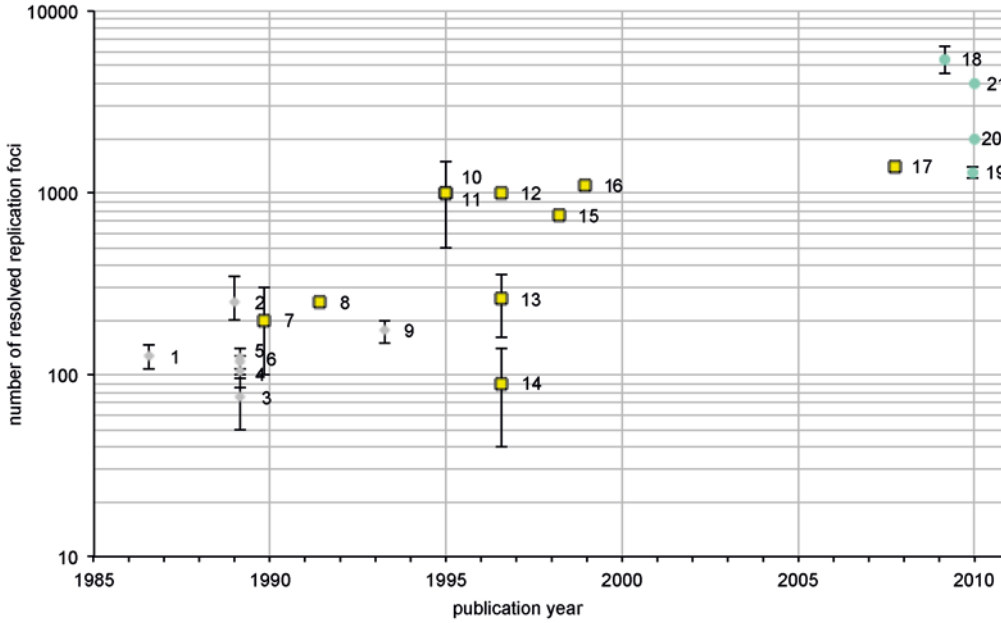
At the cellular level, active sites of ongoing DNA synthesis are manifested by focal incorporation of the nucleotides and accumulation of the replication machinery proteins and referred to as replication foci—RFi [3, 6, 7]. Quantification and analysis of RFi characteristics have been widely used in DNA replication studies (*see* Fig. 1 and references therein [3, 8–12, 4, 13–20]). From such studies, it was established that genome replication follows a series of subnuclear spatial RFi patterns, which roughly trail the chromatin epistate, with the condensed constitutive heterochromatin replicating in the second half of S-phase [21, 8, 9, 7].



#	replication foci numbers	year, month	cell line		reference	imaging method
			name	origin/description		
1	126+-18,8	1986, Aug	3Y1-B (IMR-90|100T)	rat embryonic fibroblast	Nakamura et al.	wide field
2	150-300 (~250)	1989, Jan	3T3	mouse fibroblast cells	Nakayasu and Berezney	wide field
3	<100 (S5)	1989, Mar	MCF-7 | cov362c14 | cov86044 (S5)	human cancer cell	van Dierendonck et al.	wide field
4	85-127 (S1)	1989, Mar	MCF-7 (S1)	human cancer cell	van Dierendonck et al.	wide field
5	107-141 (S1)	1989, Mar	cov362c14 (S1)	human cancer cell	van Dierendonck et al.	wide field
6	96-138 (S1)	1989, Mar	cov86044 (S1)	human cancer cell	van Dierendonck et al.	wide field
7	100-300	1989, Nov	Xenopus (sperm nuclei in egg extract)	in vitro fertilized Xenopus eggs	Mills et al.	CLSM
8	250	1991, Jun	3T3 (S1)	mouse fibroblast cells	Fox et al.	CLSM
9	150-200	1993, Apr	HeLa	human cervical cancer cell	Hozák et al.	wide field
10	500-1500	1995, Jan	K562	human myelogenous leukemia	Tomilin et al.	CLSM
11	500-1500	1995, Jan	MCF-7	human breast cancer	Tomilin et al.	CLSM
12	>1000	1996, Aug	3T3	mouse fibroblast cells	Berezney et al.	CLSM
13	160-360 (SE)	1996, Aug	V79 (SE)	chinese hamster fibroblasts	Manders et al.	CLSM
14	40-140 (SL)	1996, Aug	V79 (SL)	chinese hamster fibroblasts	Manders et al.	CLSM
15	750	1998, Mar	HeLa	human cervical cancer cell	Jackson and Pombo	CLSM
16	1100	1998, Dec	3T3	mouse fibroblast cells	Ma et al.	CLSM
17	1400	2007, Oct	GM05389	human fetal lung fibroblasts	Gotoh	CLSM
18	5460 ± 923	2009, Mar	HeLa	human cervical cancer cell	Ligasová et al.	EM
19	~1200-1400 (SE)	2009, Dec	MRC5	normal human fetal lung fibroblast	Cseresnyes et al.	STED
20	~2000 (SL)	2010, Jan	C2C12 (SL)	mouse myoblast cells	Baddeley et al.	SMI
21	4000 (SL)	2010, Jan	C2C12 (SL)	mouse myoblast cells	Baddeley et al.	3D-SIM

Fig. 1 Historical progress in DNA replication foci quantification

Attempts to quantify RFi numbers in cells and to relate them with estimations of total replicons per genome by DNA fiber analysis ([22]; reviewed in [23]) were initiated in the mid-1980s and are summarized chronologically in Fig. 1. With the advent of laser scanning confocal microscopy as well as digital imaging and image analysis in the mid-1990s, the numbers of RFi increased severalfold. In the last years, the development of super-resolution fluorescence microscopy has once again boosted the numbers of RFi (Fig. 1). The precision of RFi numbers analysis can be improved by (1) changing physical resolution of the cellular preparations [24], (2) increasing optical resolution of the cellular images [20], and (3) improving accuracy of RFi quantification [16]. In general, advances in each of those aspects of RFi analysis have led to higher numbers of RFi identified (Fig. 1). At the highest precision of quantification, RFi numbers were comparable with the theoretical estimated numbers of active replicons [20]. Quantification of RFi, therefore, can provide detailed information regarding organization of genome duplication at a molecular level.

