nature chemistry

SUPPLEMENTARY INFORMATION

https://doi.org/10.1038/s41557-018-0046-3

In the format provided by the authors and unedited.

I-motif DNA structures are formed in the nuclei of human cells

Mahdi Zeraati[1,2], David B. Langley[1], Peter Schofield[1], Aaron L. Moye[3], Romain Rouet[1], William E. Hughes[1,2], Tracy M. Bryan[3], Marcel E. Dinger[1,2*] and Daniel Christ[1,2*]

[1]Kinghorn Centre for Clinical Genomics, Garvan Institute of Medical Research, Darlinghurst, Sydney, New South Wales, Australia. [2]St Vincent's Clinical School, Faculty of Medicine, University of New South Wales, Kensington, Sydney, New South Wales, Australia. [3]Children's Medical Research Institute, University of Sydney, Westmead, Sydney, New South Wales, Australia. *e-mail: m.dinger@garvan.org.au; d.christ@garvan.org.au

I-motif DNA structures are formed in the nuclei of human cells

Mahdi Zeraati[1,2], David B. Langley[1], Peter Schofield[1], Aaron L. Moye[3], Romain Rouet[1], William E. Hughes[1,2], Tracy M. Bryan[3], Marcel E. Dinger[1,2*] and Daniel Christ[1,2*]

[1] Kinghorn Centre for Clinical Genomics, Garvan Institute of Medical Research, Darlinghurst, Sydney NSW 2010, Australia

[2] St Vincent's Clinical School, Faculty of Medicine, University of New South Wales, Kensington, Sydney NSW 2010, Australia

[3] Children's Medical Research Institute, University of Sydney, Westmead, Sydney NSW 2145, Australia

*Corresponding authors: m.dinger@garvan.org.au and d.christ@garvan.org.au

Table of Contents

Materials and Methods **3**
Selection of an antibody fragment by phage display 3
Production and purification of selected antibody fragment 3
In vitro characterization of iMab 3
CD spectroscopy 5
Cell culture 6
Microscopy 9
Counting the number of foci 9

Supplementary Table 1. List of the oligonucleotides used in this study **10**

Supplementary Figure 1. Investigating the stability of iMab across different pH ranges **11**

Supplementary Figure 2. CD spectra of hTelo i-motif in the presence or absence of iMab across different pH ranges **12**

Supplementary Figure 3. Evaluation of the specificity of antibodies targeting i-motif and G-quadruplex structures using biolayer interferometry **13**

Supplementary Figure 4. Competition between iMab and BG4 for binding to i-motifs determined by biolayer interferometry **14**

Supplementary Figure 5. Visualization and quantification of i-motif structures during cell-cycle progression in MCF7 cells **15**

Supplementary Figure 6. Investigation of the effect of pH variation on the formation of i-motif structures in HEK 293T cells **16**

Supplementary Figure 7. Evidence of i-motifs formation in the telomeric regions **17**

Supplementary Figure 8. Comparing sensitivity of three different methods for detecting folded hTelo i-motif **18**

Supplementary method 1. Selection of an i-motif specific antibody fragment using Phage display **19**

Supplementary Table 2. Details of phage display procedure used to select a hTelo i-motif specific antibody fragment **20**

Supplementary method 2. Counting foci in the nucleus using FIJI **21**

References **29**

Materials and Methods

Selection of an antibody fragment by phage display

Phage display was performed as previously described [1] with specific modifications described in the "Selection of an i-motif specific antibody fragment using Phage display" section of the supplementary information.

Production and purification of selected antibody fragment

Production and purification of the best hit (iMab) were performed as described earlier [2]. Briefly, electrocompetent *E. coli* HB2151 cells were transformed with the pHEN1 vector containing iMab and myc-tag genes and grown overnight. The supernatant was cleared by centrifugation followed by filtering. Antibody fragment was purified using protein L beads (ML-0422-03, ACRO Biosystems). Purified iMab-myc was used for the *in vitro* characterizations. For immunofluorescence staining, iMab gene was cloned into the pSANG10-3F vector (39264, Addgene), which contains three tandem FLAG tags. Electrocompetent *E. coli* BL21-Gold (DE3) were transformed with this vector and the same procedure of antibody fragment production and purification as described above were followed. The purified iMab-x3FLAG was used for immunostaining.

In vitro characterization of iMab

Detection of antibody binding by ELISA was performed as previously described [2]. Briefly, 5' biotinTEG-oligonucleotides (Integrated DNA Technologies) (Supplementary Table 1) or biotinylated proteins were immobilized on the streptavidin or neutravidin coated MaxiSorp plates (442404, Thermo Scientific) followed by iMab incubation for 45 minutes at room temperature. Attached iMab were detected by anti-myc horseradish peroxidase (HRP) antibody

(CMYC-45P, ICL) incubation for 45 minutes at room temperature and the signal was developed by 3,3',5,5'-tetramethylbenzidine (TMB) (BD Biosciences) and measured at 450 nm using CLARIOstar microplate reader (BMG Labtech). Apparent K_D was calculated by the saturation binding model (one site - specific binding) using GraphPad Prism software version 7.

Binding kinetics of iMab and hTelo i-motif oligonucleotide was determined by biolayer interferometry assay using a BLItz system (Pall ForteBio LLC) at room temperature. Biotinylated hTelo i-motif oligonucleotide (Supplementary Table 1) was annealed in Tris-AcOH pH 6, 7, 8 or 9 and 4 μL of 200 nM annealed oligonucleotide was used to load the streptavidin biosensor for one minute to reach ~ 0.5 nm of signal. The pH dependency of iMab binding to hTelo i-motif was investigated at an antibody concentration of 1 μM. Association and dissociation steps were run for two minutes. Global fitting curves were analyzed using BLItz Pro software version 1.2.

Biolayer interferometry was used to examine the binding of iMab, BG4 and DP47DPK9 to a range of G4 and i-motif oligonucleotides (Supplementary Table 1). G4s were annealed in Tris-HCl pH 7.4 supplemented with 150 mM KCl and i-motifs were annealed in Tris-AcOH pH 6. Biotinylated oligonucleotides were prepared at 200 nM concentration and 4 μL was used to load the streptavidin biosensor for one minute to reach ~ 0.5 nm binding. DP47DPK9 and iMab were produced as described above and diluted to 1 μM in PBS. BG4 (Ab00174-1.1, Absolute Antibody) was diluted 1:50 in PBS. Association and dissociation steps were run for 90 seconds each in PBS. For iMab - BG4 competition assay, biotinylated i-motif oligonucleotides were prepared and loaded as described above. First association step was performed either using BG4 (1:50 dilution in PBS) or PBS only for one minute. Second association step was performed

using 1 μM iMab diluted in PBS for one minute. Dissociation step was performed using PBS only for one minute.

To examine the effect of pH on the stability of the antibody, purified iMab was dialyzed against 50 mM Tris-AcOH buffer adjusted to the appropriate pH (6, 7, 8 or 9) using a SnakeSkin 10K MWCO dialysis membrane (68100, ThermoFisher). Samples were then filtered using Spin-X 0.2 μm filter units (8160, Costar), 100 μL of 150 μg / mL samples transferred to a quartz cuvette and turbidity (at 360 nm) was recorded over 12 hours at 25 °C using a Cary 100 spectrophotometer (Agilent Technologies). For analyses of iMab heat-resistance, sample temperatures were increased at a rate of 1 °C per minute from 20 °C to 45 °C and turbidity was measured as described above.

CD spectroscopy

DNA oligonucleotides were prepared at 5 μM in 50 mM Tris-AcOH pH 6 and annealed by heating at 90 °C for 10 minutes, and cooling down to room temperature at the rate of 1 °C per minute. CD spectra were recorded using Aviv 215S circular dichroism spectrometer equipped with a Peltier temperature controller at 25 °C. Four scans were gathered over the wavelength ranging from 220 to 320 nm in a 0.1 cm path length cell at the standard sensitivity, data pitch 0.1 nm, continuous scanning mode, scanning speed 100 nm min^{-1}, response 4 sec, and bandwidth 1 nm. 50 mM Tris-AcOH pH 6 were scanned as a buffer sample and its spectra subtracted from the average scans for each sample. CD spectra were collected in millidegrees, normalized to the total species concentrations and stated as molar ellipticity units (deg cm^2 $dmol^{-1}$). Each CD spectrum was smoothed by averaging ten neighbour points using GraphPad Prism software version 7.

