

Legends to Supplementary figures and videos:

Figure S1: The small ribosomal subunit and mRNA are present in stress granules in contrast with the large ribosomal subunit.

- A) NRK cells were exposed to 300 μ M arsenite for 45 min. Cells were then labeled with antibodies raised against indicated proteins (YB-1, S6, RPL10). As previously published (1), we found that S6, a protein of the small ribosomal subunit, is more concentrated in stress granules than in the rest of the cytoplasm, even though to a lesser extent than YB-1 an mRNA-binding protein. On the other hand, RPL10, a protein of the large ribosomal subunit, is rather excluded from stress granules.
- B) NRK cells were partially enucleated and exposed to arsenite (300 μ M for 45 min). In situ hybridization was then performed to reveal Poly(A) mRNA and the 40S and 80S ribosomal subunits in arsenite-treated NRK cells as follows. Cell fixed with 4% PFA (in PBS-sucrose) for 30 min at 37° C. Cells were incubated with 100% ice-cold methanol for 10 minutes at -20° C, after that in ice-cold 70% ethanol for 10 minutes at -20° C, and then 1 M Tris pH 8 for 5 minutes, before addition of a Cy3-conjugated oligonucleotides (Sigma) in the hybridization buffer (0.005% BSA, 1 mg/mL yeast RNA, 10% dextran sulphate, 25% formamide in 2XSSC) at 1 μ g/ μ L. Ploy(T) of 40 nucleotides, 5'-AAGGATTTAAAGTGGACTCATTCCAATTAC and 5'GGATTCTGACTTAGAGGCGTTCAGTCATAA probes, were used to detect mRNA and the 18S (40S subunit) and 28S (80S subunit) rRNA in NRK cells respectively. Slides were then placed in a humidity chamber for 1 hour at 37°C and shacked. Following hybridization, cells were washed once with 4X SSC and once with 2XSSC. NRK cells were also labeled with anti-YB-1 antibody to detect stress granules.

The line profiles of fluorescent intensity over typical stress granules (SG) indicate a significant increase of the mRNA concentration and a moderate increase of the 40S subunit concentration. No increase of the 80S subunit concentration was detected in stress granules.

Figure S2: Quercetin induces stress granule assembly in the presence of puromycin and polyubiquitinated proteins were detected in stress granules.

- A) Anti-YB1-labeled NRK cells were treated with quercetin (25 μ M) in the presence or absence of puromycin (2.5 μ g/mL). YB-1 is an mRNA-binding proteins which is used here as marker of mRNA.
- B) NRK cells were treated with arsenite (300 μ M for 45 min), 10 μ M MG132 for 3 h in the presence or absence of puromycin. NRK cells were then immune-stained with anti-polyubiquitin. The results show that cytoplasmic aggregates containing polyubiquitinated protein appear after arsenite or puromycin/MG132 treatments. These aggregates are co-localized with stress granules (see figure 2).

Figure S3: Puromycin induces stress granule assembly in MG132-treated NRK cells.

- A) NRK cells were continuously exposed to 10 μ M MG132 for 4 h. Except for control, cells also received either cycloheximide or puromycin during the first 3h and then cycloheximide or puromycin for the next hour, as indicated. Anti-YB-1 labeling indicates that stress granules only appeared when puromycin was present during the last hour. Washing out puromycin during the last hour readily dissociates stress granules. Results are mean \pm SD obtained on three different samples. ** p < 0.005 by t test. Puro, puromycin (2.5 μ g/mL); Cyclo, cycloheximide (20 μ g/mL).
- B) NRK cells were continuously exposed to 10 μ M MG132 for 4 h or to arsenite (300 μ M for 45 min). The level of eIF2 α phosphorylation at serine 51 was then probed using a specific anti-phosphorylated eIF2 α antibody. We found that the phosphorylation level was significantly lower in MG132-treated than arsenite-treated cells. In a previous report, MG132 was shown to trigger stress granule assembly by itself but the initiation factor, eIF2 α , was then significantly phosphorylated thus leading to polysome dissociation (2). This is apparently not the case for NRK cells under the condition presented in A), which explains why puromycin was necessary to trigger stress granule assembly after MG132 treatment.