In this chapter, we describe in detail approaches for labeling and for statistically sound quantification of DNA replication units at different levels of optical and/or physical resolution. Sequential labeling with different nucleotide analogs or direct analysis of RFi in live cells expressing fluorescent replication factors [25] and/or after incorporation of fluorescent nucleotide analogs can be used to elucidate dynamic aspects of the DNA replication process. Depending on the particular question to be addressed, the researcher can select an optimal combination of steps of the protocols presented to quantitatively analyze DNA replication units with the necessary level of accuracy, while minimizing experimental material and time. The protocols presented can be adapted and used in the analysis of intracellular distribution of a variety of biomolecules involved in localized molecular processes, e.g., DNA repair or transcription.

2 Materials

2.1 (A)Synchronously Growing Cell Cultures

1. Standard cell culture equipment.

2.2 RFi Labeling by Cell-Permeable Nucleotide Incorporation

1. 1,000× stock solution of halogenated nucleotide analog, e.g., BrdU (5-bromo-2′-deoxyuridine): 10 mM BrdU in ddH_2O, filter sterilized, aliquoted, and stored at –20 °C; *or* 1,000× stock solution of alkyne labeled nucleotide, e.g., EdU (5-ethynyl-2′-deoxyuridine): 10 mM EdU in DMSO (*see* **Note 1** for advantages and disadvantages of different nucleotide analogs).
2. Standard cell culture media and supplements.

2.3 RFi Labeling by Cell-Impermeable Nucleotide Incorporation

1. Borosilicate coverslips, round or square, 0.152 mm thick.
2. Standard cell culture media and supplements.
3. Parafilm.
4. "Humid chamber": Petri dish diameter 150 mm wrapped with aluminum foil and having a piece of wet absorbent paper inside [29].
5. Nucleotides: 10 mM stock solution of fluorescently labeled nucleotide, e.g., Cy3-dUTP**.**
6. A hypodermic needle.
7. Forceps.
8. Inverted microscope.

2.4 3D-Preserved Cell Preparations

1. PBS, 1×: (8 g NaCl , 0.2 g KCl_2, 17 g $Na_2HPO_4 \times 7H_2O$, 0.2 gKH_2PO_4 per liter of ddH_2O, pH ~ 6.8, prepare from autoclaved 10× stock solution).
2. 0.05–0.1 % Triton X-100 solution in PBS.
3. CSK buffer: 10 mM PIPES-KOH, pH 7.0, 100 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$.
4. 0.1 % TritonX-100 solution in CSK buffer.
5. 36.5–38 % formaldehyde in H_2O.
6. PBST: 1× PBS, 0.01 % Tween.

2.5 Hypotonically Resolved RFi

1. PBST: 0.01 % Tween in 1× PBS.
2. Trypsin/EDTA solution: 0.025 Trypsin, 0.01 % EDTA in PBS.

2.5.1 Harvesting Cells

3. Cell culture medium or fetal calf serum.
4. 15 ml conical tube.

2.5.2 Hypotonic Treatment

1. Hypotonic solution: 50–75 mM KCl in ddH_2O. Prepare fresh.
2. 99.8 % methanol.
3. ≥99.7 % acetic acid.

2.5.3 Fixation

1. MeAA solution: three volumes of cold methanol and one volume of acetic acid.

2.5.4 Slide Preparation

1. Pre-cleaned microscope slides.
2. MeAA solution (Subheading 2.5.3, **item 1**).
3. Pasteur pipette.
4. Water bath heated to 60–75 °C.
5. Heating table with regulated temperature or Bunsen burner or alcohol lamp.

2.6 RFi Detection

Protein components of the replication machinery can be labeled by expressing fluorescently tagged proteins, e.g., GFP-PCNA using:

2.6.1 RFi Detection by Tagged Replication Factors

(a) Transient expression: Corresponding expression construct (vector) and conventional (PEI, $CaPO_4$) or commercially available transfection protocols/kits for introducing vectors into cells.

(b) Stable expression: A number of mammalian cell lines stably expressing replication proteins have been reported [7, 26].

2.6.2 RFi Detection of Native (Untagged) Replication Proteins

1. Borosilicate coverslips, round or square, 0.152 mm thick.
2. Standard cell culture media and supplements.
3. Parafilm.
4. "Humid chamber": Petri dish diameter 150 mm wrapped with aluminum foil and having a piece of wet absorbent paper inside [29].
5. 0.1 % Triton X-100 solution in PBS (*see* Subheading 2.4, **item 2**).
6. 1 % BSA or 0.2 % fish skin gelatin in PBS.
7. PBST: 1× PBS, 0.01 % Tween.
8. Primary antibodies against the protein, e.g., anti-PCNA monoclonal antibody [27, 28] or anti-DNA ligase I rabbit polyclonal antibody [6].
9. Secondary fluorochrome-conjugated antibodies specific to the Ig of the primary antibodies *or* primary or secondary antibodies tagged to biotin and (strept)avidin conjugated to a fluorescent moiety (*see* **Note 2**).

2.6.3 RFi Detection of Cell-Permeable Nucleotide Analogs

1. Borosilicate coverslips, round or square, 0.152 mm thick.
2. Standard cell culture media and supplements.
3. Parafilm.
4. "Humid chamber": Petri dish diameter 150 mm wrapped with aluminum foil and having a piece of wet absorbent paper inside [29].
5. 0.1 % Triton X-100 solution in PBS (*see* Subheading 2.4, **item 2**).
6. 1 % BSA or 0.2 % fish skin gelatin in PBS.
7. PBST (*see* Subheading 2.6.2, **item 7**).
8. 1,000× DNaseI: 1 mg/ml DNaseI in 50 % glycerol. Stored at −20 °C and 2× denaturation buffer: 60 mM Tris–HCl (pH 8.1), 0.66 mM $MgCl_2$, 1 mM mercaptoethanol in ddH_2O (enzymatic denaturation); *or* 4 N HCl (acidic denaturation). These reagents are not necessary for click chemistry-based detection.

9. *BrdU*: mouse anti-BrdU antibody (BD, clone B44, Cat no: 347580, or clone IU-4, CALTAG Labs) and secondary anti-mouse Ig antibodies conjugated with Alexa dyes of appropriate emission wavelength; *or EdU*: click reagents labeled with Alexa dyes of appropriate emission wavelength (Baseclick or Invitrogen).