To examine the effect of iMab binding, spectra were recorded after adding equimolar (5 μM final concentration) of purified iMab, which was dialyzed against 50 mM Tris-AcOH buffer adjusted to the appropriate pH (6, 7, 8 or 9), and incubating for 45 min at room temperature. For each pH condition, 5 μM iMab alone in appropriate pH was scanned and its spectra was subtracted from the corresponding iMab plus hTelo i-motif scan.

Cell culture

MCF7, U2OS, HeLa and HEK 293T cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (11995, ThermoFisher) supplemented with 10 % heat-inactivated fetal bovine serum (FBS) (HyClone) and 1 % penicillin / streptomycin (Gibco) at 37 °C and 5 % CO_2.

To alter pH, CO_2 concentration was changed to either 2 % or 8 %. After 2.5 hours incubation, the pH of the culture was measured using a pH meter (Eutech Instruments pH 700) immediately after taking the flask out of the incubator. Relative intracellular pH was measured using either pHrodo Red or pHrodo Green (P35380, ThermoFisher) according to the manufacturer's instructions. Briefly, cells were grown in a black 96-wells plate (CLS3603, Sigma) until they reached ~ 80 % confluency. After incubating at different CO_2 concentrations for 2.5 hours, cells were washed twice with live cell imaging solution (LCIS) (A14291DJ, ThermoFisher), pHrodo red or green were added and cells were incubated for another hour in an appropriate CO_2 concentration. Cells were then washed twice with LCIS and fluorescent intensity was measured at the excitation / emission recommended by the manufacturer for each dye using CLARIOstar microplate reader (BMG Labtech) while 100 μL LCIS was kept in each well.

For confocal microscopy, cells were grown on poly-L-lysine (P4707, Sigma) coated glass coverslips (# 1.5, Menzel) in a 12-well plate until they reached 70 - 80 % confluency. Cells

were then pre-fixed by adding equivolume of 2 % paraformaldehyde (P6148, Sigma) / PBS (14190144, ThermoFisher) to the media and incubating for two minutes at room temperature. Cells were then washed with ice-cold PBS and fixed by adding 2 % paraformaldehyde / PBS and incubating for 30 minutes at 4 °C. Next, cells were washed once with ice-cold PBS and permeabilized using 0.1 % triton X-100 (Sigma) / PBS and incubating for 30 minutes at 4 °C. After blocking with SuperBlock (37515, ThermoFisher) supplemented with 1 % Marvel (Sigma) for overnight at 4 °C, immunofluorescent staining was performed. This was carried out by incubating cells with iMab-FLAG diluted in SuperBlock supplemented with 1 % Marvel for overnight at 4 °C followed by incubating with rabbit anti-FLAG antibody (14793S, Cell Signaling Technology) and anti-rabbit Alexa 488-conjugated antibody (711-546-152, Jackson ImmunoResearch) both diluted in PBS supplemented with 2 % gelatin from cold water fish skin (G7041, Sigma), 0.5 % BSA and 0.1 % Tween 20 for 45 minutes at room temperature for each of the secondary and tertiary antibodies. After each antibody incubation, three washing steps were performed using ice-cold PBS supplemented with 0.1 % Tween 20. Coverslips were mounted using ProLong gold antifade reagent with DAPI (8961S, Cell Signaling Technology).

For colocalization assays using confocal microscopy, i-motifs were visualized as described above. To visualize TRF2 and E12/E47, mouse anti-TRF2 (ab13579, Abcam) and mouse anti-E12/E47 (sc-133075, Santa Cruz Biotechnology) antibodies were used as primary antibodies and anti-mouse Alexa 594-conjugated antibody (A11005, ThermoFisher) was used as a secondary antibody. For STED microscopy, i-motifs were visualized using iMab-FLAG followed by rabbit anti-FLAG antibody (14793S, Cell Signaling Technology) and anti-rabbit Alexa 594-conjugated antibody (A11037, ThermoFisher). Mouse anti-TRF2 (ab13579, Abcam) and anti-mouse Alexa 488-conjugated antibody (715-546-150, Jackson

ImmunoResearch) were used to visualize TRF2. For STED, coverslips were mounted using ProLong diamond antifade reagent (P36962, ThermoFisher).

DNase I treatment was performed by incubating the fixed and permeabilized cells with 200 units of the enzyme (M0303S, NEB) for one hour at 37 °C. RNase A treatment was performed by incubating the fixed and permeabilized cells with 50 μg / mL of the enzyme (R4875, Sigma) for 30 minutes at room temperature. Pre-incubation of iMab was performed by adding five-fold molar excess of folded hTelo i-motif oligonucleotide in 50 mM Tris-AcOH pH 6 to iMab-FLAG and incubating for one hour at room temperature followed by diluting in the blocking buffer and immunostaining as described above. DP47DPK9-FLAG antibody was produced as described for iMab and used as a control for the staining following the same protocol used for iMab. For the oligonucleotide transfection control experiment, 1, 2 and 4 μg of hTelo i-motif and KIT2 G4 (Supplementary Table 1) were diluted and annealed in Tris-AcOH pH 5 and Tris-HCl pH 7.4 (supplemented with 150 mM KCl), respectively. HeLa cells were transfected with folded oligonucleotides using Lipofectamine 3000 (L3000008, ThermoFisher), according to the manufacturer's protocol. After 16 hours of incubation, cells were fixed and stained as described above.

Cell synchronization at G0/G1 phase was obtained by incubating cells in the serum-free media for 24 hours. For synchronization at G1/S boundary, cells were grown for 16 hours in the complete media supplemented with 300 μM mimosine (M0253, Sigma). Early S phase synchronization was obtained by growing cells in complete media without mimosine for three hours after mimosine-induced blockade. To confirm cell-cycle stages, propidium iodide (P4170, Sigma) staining was performed using standard methods followed by flow cytometry and data were analyzed using FlowJo software version 9.

Microscopy

Confocal microscopy was performed on a Leica SP8 System with a DMI 6000 stand equipped with 63x/1.40 (HC PL APO CS2), 405nm, 488nm, 552nm and 638nm excitation lasers, running LAS X software (Leica, Wetzlar, Germany).

Deconvolution was performed on confocal image stacks generated on a Leica SP8 System with Hybrid GaAsP (HyD) detectors. A DMI6000 stand was used, equipped with 63x/1.2 (HC PL APO W motCORR CS2) with 405nm, 488nm (from ArKr laser), 561nm and 633nm excitation lasers running LAS X software (Leica, Wetzlar, Germany). Image z stacks were obtained, using the 'pinhole' set to 0.55 Airy units and oversampling more than 2x in x-y and z. Deconvolution was performed with Huygens Professional software (Scientific Volume Imaging, Hilversum, The Netherlands). Images were analyzed by FIJI [3] and Imaris 8 (BITPLANE) softwares.

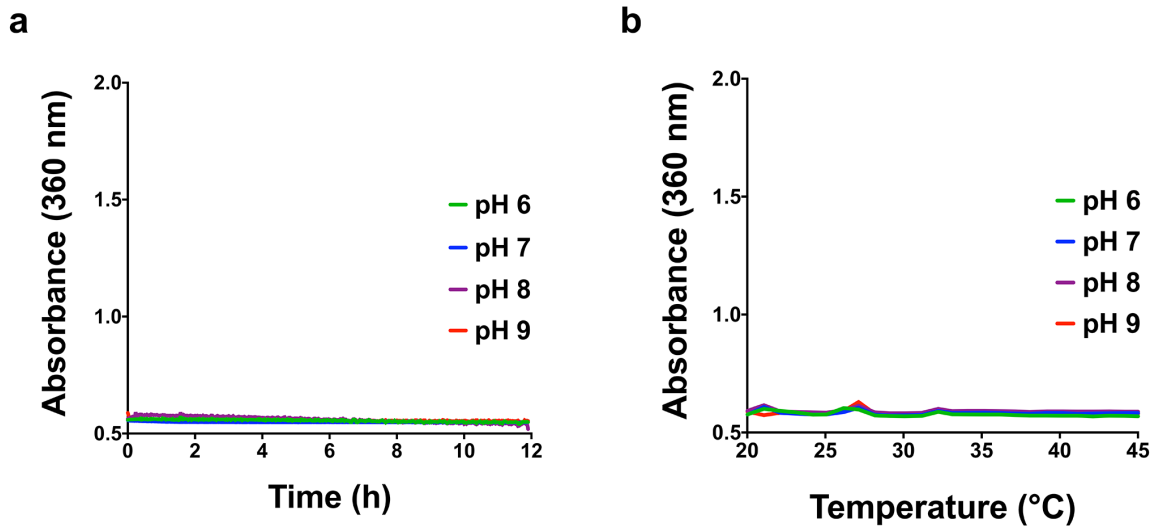
Super resolution microscopy was performed on cells mounted in ProLong Diamond Antifade Mountant (P36962, ThermoFisher). STED microscopy was performed on a Leica TCS SP8 STED 3× system with Hybrid GaAsP (HyD) detectors with a DMi8 stand. The stand was equipped with 100x / 1.40 oil (HC PL APO STED) and a tunable pulsed white light laser, and 775 nm and 592 nm depletion lasers running LAS X software (Leica, Wetzlar, Germany).