C) NRK cells were exposed to 10 μ M MG132 for indicated time and imuno-stained with anti-YB-1 (green) and anti-Vimentin (red). After 10 h exposure, the aggregation of vimentine, a typical marker of aggresomes, can be observed in the centrosomal area. The formation of aggregates in the centrosomal region made of misfolded proteins called aggresomes after MG132 treatment is a control that this chemical indeed targets the proteasome activity (3).

Figure S4: Effect of the mRNA concentration on stress granule formation due to mRNA transfection and kinetic of stress granule formation after mRNA transfection.

- A) NRK cells were transfected with α -globin mRNA at varying concentration in the presence of 2.5 μ g/mL puromycin using lipofectamine (Lipo) and cells were then fixed after 3 h for the immunofluorescence detection of stress granules.
- B) Graph showing the appearance of stress granules with time. 1 h 30 are required to observe the first stress granules in the presence of puromycin. (1 μ g/mL α -globin mRNA)

Figure S5: Effect of YB-1 silencing and GFP-YB-1 expression on stress granule assembly.

- A) NRK cells were transfected with control or YB-1 siRNA duplexes for 36 h. NRK cells were then fixed (upper panel) or exposed to 300 μ M arsenite for 45 min (lower panel). Anti-HuR labeling revealed the presence of stress granules in both control and YB-1 siRNA-treated cells (the cytoplasm of YB-1-silenced cells appears red in the merge, see arrows).
- B) NRK cells were transfected with GFP-YB-1 or GFP plasmids for 36 h. NRK cells were then exposed to puromycin (2.5 μ g/mL)/VER-155008 (10 μ M) for 3 h (lower panel). Anti-YB-1 (endogenous and GFP-labeled YB-1) or anti-HuR labeling revealed that stress granule assembly was impaired in GFP-YB-1 expressing cells, in contrast with GFP-expressing cells. Let us recall that VER-155008 is a potent inhibitor of HSP70.

Figure S6: The enucleation process has no significant effect on the location of shuttling proteins (HuR, TIA-1) in NRK cells.

- A) NRK cells were exposed to 1 μ M cytochalasin D for 1 h and then cytochalasin D was washed away for 1 h. Anti-TIA-1 labeling show that these nucleocytoplasmic proteins did not significantly shuttle in the cell cytoplasm.
- B) NRK cells were enucleated as described in Materials and Methods and labeled with anti-TIA-1 and DAPI as indicated. We then measured the mean anti-TIA-1 cytoplasmic fluorescence in both non enucleated and enucleated cells. Results are mean \pm SD obtained on three different samples. No significant difference was found between enucleated and non enucleated cells
- C) NRK cells were enucleated and labeled with anti-HuR. Note that the cytoplasmic level of HuR is similar in both non enucleated or enucleated cells.
- D) NRK cells were partially enucleated and exposed to arsenite (300 μ M for 45 min). Hybridization in situ was then performed to reveal Poly(A) mRNA, as previously described (Figure S1A). Stress granules were indeed observed in both enucleated and non enucleated cells.

Figure S7: Enucleated cells form stress granules excluded from the center of the cytoplasm and stress granules are maintained after enucleation.

- A) NRK cells were enucleated, exposed to 10 μ M VER-155008 plus 2.5 μ g/mL puromycin for 1 h 30 min and immune-stained with anti-YB-1 and anti-tubulin. Stress granules formed in enucleated cells are located at the cell periphery and tend to avoid the enucleated cell center.
- B) NRK cells were treated with VER15508 plus puromycin 1 h prior to and during the enucleation procedure. VER15508 plus puromycin was also present in the incubation buffer after the cytochalasin D removal for 1 h. Coexisting enucleated and non enucleated cells were then immune-stained with DAPI (blue) and anti- YB-1(green). Stress granules can be observed in both enucleated and non enucleated cells.