2.6.4 DNA Counterstaining

1. PBST (Subheading 2.6.2, **item 7**).
2. 1,000× DNA counterstaining stock solution: 1 mg/ml Hoechst 33258 in ddH_2O (350/461 nm excitation/emission maxima, respectively) *or* 1 mg/ml 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) (350/461 nm) in ddH_2O *or* 1 mM solution Molecular Probes TO-PRO®-3 Iodide (642/661 nm) in DMSO. Aliquot and store frozen.

2.7 Mounting

1. Pre-cleaned microscope slides.
2. Mounting medium: nonhardening antifadents, AF, Citifluor Ltd. CFM, Vector Laboratories Inc. Vectashield®; *or* hardening antifadents, Moviol [25], Molecular Probes ProLong® Gold.
3. Absorbent paper (lint-free).
4. Pigment-free nail polish.

2.8 Fluorescence Microscopy

1. Microscopy setup (*see* Subheading 3.8).

2.9 RFi Quantification

1. ImageJ software, version 1.43 or later.

2.9.1 Confocal or Wide-Field Images: Segmentation of Replication Foci

2.9.2 3D-SIM Images

1. ImageJ and Perkin Elmer Volocity 5 software.

3 Methods

Procedures with live cells are carried out in a tissue culture room equipped with a laminar flow biosafety cabinet and CO_2 incubator. All procedures with coverslips utilizing small volumes of solutions are carried out on a piece of Parafilm in the "humid chamber." (Bio)material disposal regulations should be diligently followed.

3.1 (A)Synchronously Growing Cell Cultures

Having an actively proliferating culture is a prerequisite for all protocols of replication sites detection. Generally, cells should be no more than 80 % confluent. Asynchronously growing cell cultures,

where cells in all stages of the cell cycle and, importantly, S-phase are present, can be obtained by regularly subculturing the cells to a lower density. For primary adherent cultures, enrichment of cells in particular periods of S-phase can be achieved by splitting the cultures after they become contact inhibited and obtaining samples at specific time points (typically starting 8–12 h after the cells are split). Alternatively, a chemical synchronization protocol can be applied [30, 31], though this may alter cellular metabolism [32].

Visually inspect the cells. An actively growing culture will be characterized by a sufficient number of mitotic cells and low amount of cell debris in the medium.

3.2 RFi Labeling by Cell-Permeable Nucleotide Incorporation

1. If cells are supposed to be grown further after the labeling (e.g., in pulse-chase-pulse experiments), take half of the conditioned medium from the dish with the cells and keep it in the incubator. Absence of differences in the medium characteristics will ensure unaltered S-phase dynamics.
2. Add nucleotide to the cell culture medium to a final concentration of 10 μM (*see* **Notes 3** and **4**).
3. Place the cells back into the CO_2 incubator and incubate for the desired period of time (*see* **Note 5**).
4. If further in vivo procedures are planned:
   (a) Collect and discard the medium containing the nucleotide.
   (b) Wash the cells with pre-warmed medium and discard wash medium.
   (c) Add standard volume of pre-warmed and conditioned culture medium.

3.3 RFi Labeling by Cell-Impermeable Nucleotide Incorporation [5]

1. Grow cells on coverslips to the highest density at which the cells still proliferate. A Petri dish containing several coverslips can be used.
2. Prepare the labeled nucleotide solution in pre-warmed cell culture medium (10–20 μl per 18–24 mm coverslips, final concentration 10 μM of the labeled nucleotide).
3. Prepare a hypodermic needle.
4. Using forceps, take a coverslip with the cells out of the Petri dish.
5. Remove the extra medium by touching the side of the Petri dish with the edge of the coverslip.
6. Place the coverslip into a new Petri dish of a size suitable to accommodate the coverslip.
7. Press the coverslip to the dish using the needle and add 10–20 μl of the labeled nucleotide solution.

8. While observing the cells on an inverted microscope, make a series of parallel scratches with the tip of the needle. Touching the cells with the needle will cause transient plasma membrane disruption and penetration of the diluted nucleotide into the cell's cytoplasm.
9. Put the Petri dish with the coverslip into the CO_2 incubator for 1–3 min.
10. Cover the coverslip with 1–2 ml of the warm conditioned cell culture medium taken from the "parent" Petri dish.
11. Incubate for the desired period of time (*see* **Note 6**).
12. Proceed for further treatments or fixation.

3.4 3D-Preserved Cell Preparations

1. Remove culture medium avoiding drying at any stages (*see* **Note 7**).
2. Wash the coverslips with the cells with PBS (*see* **Note 8**). Alternatively, coverslips with the cells can be placed into a new Petri dish with the PBS solution.
3. To reduce the background staining/signal from the non-bound fraction of the replication proteins, the following pre-treatment procedures can be used prior to fixation. If this step is not required, proceed directly to **step 6**.
4. (Optional) Extraction before fixation: incubate coverslip in 0.05–0.1 % Triton X-100 solution in PBS for 3 min at RT or incubate coverslip in 0.1 % TritonX-100 solution in CSK buffer [33] (*see* **Note 9**) 1–5 min at RT. This extraction can also be performed on ice, which is useful not to loose cells that detach easily.
5. Aspirate the pre-extraction solution.
6. Wash coverslips with PBS.
7. Remove the wash buffer.
8. Cover the cells with freshly prepared 3.7 % formaldehyde solution (*see* **Note 10**).
9. Incubate for 10–15 min at RT protected from light.
10. Remove formaldehyde and wash thoroughly with PBST at least twice (*see* **Note 11**).
11. Proceed to RFi staining (Subheading 3.6, *see* **Note 12**).

3.5 Hypotonically Resolved RFi

Hypotonic treatment leads to swelling of the cells (*see* **Note 13**). The consequent fixation with the mixture of methanol and acetic acid leads to extraction of many cellular proteins including histones. Drying the resulting cells/nuclei preparations on the glass slides leads to considerable flattening of the nuclei and enhanced physical resolution of the DNA replication foci (Fig. 2).