Counting the number of foci

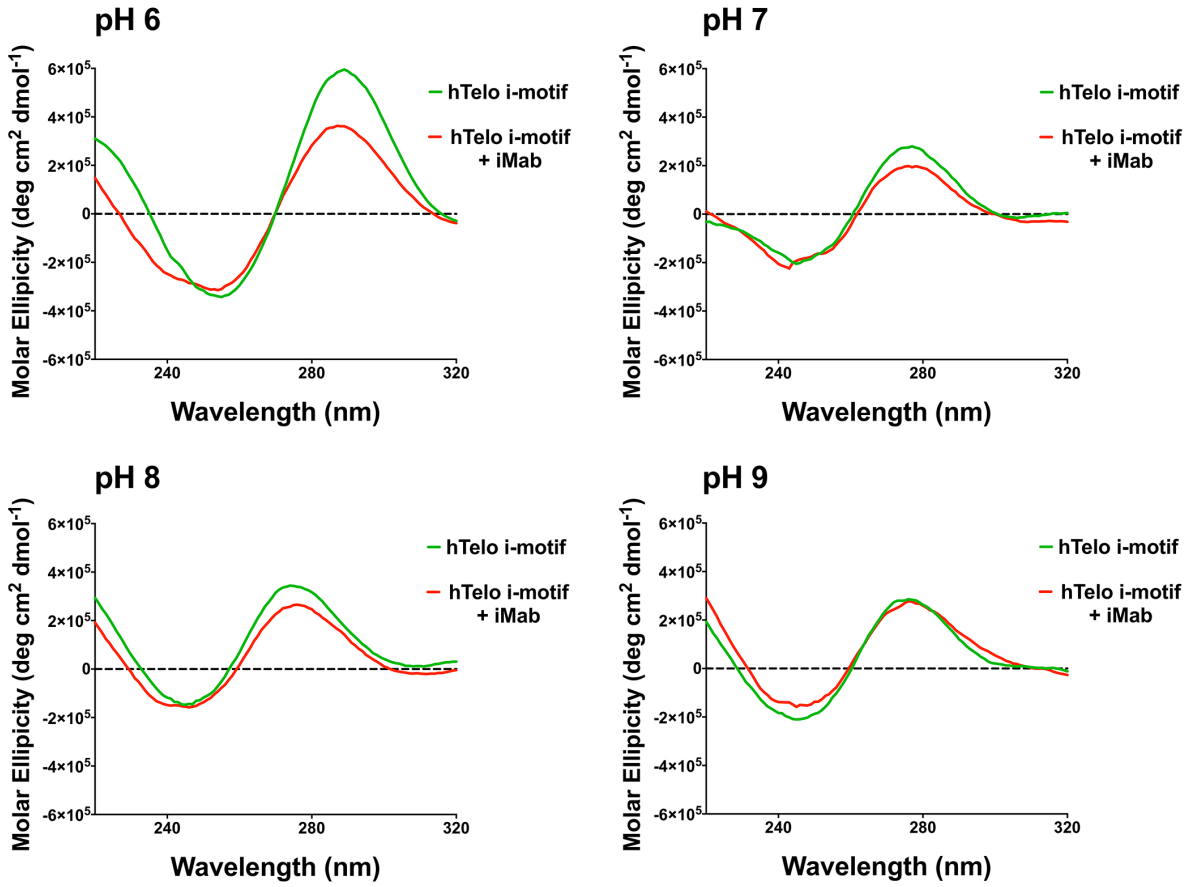
The procedure used to quantify the foci are outlined in the "Counting foci in the nucleus using FIJI" section of the supplementary information with statistical analyses performed using GraphPad Prism version 7.

Supplementary Table 1. **List of the oligonucleotides used in this study.**

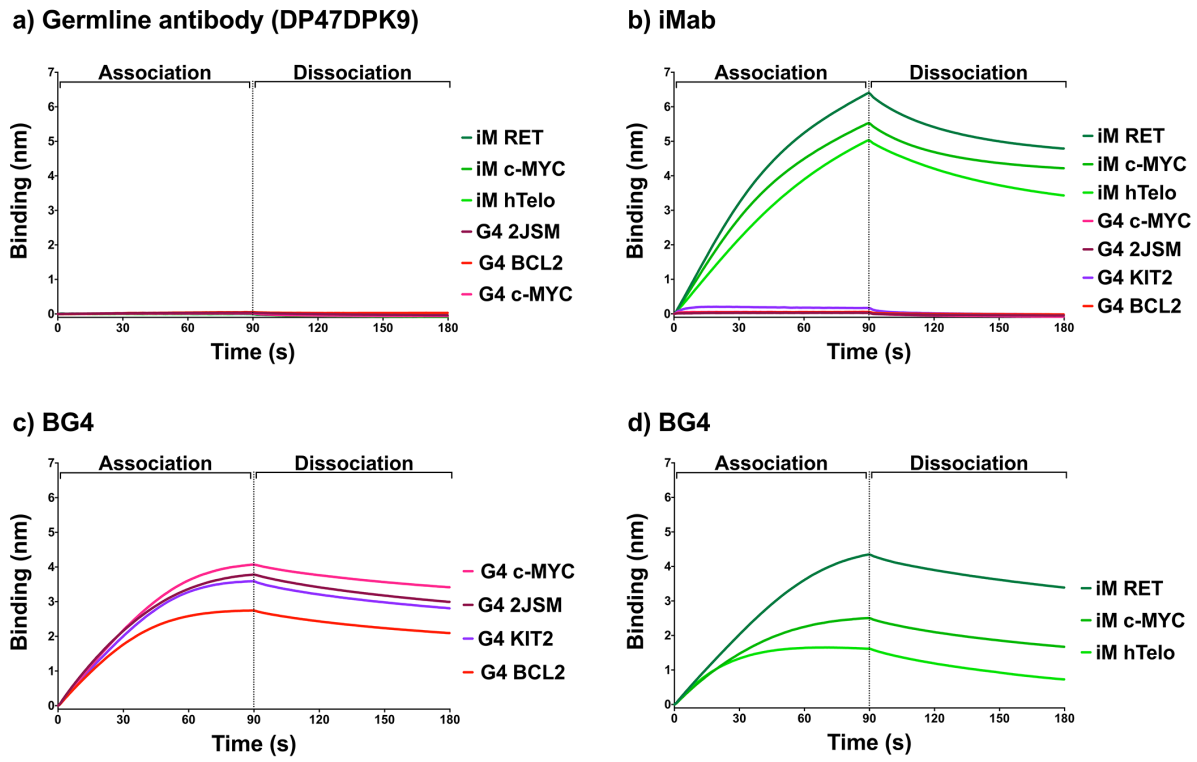
Name	**Sequence (5' → 3')**	**PDB ID**
iM hTelo	CCCTAACCCTAACCCTAACCCT	1EL2
iM hTelo x3 C-runs	CCCTAACCCTAACCCTAA	-
iM hTelo x2 C-runs	CCCTAACCCTAA	-
iM hTelo mut	CGGTAACGGTAACGGTAACGGT	-
iM c-MYC	CCCCACCTTCCCCACCCTCCCCACCCTCCCC	-
iM 1A83	CCTTTCCTTTACCTTTCC	1A83
iM Nano	CCCCCCCCTTTCCCCCCCC	-
iM 1G22	CCATTCCATTCCTTTCC	1G22
iM RET	CCCCGCCCCGCCCCGCCCCTA	-
iM VEGF	GACCCCGCCCCCGGCCCGCCCCGG	-
G4 c-MYC	TGAGGGTGGGTAGGGTGGGTAA	1XAV
G4 TBA	GGTTGGTGTGGTTGG	148D
G4 BCL2	GGGCGCGGGAGGAATTGGGCGGG	2F8U
G4 2JSM	TAGGGTTAGGGTTAGGGTTAGGG	2JSM
G4 2GKU	TTGGGTTAGGGTTAGGGTTAGGGA	2GKU
G4 VEGF	CGGGGCGGGCCTTGGGCGGGGT	2M27
G4 KIT2	CGGGCGGGCGCTAGGGAGGGT	2KYP
DNA hairpin	AGTCAGTCATGACTGACT	-
dsDNA	Sense: GATCGTTAACTAGTTAC Anti-Sense: GTAACTAGTTAACGATC	-
miR-21	r(UAGCUUAUCAGACUGAUGUUGA)	-



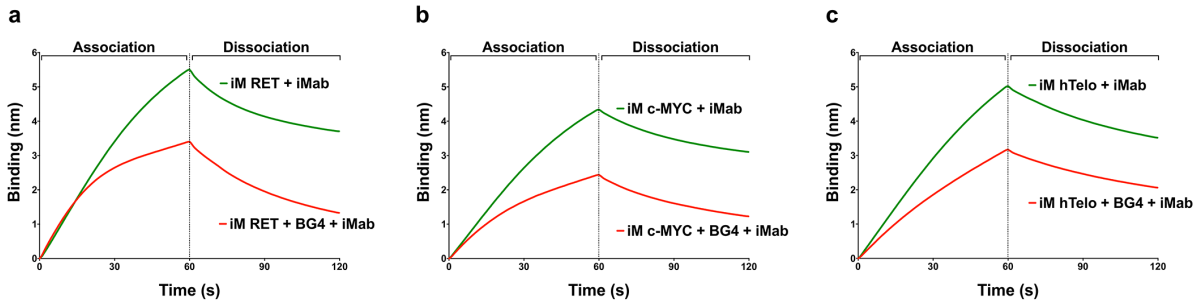
Supplementary Figure 1. **Investigating the stability of iMab across different pH ranges.** iMab does not aggregate under a wide range of pH (a) after incubation for 12 hours at 25 °C and (b) at higher temperature conditions (as indicated by turbidity at 360 nm). 100 µL of 150 µg / mL (~ 5.5 µM) purified iMab was used for each condition.



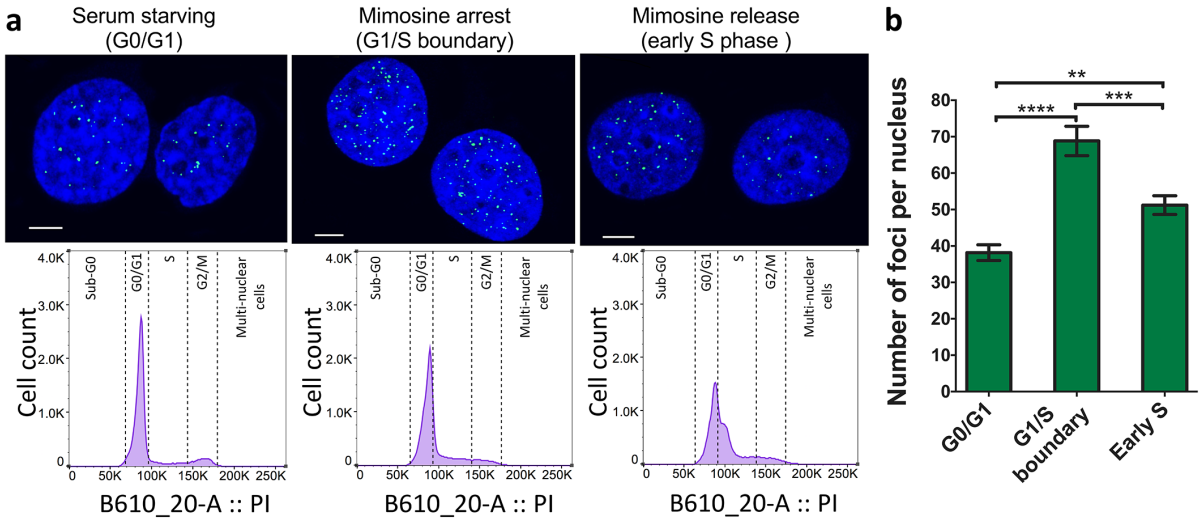
Supplementary Figure 2. **CD spectra of hTelo i-motif in the presence or absence of iMab across different pH ranges.** Presence of equimolar amount of iMab does not result in wavelength shift in the CD spectra of hTelo i-motif under different pH conditions. CD spectra were recorded for either 5 µM hTelo i-motif alone or after adding 5 µM iMab for each pH condition.



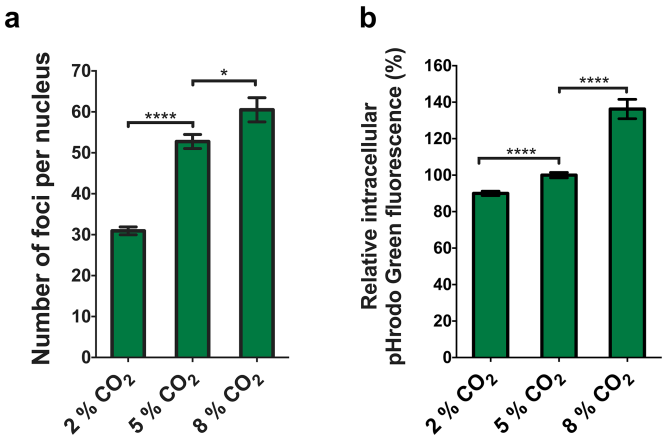
Supplementary Figure 3. **Evaluation of the specificity of antibodies targeting i-motif and G-quadruplex structures using biolayer interferometry.** (a) DP47DPK9 germline antibody does not detectably bind to i-motifs and G4 quadruplex structures. (b) iMab binds to i-motifs while it does not recognize G4s. (c) BG4 binds to G4s. (d) BG4 binds to i-motifs. G4s (folded in Tris HCl pH 7.4, 150 mM KCl) and i-motifs (folded in Tris AcOH pH 6) were loaded on the sensor to reach ~ 0.5 nm. 1 µM germline antibody, 1 µM iMab and 1:50 diluted BG4 (all in PBS) were used during association step. Dissociation step were run using PBS only.



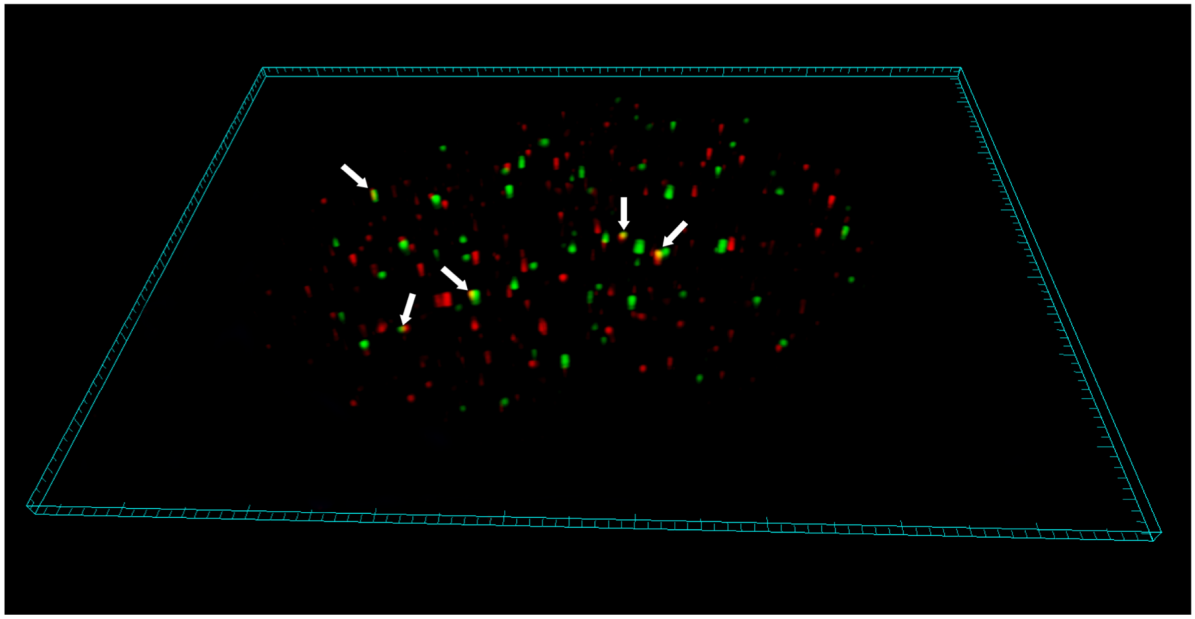
Supplementary Figure 4. **Competition between iMab and BG4 for binding to i-motifs determined by biolayer interferometry.** BG4 decreases the binding capacity of iMab to (a) RET i-motif, (b) c-MYC i-motif and (c) hTelo i-motif. Folded i-motifs in pH 6 were loaded on the sensor to reach ~ 0.5 nm binding followed by either loading BG4 (1:50 dilution in PBS) or PBS only. Association step were run using 1 μM iMab in PBS. Dissociation step were run using PBS only.