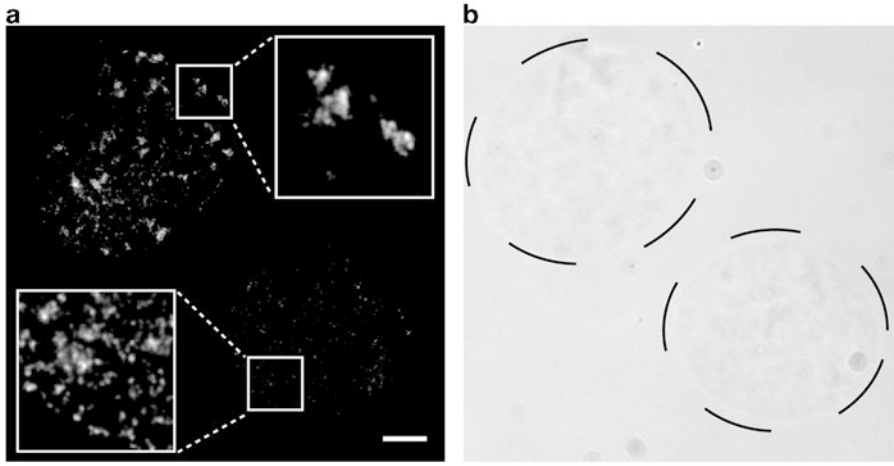


Fig. 2 Hypotonically resolved DNA replication foci. Mouse myoblasts growing at 70 % confluency were incubated with 10 μM BrdU for 15 min and processed as described under Subheadings 3.5–3.8. (**a**) Late S-phase (*upper*) and early S-phase (*lower*) RFi distribution patterns are presented with corresponding phase contrast images (**b**). Scale bar: 5 μm

3.5.1 Harvesting Cells

1. Replace culture medium with cold PBST. Wash twice.
2. Add pre-warmed trypsin/EDTA solution (0.4–0.5 ml per 25 cm^2) and incubate for 3–5 min at 37 °C.
3. Observe cells at the microscope.
4. When most of the cells become round, bump at the side of dish with your palm.
5. Check that the cells have detached and are floating.
6. Add a small amount of the cell culture medium or fetal calf serum to stop the trypsin action.
7. Collect cell suspension and place it into a 15 ml conical tube.
8. Spin down the cells at ~300 × *g* for 5 min at 4 °C.
9. Remove supernatant as completely as possible, leaving about 20–50 μl of the medium.
10. Resuspend the cells by gently tapping against the tube.

3.5.2 Hypotonic Treatment

1. Add 1–3 ml of pre-warmed hypotonic solution (*see* **Note 14**).
2. Incubate in a water bath at 37 °C for 15–30 min.
3. While incubating in the hypotonic solution, mix three volumes of cold methanol and one volume of acetic acid (3:1 MeAA solution, Carnoy's fixative) and place it into the fridge until use.
4. Centrifuge the cells at ~300 × *g* for 5 min at 4 °C (*see* **Note 15**).
5. Remove supernatant as completely as possible.
6. Resuspend cells in the remaining volume (20–50 μl) by gently tapping the tube. Do NOT resuspend the cells by pipetting.

3.5.3 Fixation

1. *Slowly* add several drops of freshly prepared ice-cold MeAA solution (*see* **Note 16**).
2. Incubate for 5 min at RT.
3. Add 0.5 ml more of MeAA solution and incubate for 10 min at RT.
4. Centrifuge at ~300 × *g* for 5 min at 4 °C.
5. Replace the solution with excess volume (1–2 ml) of fresh ice-cold MeAA.
6. Incubate for at least 30 min at 4 °C.
7. Centrifuge at ~300 × *g* for 5 min at 4 °C and replace the solution with excess volume (1–2 ml) of fresh ice-cold MeAA. Keep it at −20 °C for up to several weeks or proceed to the next step.
8. Centrifuge at ~300 × *g* for 5 min at 4 °C and resuspend the cells in a smaller volume (300–500 μl) of MeAA to get the desired concentration. The cells are preferably resuspended by tapping the tube against the table. Only very slow pipetting is acceptable.

3.5.4 Slide Preparation

1. Use pre-cleaned microscope slides or wash the slides thoroughly with mild detergent, rinse several times with ddH_2O, and air dry.
2. Immerse pre-cleaned slides in MeAA at least 15 min prior to use, and wipe slides dry with a lint-free tissue.
3. Drop 1–2 small (~15–20 μl) drops of cell suspension from Subheading 3.5.3, **step 8** above onto slide surface with a Pasteur pipette.
4. Allow the drops to spread.
5. Pass the slide through vapor of a water bath heated to 60–75 °C.
6. Dry it at RT or at 40 °C on the heated table or pass the slide through a flame or put the slide on a slightly tilted surface and let it air dry at RT.
7. Inspect the slide for the flatness of the nuclei.
8. The slides can be kept dry at 4 °C for several days before staining.

3.6 RFi Detection

It is recommended to use fluorescent groups with increased photostability, e.g., Alexa or Atto dyes. Signal from GFP-tagged protein can be additionally enhanced using anti-GFP antibodies [34]. For cells with RFi labeled by fluorescently tagged (e.g., GFP-tagged) proteins or by fluorescently tagged nucleotide incorporation, proceed directly to DNA counterstaining (Subheading 3.6.4).

3.6.1 RFi Detection by Tagged Replication Factors

Protein components of the replication units can be visualized by expressing fluorescently tagged replication proteins. For transient transfection with fluorescent replication factors, transfect the cells by following the steps of an optimal protocol for the cell type in question or following the manufacturer's instructions (*see* **Note 17**). Alternatively, cells stably expressing fluorescent replication at a level, which does not interfere with replication dynamics, can be used (*see* **Note 18**).

3.6.2 RFi Detection of Native (Untagged) Replication Proteins

1. Permeabilize cells by incubating in 0.1 % Triton X-100 solution for 10–20 min at RT (*see* **Note 19**).
2. Wash twice with PBS.
3. To block, incubate with 1 % BSA or 0.2 % fish skin gelatin in PBS for 20 min at RT.
4. Incubate with primary antibodies specific to the replication protein for an hour at RT.
5. Wash coverslip with PBST three times.
6. Incubate with secondary antibodies tagged to a fluorescent group.

3.6.3 RFi Detection of Incorporated Cell-Permeable Nucleotide Analogs

In case of halogenated nucleotide analogs, cells must be permeabilized and DNA denatured prior to incubation with primary antibodies (**steps 1–4** below). EdU detection requires only permeabilization of the cells (**steps 1, 2, 3** (optional), and **7**, below). Detailed protocols for nonfluorescently tagged nucleotides detection are presented in [25].

The outline of the procedure is as follows:

1. Permeabilize cells by incubating in 0.1 % Triton X-100 solution for 10–20 min at RT (*see* **Note 19**).
2. Wash twice with PBS.
3. To block, incubate with 1 % BSA or 0.2 % fish skin gelatin in PBS for 20 min at RT.
4. (Skip this step in case of EDU detection and proceed directly to **step 7**)

   Perform acidic denaturation of DNA by incubation with 4 N HCl 30 min at RT, wash the cells thrice with PBST, and then incubate with primary antibodies specific to the incorporated nucleotide for 30–60 min at RT *or* enzymatically expose and detect the epitope by incubation in a 1× DNase buffer solution containing DNase I (20 U/ml) and anti-BrdU mouse monoclonal antibody for 30–60 min at 37 °C.
5. Wash coverslip with PBST three times.
6. Incubate with secondary antibodies tagged to a fluorescent group.

7. Detect the incorporated EdU by incubation with a fluorescent azide in the presence of Cu(I) according to the manufacturer's instructions.

3.6.4 DNA Counterstaining

1. Rinse coverslips with PBST.
2. Add 20–50 μl of 1× DNA counterstaining solution and incubate for 5 min at RT.

3.7 Mounting

1. Put a small drop of the mounting medium (*see* **Note 20**) on the microscope slide (for coverslip-grown cells).
2. If cells were grown and/or stained on a slide, proceed to (Subheading 3.7, **step 5**).
3. Wash salts away by dipping coverslips briefly in ddH_2O.
4. Remove excess water by touching absorbent paper with the edge of the coverslip.
5. Place the coverslip over the drop of mounting medium with the cells facing the mounting medium (*see* **Note 21**).
6. When mounting with hardening medium, let it solidify by incubating in the dark overnight at RT.
7. For liquid mounting medium, carefully put slides with coverslip down on a paper towel and wait for 5 min for excess media to be absorbed.
8. Seal coverslip with nail polish all-around.
9. Proceed for imaging.

3.8 Fluorescence Microscopy

Imaging of the labeled RFi can be performed using commercially available or custom-made microscopy setups (*see* **Note 22**). DNA replication foci in 3D-preserved nuclei can be imaged using confocal systems (laser scanning confocal systems or spinning (Nipkow) disk-based microscopes) or super-resolution setups, which allow 3D imaging. Using live-cell RFi labeling and confocal high temporal resolution microscopy or pulse-chase-pulse labeling approach [25] and multicolor super-resolution microscopy allows temporal analysis of DNA replication (*see* **Note 23**). Multicolor structured illumination microscopy (3D-SIM) [35] is the most promising multicolor super-resolution imaging approach, which has been successfully applied to RFi analysis [20, 36]. Due to their utmost flatness, RFi on the hypotonically treated nuclei preparations can be analyzed by the whole spectrum of microscopy techniques, including wide-field microscopy, confocal microscopy (*see* **Note 24**), and 2D super-resolution microscopy techniques [20, 37].

3.9 RFi Quantification

There are two major steps in object-counting procedures: first, the images are processed and/or segmented to discriminate between signal and background pixels, and, then, individual objects in the signal segment of the image are identified and counted. The different steps are summarized in Figs. 3 and 4.

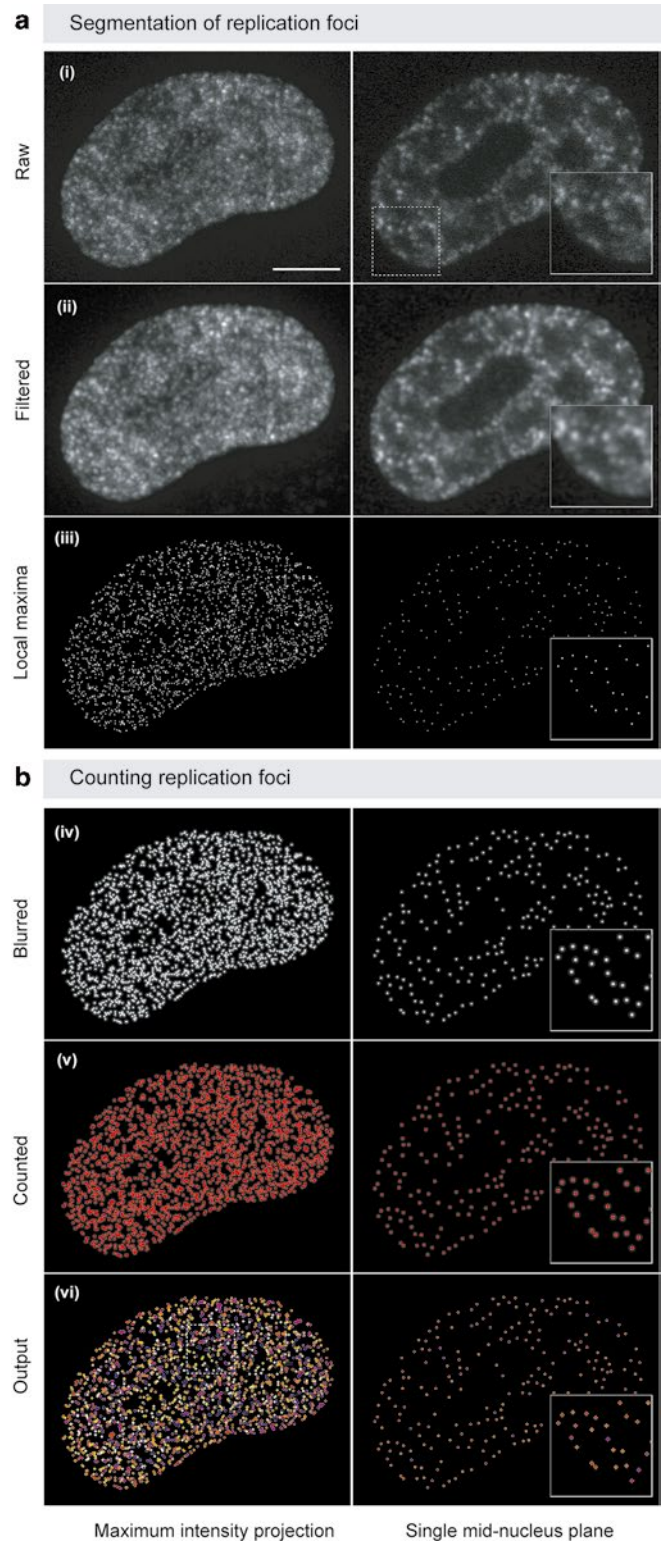


Fig. 3 Quantification of replication sites on conventional fluorescent microscopy images. An overview of the intermediate results of RFi quantification on 3D stacks of confocal images (**a**, **b**) described in Subheading 3.9.1. To quantify RFi in hypotonically flattened nuclei, the steps of part (**a**) suffice. Scale bar: 5 μm