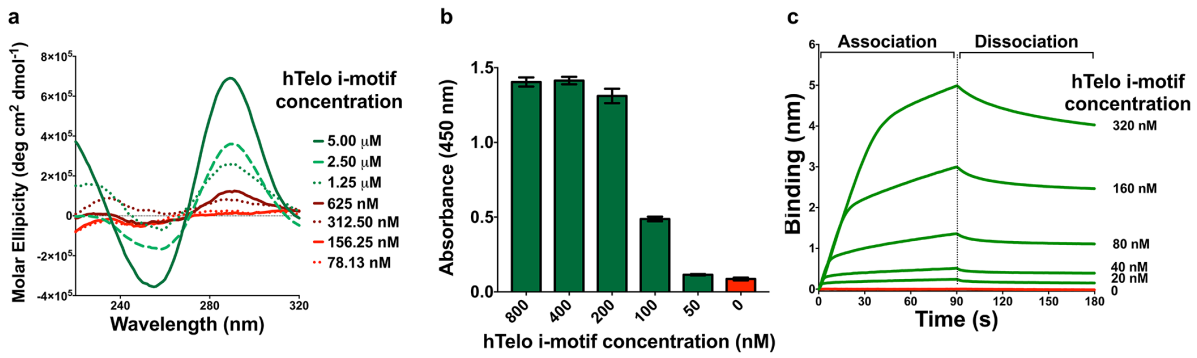
Supplementary Figure 5. **Visualization and quantification of i-motif structures during cell-cycle progression in MCF7 cells.** (a) Imaging of iMab foci in the nuclei of MCF7 cells using confocal microscopy. Cells were synchronized at the G0/G1, G1/S boundary and early S phases. Nuclei were counterstained with DAPI. Scale bars represent 5 µm. Flow cytometry graphs outline the population of synchronized and propidium iodide stained cells in each phase. (b) Quantification of the iMab foci in the nuclei of MCF7 cells synchronized at the G0/G1, G1/S boundary and early S phases. iMab foci of 300 - 400 nuclei were counted per condition for each biological replicate. Error bars represent mean ± SEM of at least two biological replicates. **P < 0.01, ***P < 0.001, ****P < 0.0001; statistical significance was assessed by one-way ANOVA using Tukey's multiple comparisons test.



Supplementary Figure 6. **Investigation of the effect of pH variation on the formation of i-motif structures in HEK 293T cells.** (a) Quantification of iMab foci in the nuclei of HEK 293T cells after 2.5 hours variation of CO_2 concentration. iMab foci of 300 - 400 nuclei were counted per condition for each biological replicate. Error bars represent mean ± SEM of at least two biological replicates. *P < 0.05, ****P < 0.0001; statistical significance was assessed by one-way ANOVA using Tukey's multiple comparisons test. (b) Relative intracellular pHrodo Green fluorescence across different CO_2 concentrations in HEK 293T cells. Error bars represent mean ± SEM of three biological replicates. ****P < 0.0001; statistical significance was assessed by one-way ANOVA using Tukey's multiple comparisons test.



Supplementary Figure 7. **Evidence of i-motifs formation in the telomeric regions.** Stimulated emission depletion (STED) image outlining the colocalization of iMab (red foci) and TRF2 (green foci) at telomeres in the nucleus of an U2OS cell. The image was deconvoluted using Huygens software. Each tick-mark represents 0.2 μm. White arrows indicate colocalization.



Supplementary Figure 8. **Comparing sensitivity of three different methods for detecting folded hTelo i-motif.** (a) CD spectroscopy; CD spectra were acquired using 300 µL of different concentrations of folded hTelo i-motif in Tris AcOH pH 6. (b) ELISA; 60 µL of 25 nM iMab were used to detect different concentrations of folded hTelo i-motif in Tris AcOH pH 6. Each bar represents the mean ± SEM from a set of three replicates. (c) biolayer interferometry; 4 µL of 500 nM iMab was used to detect different concentrations of folded hTelo i-motif in Tris AcOH pH 6.

Supplementary method 1. **Selection of an i-motif specific antibody fragment using Phage display.**

To select an i-motif specific antibody fragment, we utilized Garvan-2 scFv library, which has been generated by randomizing the complementarity-determining regions (CDR) of the DP47DPK9 germline antibody fragment. This library consists of approximately 2 x 10^8 variants.

We used human telomere i-motif (hTelo i-motif) as an antigen (Supplementary Table 1 and Fig. 1b). We performed three rounds of selection to isolate hTelo i-motif binders. Following strategies have been implemented to enrich the specific binders during successive rounds of selection:

- Alternating between neutravidin and streptavidin for coating MaxiSorp plates.
- Decreasing the concentration of target during selection.
- Increasing the stringency of washing steps.
- Counter-selection against several G-quadruplex structures.

Details are provided in Supplementary Table 2.

After three rounds of selection, polyclonal phage ELISA was performed and the result showed a considerable enrichment for hTelo i-motif specific binders. We performed monoclonal soluble fragment ELISA to identify the lead hTelo i-motif binders. Based on the intensity of signal on ELISA, which were further ranked by off-rate (k_d) using bio-layer interferometry (BLI), we identified a clone with a particularly slow dissociation rate (which we termed iMab).

Supplementary Table 2. **Details of phage display procedure used to select a hTelo i-motif specific antibody fragment.**

Selection Round	**MaxiSorp plate coating**	**Phage**	**Plate blocking reagent**	**Phage blocking reagent**	**Competitors added to the phage mixture**	**Counter-selection**	**Antigen**	**Washing**
1st	Neutravidin	2 mL of 1E12 phage per mL	5 % BSA in PBS O/N at 4 °C	5 % BSA and 0.1 % Tween-20 (T) in 50 mM TrisAcOH pH6 1 hour at room temperature (RT)	- 50 µg/mL (final concentration) Salmon Sperm (SaSp) DNA - 50 µg/mL (final conc.) yeast tRNA	- 3x Neutravidin coated wells for 30 min at RT	1 µM hTelo i-motif diluted and annealed in TrisAcOH pH 6	10x PBST 2x PBS
2nd	Streptavidin	2 mL of 5E11 phage per mL	Same as above	Same as above	- 100 µg/mL (final conc.) SaSp DNA - 100 µg/mL (final conc.) yeast tRNA	- 3x Streptavidin coated wells for 30 min at RT - 100 nM TBA G4 for 30 min at RT	0.5 µM hTelo i-motif diluted and annealed in TrisAcOH pH 6	15x PBST 2x PBS
3rd	Neutravidin	2 mL of 5E11 phage per mL	Same as above	Same as above	Same as above	- 3x Neutravidin coated wells for 30 min at RT - 100 nM KIT2 G4 for 30 min at RT - 100 nM 2JSM G4 for 30 min at RT	0.25 µM hTelo i-motif diluted and annealed in TrisAcOH pH 6	20x PBST 2x PBS

Supplementary method 2. **Counting foci in the nucleus using FIJI.**

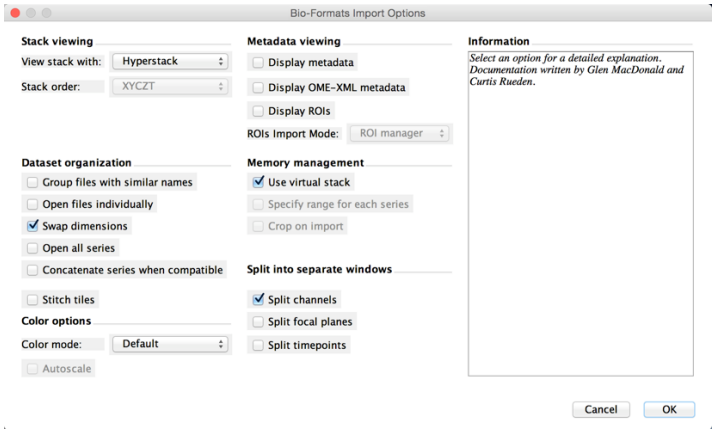
Notes:

1) The following procedure is based on the Mac version of FIJI. However, it can easily be adjusted to the Windows version.

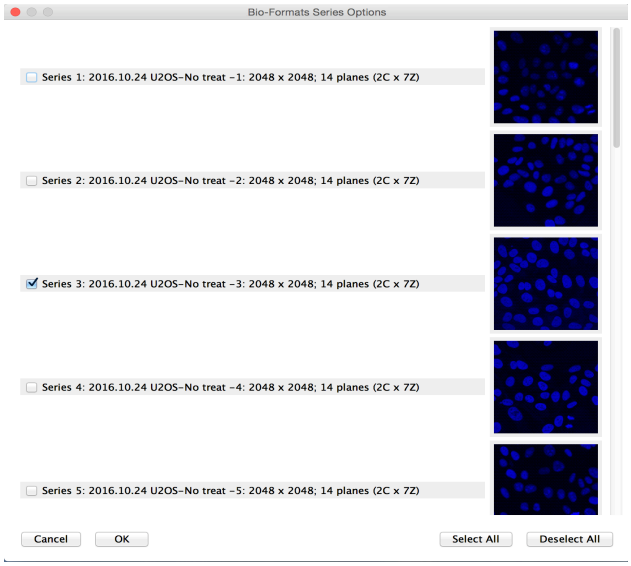
2) The image accompanied by each step is just an example that helps user follow the procedure smoothly.

3) This procedure assumes that the nuclei are counterstained by a fluorescent DNA binding dye such as DAPI.

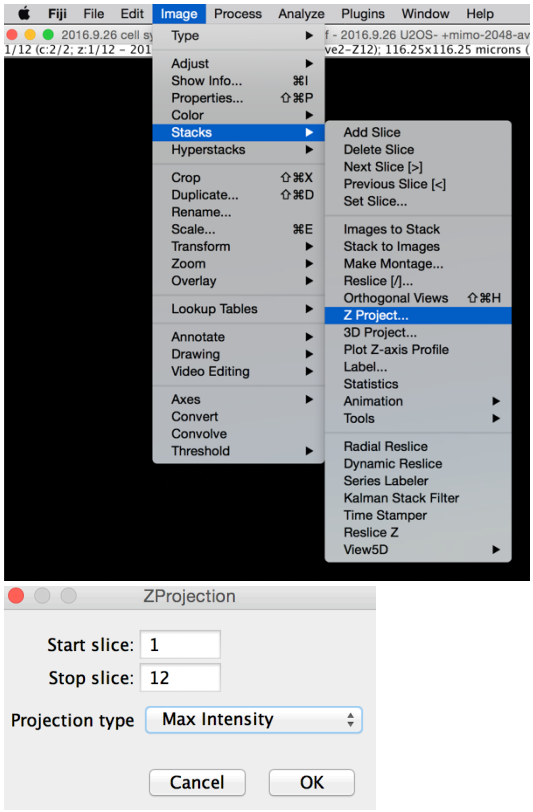
- Open the microscopy file in FIJI.

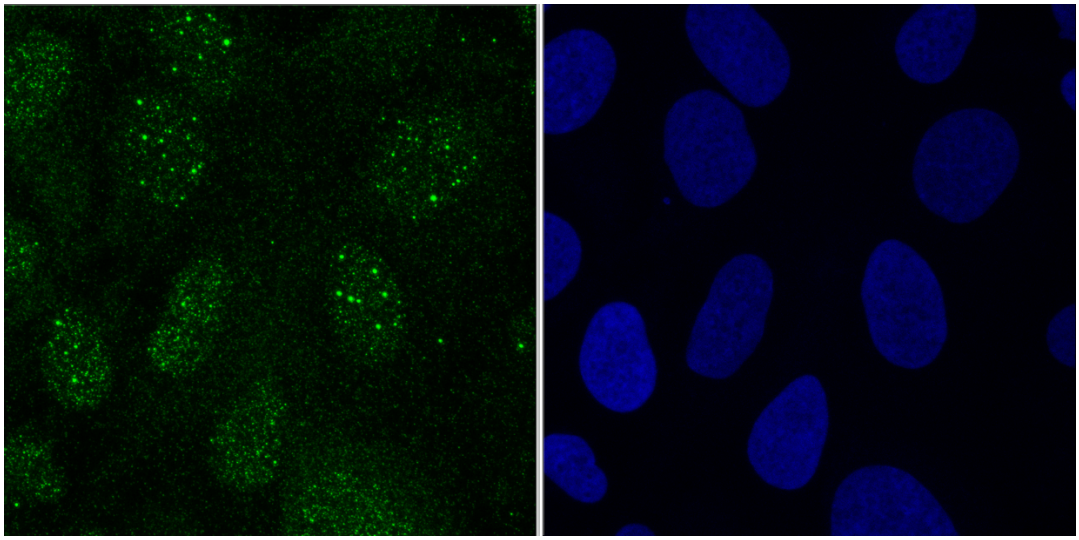


- If the file contains more than one image, choose the appropriate image.

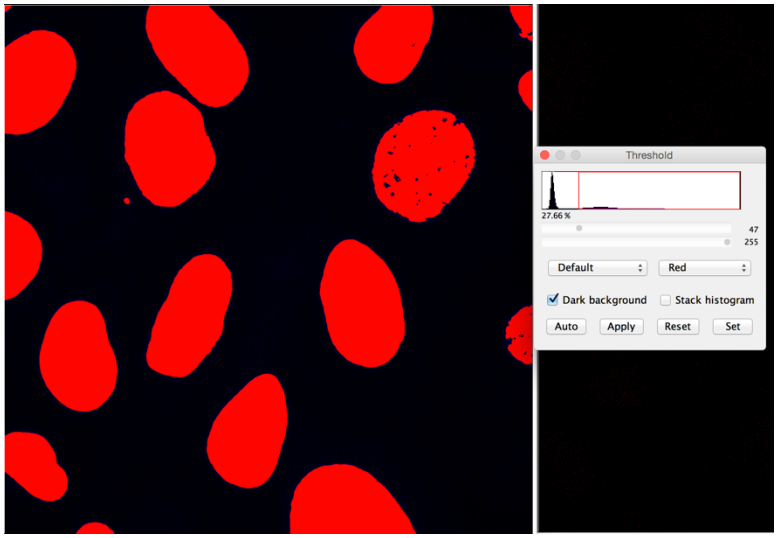


- If the image has more than one stack, make the maximum intensity for each channel.
  → Image → Stacks → Z Project → choose Max Intensity



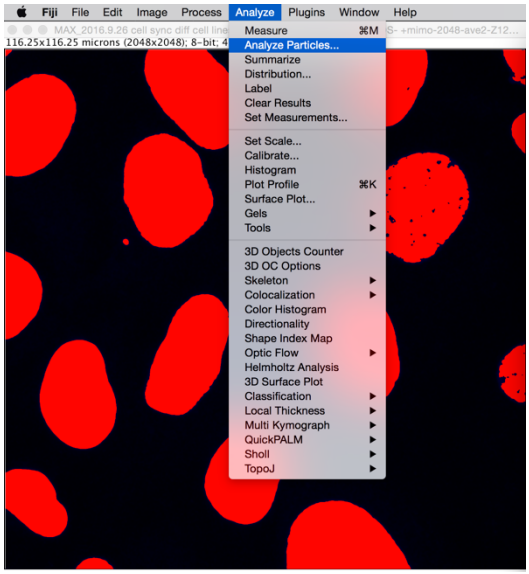


- From now on, just continue working on the max intensity images.
- Adjust the threshold on the DAPI channel so that all the nuclei become completely red
  → Command + Shift + T