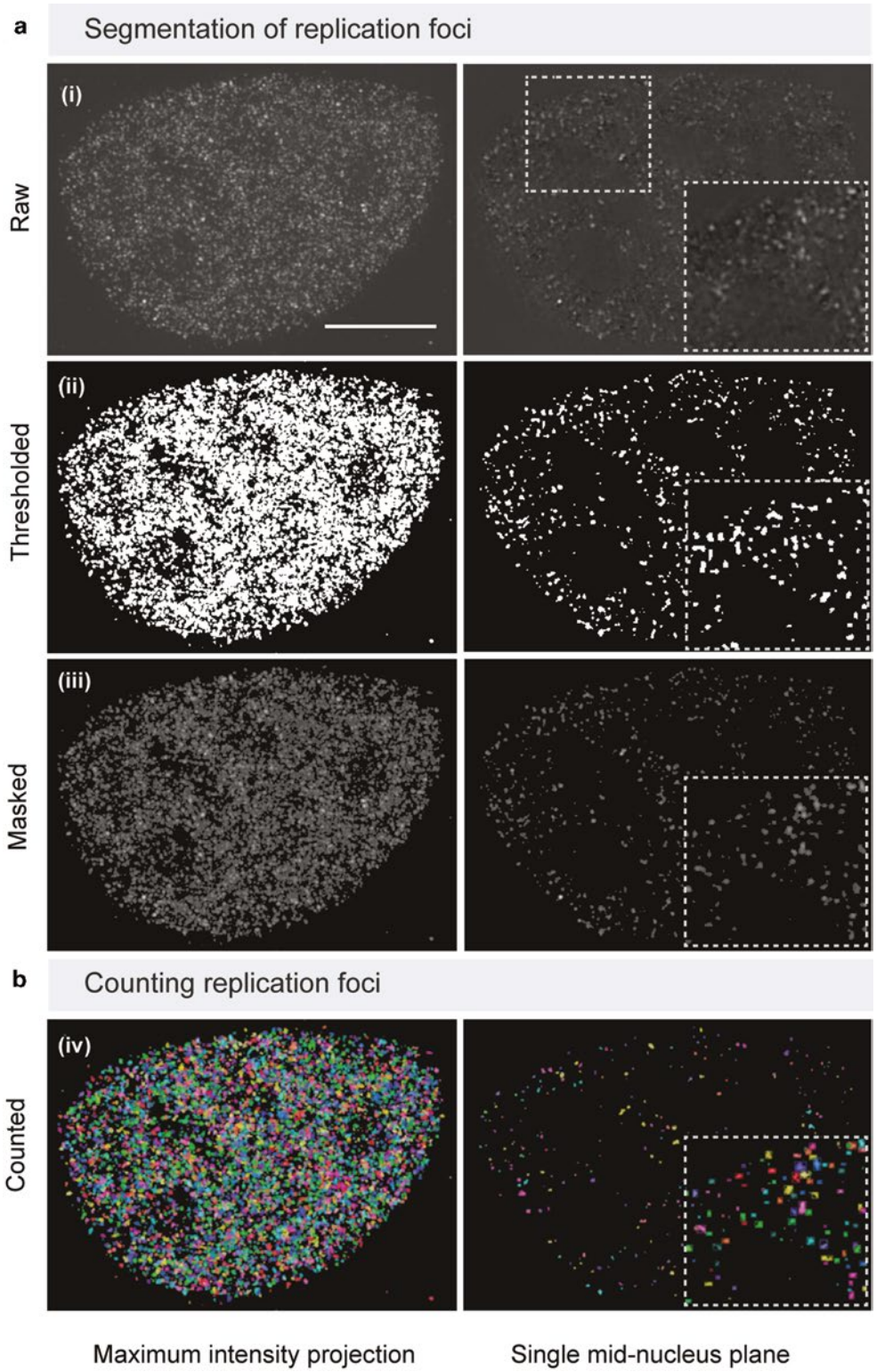


Fig. 4 Quantification of replication sites on super-resolution fluorescent microscopy images. An overview of the intermediate results of RFi quantification on 3D stacks of 3D-SIM images (**a**, **b**) described in Subheading 3.9.2. Scale bar: 5 μm

3.9.1 Confocal or Wide-Field Images

The procedure presented (Fig. 3) relies on the identification of local maxima of intensity. Accordingly, the influence of random noise, which is represented as 1 pixel spikes in intensities, on local maxima identification should be reduced. For that a smoothing filter is used having the kernel size of 1–2 pixels. Larger kernel sizes will lead to considerable reduction in image contrast and failure to identify closely located maxima as separate ones.

Segmentation of Replication Foci

1. Open the image stack in ImageJ. Remove slices without signal or having unfocused image of the nucleus (Fig. 3a (i)).
2. To filter one pixel noise, go to "Process" menu>Filter and select a smoothing filter. For the selected filter, e.g., "Mean" set the kernel size. Run the filter to process all images in the stack. Smoothing the images shifts the image histogram to the region of lower intensities. For standard threshold detection during further steps of image processing, images are normalized.
3. To normalize image histogram, go to "Process" menu>Enhance contrast. Choose linear stretching of the histogram: select "Normalize" and process all slices. Use stack histogram during the normalization and avoid introducing saturated pixels (Fig. 3a (ii)).
4. For identification of local maxima, go to "Process" menu>Find maxima and select "Preview point selection." Identified local maxima will be shown by small crosses.
5. Choose noise tolerance setting to exclude background signal from the analysis. The particular value of noise tolerance (threshold) will depend on many factors, including bit range, signal-to-noise ratio, and image quality. Use one of the central sections of the nucleus. Change noise tolerance stepwise and follow the changes in pixels being selected. Correct setting will be manifested by only a few maxima identified outside the nucleus area and, e.g., inside nucleoli. The number of identified maxima will be shown. For single plane images, calculate RFi inside nucleus using the region of interest (ROI) and skip further steps.
6. Write down or remember the selected noise tolerance.
7. To identify local maxima in 3D, run "Find Stack Maxima" macros (http://imagej.nih.gov/ij/macros/FindStackMaxima.txt) with the selected noise tolerance setting, and select "single points" as the procedure output (Fig. 3a (iii)).
8. Save the output stack.

Counting of replication foci

Local maxima are identified and marked in the output binary image as single white pixels. Counting of such single-pixel objects on the stack of images (in 3D) will lead to an overestimation of the number of objects (*see* **Note 25**). To get a conservative estimate of

the RFi number and account for RFi present in more than one slice in the image stack, replace the identified local maxima with objects of standard size by blurring the output stack using convolution with Gaussian kernel.

9. Go to "Process" menu>Filter and select "Gaussian blur."
10. Set the kernel size of 1–2 pixels and process all slices (*see* **Note 26**).
11. Normalize the processed stack (*see* Subheading 3.9.1 **step 2** and **3**) (Fig. 3b (iv)).
12. Save the resulting image stack.
13. To estimate the number of objects in 3D, run "3D object counter" plug-in (http://rsbweb.nih.gov/ij/plugins/track/objects.html).
14. Set "Threshold" in the plug-in menu to 21 (*see* **Note 27**) (Fig. 3b (v)).
15. For the output of the data analysis, select "Maps to show" = "objects" and "Results tables to show" = "Statistics" and "Summary." In addition to the numbers of RFi identified, the plug-in will return a stack with color-coded individual objects (Fig. 3b (vi)).

3.9.2 3D-SIM Images

For the two step approach used in analysis of 3D-SIM images (Fig. 4), the first step consists of the segmentation of the nuclei in ImageJ [38], and the final step uses the commercial software Volocity 5 (Perkin Elmer) and performs further segmentation of touching objects and automated counting. An intensity-based object recognition is followed by a proprietary watershed algorithm for the separation of touching objects.

Segmentation of replication foci

1. Open the image in ImageJ and crop the nuclei of interest ("Image" menu>"Crop") (*see* **Note 28**).
2. Duplicate the cropped image stack with the "Image" menu>"Duplicate">"Duplicate Stack" option (*see* **Note 29**) (Fig. 4a (i)).
3. With the duplicated image, adjust with the "Image" menu>"Adjust">"Autothreshold function."
4. Choose the "Triangle Method" (*see* **Note 30**) and following options: "Ignore black," "White objects on black background," "Stack," and "Use stack histogram." During this step, an automatic thresholding, the triangle method, is used to differentiate between background noise and replication foci based on the shape of the histogram (Fig. 4a (ii)).
5. To recombine both images, go to the "Process" menu, and select "image calculator" with the following options:

(a) Image1: Choose thresholded image.

(b) Specify method: "Min."

(c) Image2: Choose cropped original image.

(d) Select "Create New Window."

(e) Process all images from this stack.

6. Save resulting masked image as TIFF (Fig. 4a (iii)).

Separation and counting of replication foci (*see* **Note 31**).

7. Import masked image to Volocity 5 (Perkin Elmer), and generate a 3D stack by using the "Tools" menu>"Make Volumes" (*see* **Note 32**) command (*see* **Note 33**).
8. To quantify the replication foci, choose "Measurements" tab, and drag following objects to the measurement:
   (a) "Find objects," select the wheel in top right corner to specify intensities and specify "Lower" = 1.
   (b) Drag also task "Separate touching objects" to measurement window.
   (c) At this stage, a measurement will be performed by Volocity window (*see* **Note 34**) (Fig. 4b (iv)).
9. To save the measurement, go to "Measurements" menu>"Make Measurement Item" and name the item accordingly.
10. Select the Measurement item and select "File" menu> "Export">"As Comma separated Value" and save to a convenient location (*see* **Note 35**).

4 Notes

1. DNA labeling using each of the nucleotides has its own advantages and/or drawbacks. Halogenated nucleotide analogs provide for very efficient DNA labeling, but require denaturing treatment of the preparations, which may affect detection of other epitopes. Fluorescently labeled nucleotides can be used for in vivo microscopy studies but they are cell impermeable and require sophisticated procedures for their delivery into the cell. Alkyne nucleotides are easy to detect, but rely on chemical reactions for their detection and may be affected by particular, e.g., acidic treatments. EdU can also trigger DNA damage response and affect cell cycle progression in the long term [39].
2. Signal from GFP-tagged replication proteins can be further enhanced using:
   (a) anti-GFP primary antibodies
   (b) GFP enhancing nanobodies [34]

3. Reducing the volume of cell culture medium prior to nucleotide addition may be advisable when expensive or not readily available nucleotide analogs are used.
4. Best working EdU or BrdU concentrations are cell line specific. For these reasons and also to increase the intensity of RFi labeling, it may be advisable to use higher concentrations of the nucleotide.
5. Longer nucleotide incorporation times will result in brighter signal and better signal-to-noise ratio. However, increasing the time of labeling can lead to labeling of adjacent or newly activated replicons thus decreasing spatial resolution of the method. To get a snapshot of simultaneously active replicons in a particular cell, we suggest using times of incubation below characteristic replicon's "lifetime." Typically 10–20 min of incorporation with nucleotide for mouse or human cells works well (*see* **Note 4**).
6. Live cell imaging is compromised immediately after labeling due to very bright background signal from the labeled nucleotide, which has entered the cell during the scratching procedure. At about 30–60 min after labeling, the background staining drops, and cells displaying typical replication patterns can be observed live. The pattern is stably inherited to the daughter cells and the labeled RFi can be followed for several cell cycles as partially labeled segregating chromosome territories [40].
7. To avoid detachment of poorly adherent cells, coverslips can be slightly dried (until "granular" appearance of the surface), placed on Parafilm and a small volume (50–100 μl) of the Triton X-100 solution carefully placed over the coverslip.
8. Using cold PBS wash can help precisely control nucleotide incorporation time, which is especially important with short labeling times.
9. The CSK buffer is hypertonic and provides for efficient extraction of many nuclear proteins, which is, e.g., essential to detect MCM proteins bound to chromatin. However, chromatin structure and nuclear morphology are affected by this treatment.
10. To avoid detachment of poorly adherent cells, cells can be prefixed using mild formaldehyde solution (0.1 % in PBS). Using such prefixation prior to 3.7 % formaldehyde solution is also helpful when analyzing weakly bound factors.
11. Labeled cells can be stored in PBS at 4 °C up to several days prior to staining. Sealing the Petri dish with Parafilm and adding NaN_3 extends that period.
12. Some antibodies (e.g., anti-PCNA mouse and rat monoclonal antibodies) require methanol treatment of the cells. With such antibodies, the cells can be first formaldehyde fixed for better