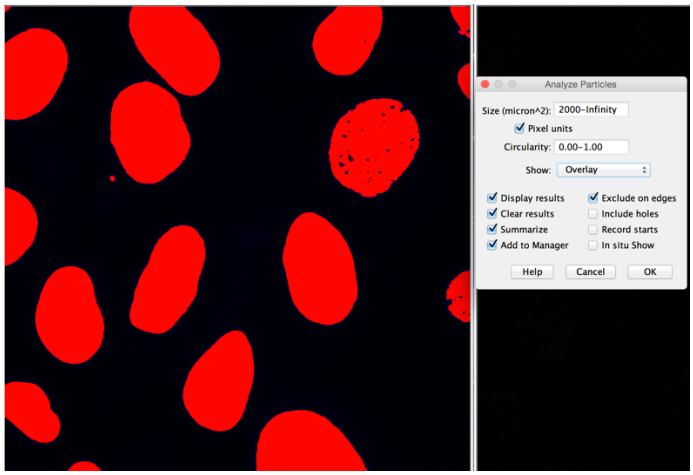


- It might be necessary to smooth the image.
  → Command + Shift + S

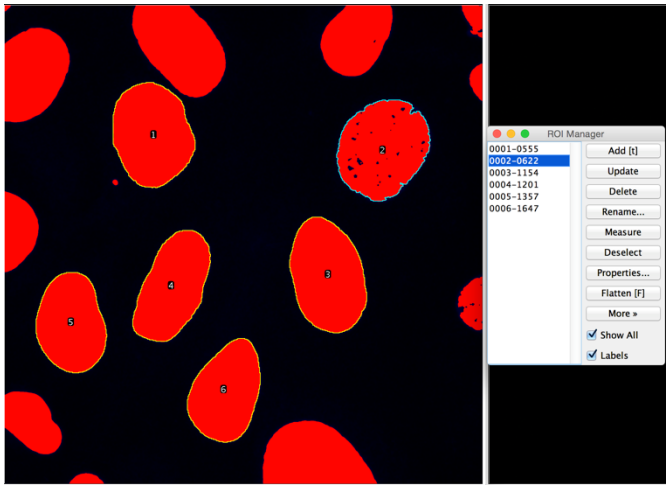
- Select the nuclei.
  → Analyze → Analyze particles



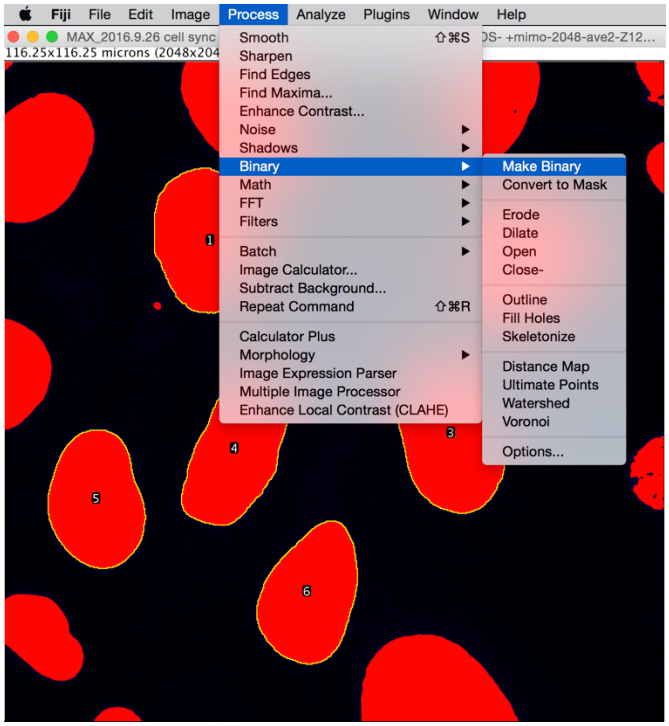
→ And choose the following parameters:



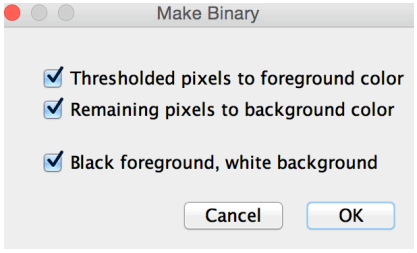
- It might be necessary to delete the overlapped nuclei or the ones that do not seem to be healthy or fully covered in red.
  → Click on the nucleus that should be deleted and use the delete button on the poped up ROI manager. For example, here, nucleus number 2 is deleted.



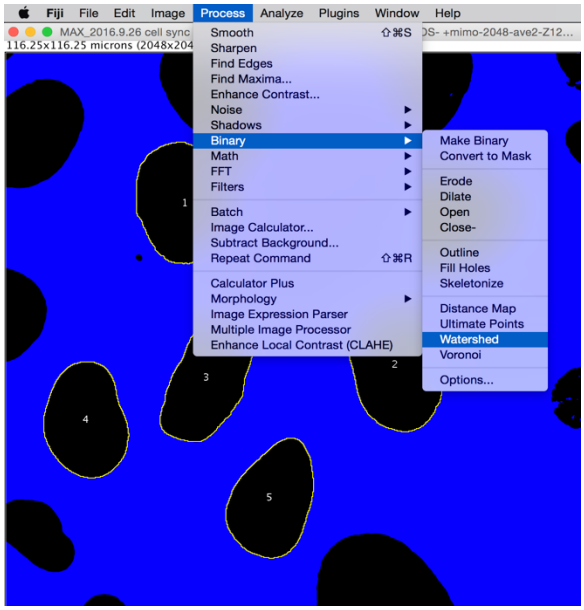
- Binarize the image.
  → Process → Binary → Make Binary



→ Choose the following parameters:

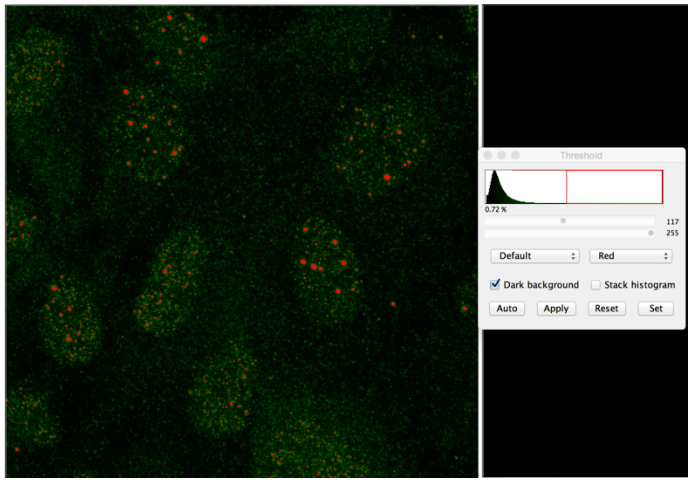


- Separate the objects by Watershed.
  → Process → Binary → Watershed

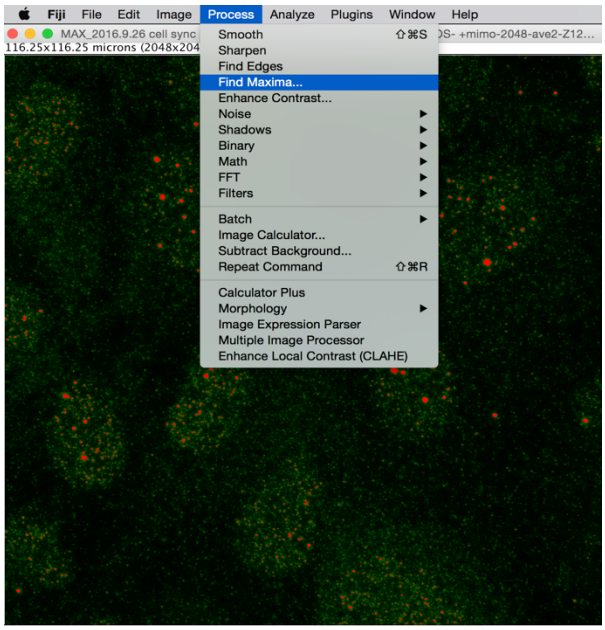


→ Alternatively, it is possible to perform Analyze particles after Binarizing and Watersheding. This helps when there are several nuclei that should be deleted.

- Go back to the max Intensity image of the foci and adjust the threshold (Command + Shift + T) so that only specific foci are in red.
  → Note: the threshold numbers are kept the same between different experiments that the number of foci is compared such as non-treatment and DNase I treatment.