preservation of the nuclear morphology and then methanol treated (postfixed) for the sake of efficient antibody detection of the epitopes of interest.

13. The hypotonic protocol cannot be combined with expression of GFP-tagged proteins because the strong acidic treatment destroys GFP fluorescence.
14. The choice of the hypotonic solution and minimal time of the hypotonic treatment is cell type and species specific. The best working solution and exact time should be experimentally selected for each cell line. In our hands, 15 min of 50 mM KCl incubation is sufficient for most cells. For rodent cells, 20–25 min incubation and 75 mM KCl/1 % $Na_2HCitrate$ give sometimes better results.
15. Successful hypotonic treatment is manifested by an increase in the pellet size as compared to the initial pellet size. We include time of centrifugation into the time of hypotonic treatment.
16. Slow and homogeneous increase in MeAA concentration is important. To obtain this, we add MeAA dropwise to the walls of the tube and flick the tube after each drop. Alternatively, the tube can be placed on a slowly rotating shaker and MeAA added dropwise.
17. Make sure that the transfection is performed one or two cell cycles before the time of observation or experiment (*see* also **Note 2**) and select cells with low to mid fluorescent protein level.
18. We suggest GFP-PCNA or GFP-DNA ligase I, which represents essential components of the replication fork [2]. For super-resolution microscopy (e.g., 3D-SIM imaging, which requires 45 times more illumination compared to conventional imaging techniques), enhancement and stabilization of GFP fluorescence are required and can be achieved using additional immunostaining (*see* **Note 2**).
19. All procedures can be carried out on a piece of Parafilm in the Petri dish covered with foil and containing a piece of wet filter paper or wet tissue, "humid chamber" [29].
20. For super-resolution microscopy, it is important to match the refractive indices of the mounting medium, coverslip, and microscope immersion as closely as possible. Nonhardening antifadents are recommended to preserve 3D structure of the cells.
21. To avoid changing cellular morphology due to excessive drying of the hardening antifading solution, it is recommended to seal the sides of the coverslip using nail polish.
22. For higher signal-to-noise ratio of the images, high numerical aperture (used commonly for total internal reflection fluorescence microscopy) objectives can be used.

23. As the energy of illumination is inversely proportional to the wavelength, when multiple fluorochromes are used to optimize the signal-to-noise ratio and minimize photobleaching, sequential acquisition of images of individual channels and starting with the longer wavelength are advisable.
24. In this case, confocal microscopy does not provide an additional advantage due to better z-resolution and elimination of the out-of-focus light. However, because of the localized sample illumination, confocal imaging leads to a sharper point spread function giving a higher x,y contrast (theoretical resolution is ~1.4-fold better) and better signal-to-noise ratio.
25. The accuracy of maxima identification is influenced by image noise, which can lead to 1–2 pixel shifts in maxima positions even after smoothing the image. The same DNA replication focus can be represented as focal signals in the neighboring stack slices, for which spatially separated local maxima can be identified. As a result, the same 3D continuous DNA replication focus will be accounted as several individual RFi in consecutive image slices.
26. A fluorescent point object will be imaged as a bell-shaped distribution of signal of a particular radius (*see*, e.g., [41]). Accordingly, there will be a minimal size of the objects in the image. Parameters of both steps (Subheading 3.9.1, **steps 2** and **6**) should be selected with account to the minimal size. The parameters applied by us can be used for 63× objectives with NA>1.4 (pixel size<~100 nm) and emission wavelengths ~500–600 nm.
27. Operations of **steps 9–11** under Subheading 3.9.1 replace local maxima marked by single pixels with standard objects representing normalized Gaussian distributions. Setting nonzero threshold in the plug-in sets the size of the objects to be counted in the stack. Importantly, the threshold also determines how many signals will overlap in the adjacent slices and, hence, attributed as belonging to the same objects (RFi) continued in 3D.
28. It is easier to make a z-max projection ("Image" menu> "Stacks">"Z-Project," select "all slices," and "Max Intensity"); select the nuclei there and transfer the mask via the ROI Manager ("Tools" menu>"ROI Manager," you can even name the ROI).
29. **Steps 2–6** can be automatized by writing a macro.
30. An implementation of this method is described in detail by Zack, Rogers, and Latt in "Automatic measurement of sister chromatid exchange frequency" [42]. Gabriel Landini has implemented the method in the "Autothreshold" ImageJ plug-in.

31. Changing the order of the protocol steps may result in a different outcome of measurements.
32. Volocity uses "Volume" as a synonym for a 3D image stack.
33. Do not forget to set the pixel size via "Edit" menu>"Properties." In most scenarios, the pixel size will otherwise be lost.
34. Batch processing of many images is possible using "Make Measurement" item.
35. A modification of the protocol can be used for RFi segmentation in very early/late S-phase cells (small number of objects over a noisy background) and/or really noisy datasets. Subheading 3.9.2 separation and counting of replication foci is performed using the complete image intensity range for thresholding (e.g., for every 8-bit images, all 255 values are chosen as individual thresholds). The number of identified objects over threshold is plotted, and an exponential curve is fitted to the graph. The best estimate threshold corresponds to the point where the first derivative of the fitted exponent curve is –1. This method is advisable only for low contrast or very dim images as it is an immensely (computational) time-intensive task, getting worse with increased bit depth.

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