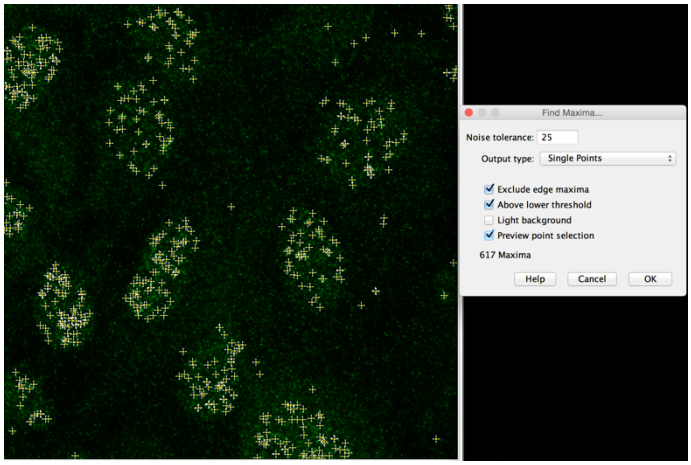


- Count the foci.
  → Process → Find Maxima

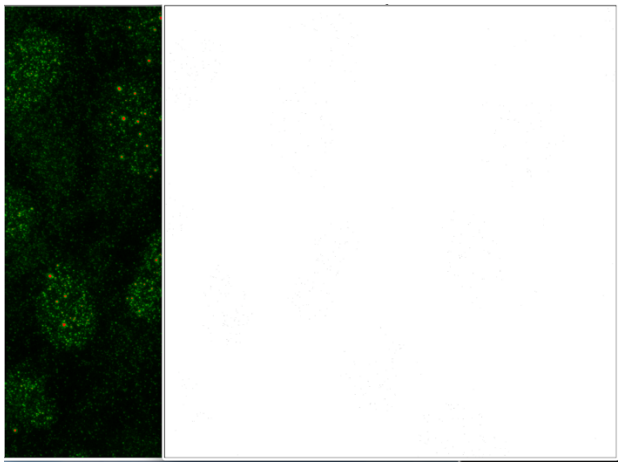


- Adjust the Noise tolerance and choose the following parameters.

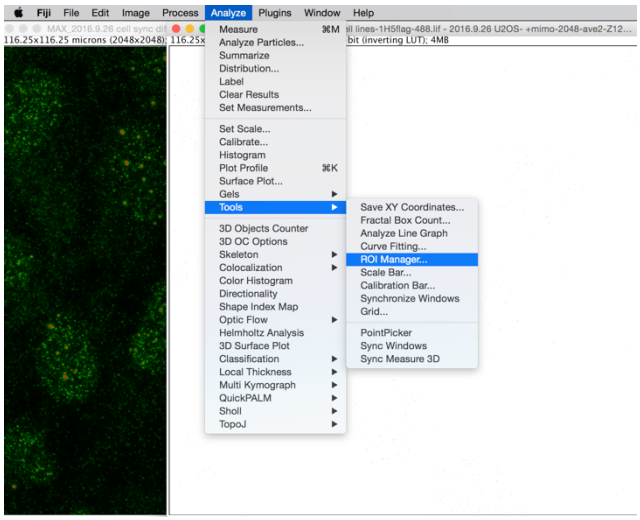
  → Note: Finding the best noise tolerance is empirical. Like the threshold numbers, the noise tolerance is kept the same between different experiments that the foci numbers are compared.



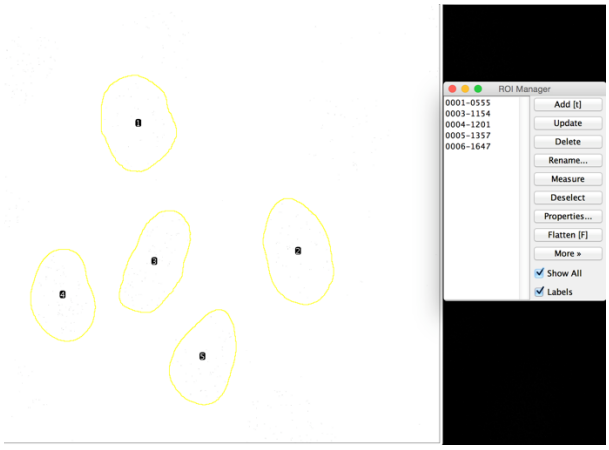
- After pressing OK, a new image containing Single Points pops up.



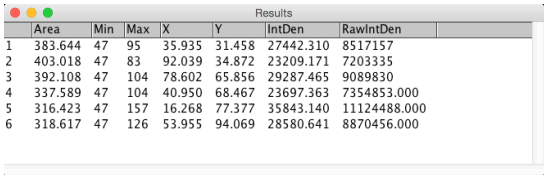
- To find the number of foci in each nucleus go to the ROI manager.



- Uncheck and Re-check the Show All.

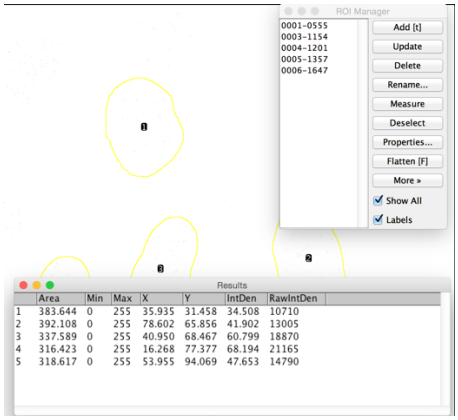


- Close previous Result window and do not save it.
  → This belongs to the max intensity image of the DAPI staining.



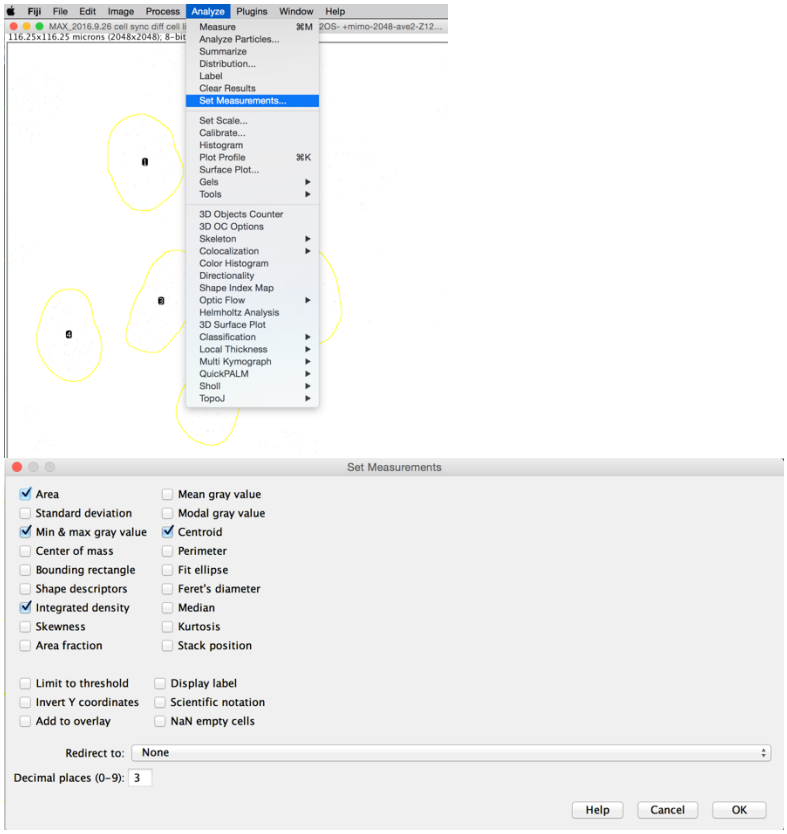
	Area	Min	Max	X	Y	IntDen	RawIntDen
1	383.644	47	95	35.935	31.458	27442.310	8517157
2	403.018	47	83	92.039	34.872	23209.171	7203335
3	392.108	47	104	78.602	65.856	29287.465	9089830
4	337.589	47	104	40.950	68.467	23697.363	7354853.000
5	316.423	47	157	16.268	77.377	35843.140	11124488.000
6	318.617	47	126	53.955	94.069	28580.641	8870456.000

- Choose Measure button in the ROI Manager. New Results pops up.



	Area	Min	Max	X	Y	IntDen	RawIntDen
1	383.644	0	255	35.935	31.458	34.508	10710
2	392.108	0	255	78.602	65.856	41.902	13005
3	337.589	0	255	40.950	68.467	60.799	18870
4	316.423	0	255	16.268	77.377	68.194	21165
5	318.617	0	255	53.955	94.069	47.653	14790

- The RawIntDen column in the Results window shows the overall intensity in each region defined by the nuclear stain. Each of the Maxima (or focal point) have the maximum value of 255 so dividing the RawIntDen number for each nucleus by 255 gives the number of detected foci in that particular nucleus.
  → If the Results does not pop up, go to the Analyze → Set measurement and check the following parameters



References

1 Lee, C. M., Iorno, N., Sierro, F. & Christ, D. Selection of human antibody fragments by phage display. *Nat Protoc* **2**, 3001-3008, doi:10.1038/nprot.2007.448 (2007).

2 Rouet, R. *et al.* Expression of high-affinity human antibody fragments in bacteria. *Nat Protoc* **7**, 364-373, doi:10.1038/nprot.2011.448 (2012).

3 Schindelin, J. *et al.* Fiji: an open-source platform for biological-image analysis. *Nat Methods* **9**, 676-682, doi:10.1038/nmeth.2019 (2012).