Figure S8: HuR is not critical for stress granule assembly and its overexpression impairs stress granule assembly.

- A) NRK cells were transfected with RFP-G3BP (left) or HuR (right) plasmid for 36 h. HuR overexpression in NRK cells, in contrast with RFP-G3BP expression, does not lead to stress granules assembly by itself. Image showing the distribution of YB-1 in the same area in HuR-overexpressing cells is provided as control.

- B) NRK cells were transfected with control or HuR siRNA duplexes for 36 h and then exposed to 300 μ M arsenite for 45 min. The presence of stress granules can be observed in both control and HuR siRNA-treated cells. (HuR-silenced cells appear with a dark nucleus in the merge see arrow)
- C) After arsenite stress, higher cytoplasmic level of HuR correlates with an inhibition of stress granule assembly, although the effect was less clear than that obtained with YB-1 (figure 4B). These results are however difficult to interpret since HuR positively regulates the expression of TIA-1 (4) and probably changes the expression of other RNA-binding proteins.
- D) NRK cells were treated with ActD (5 μ g/mL for 2 h) and labeled with anti-TIA-1. The translocation of TIA-1 from the nucleus to the cytoplasm can be observed following the ActD treatment.

Figure S9: Short ActD treatment does not significantly impact the global mRNA level and the phosphorylation of eIF2 α upon arsenite treatment.

- A) The level of mRNA was estimated via in situ hybridization using cy3-labeled Poly(T) oligonucleotides as described in figure S1. 5 μ g /mL ActD for 2 h does not significantly decrease the level of mRNA while it is sufficient to impair stress granule assembly. In line with this, the half-life of most mRNAs is comprised between 5 to 15 h (5). In addition, stress granule assembly is not inhibited in enucleated cells treated with ActD (figure 7C), which indicates that changes in the mRNA level probably not explain the observed behavior in ActD treated-cells.
- B) NRK cells were exposed to indicated treatments (arsenite 45 min, 300 μ M; ActD 5 μ g/ml). Both western blotting and immunofluorescence with antibodies directed against indicated proteins show that eIF2 α was significantly phosphorylated after arsenite exposure in ActD-pretreated cells.

Figure S10: Screening of the effect of various kinase and phosphate inhibitors on HuR translocation. NRK cells were treated for 1 h 30 with the following chemicals:

- 2 mM Vanadate, tyrosine phosphate inhibitor
- 10 μ M U1026, MEK inhibitor
- 50 mM NaF, serine/threonine phosphate inhibitor
- 10 μ M SB203580, P38 MAPK inhibitor
- 5 μ M PKRi, PKR kinase inhibitor (C16)

- 10 μ M PD0325901, MEK inhibitor
- 20 μ M SP600125, JNK inhibitor
- 0.3 μ M Okadaic Acid, serine/threonine phosphate inhibitor
- 10 μ M CDKi, CDK1/5 inhibitor
- 40 μ M Hemin, HRI kinase inhibitor
- 20 μ M H89, PKA kinase inhibitor

Cells were fixed and labeled with anti-YB-1 (green), HuR (red)

Figure S11: PKRi induces the translocation of nuclear TIA-1 and arsenite-preconditioning inhibits stress granules assembly.

- A) NRK cells were exposed to arsenite (300 μ M, 45 min) with or without PKRi (10 μ M) 1 h prior to and during arsenite treatment.
- B) NRK cells were pretreated with arsenite (200 μ M for 1 h) and then allowed to recover for 3 h. Non pretreated or pretreated cells were then exposed to arsenite and labeled with anti-YB-1 for immuno-fluorescence imaging.

Video 1: Time-Lapse videomicroscopy of stress granule assembly in puromycin-treated NRK cells after HSP70 inhibition

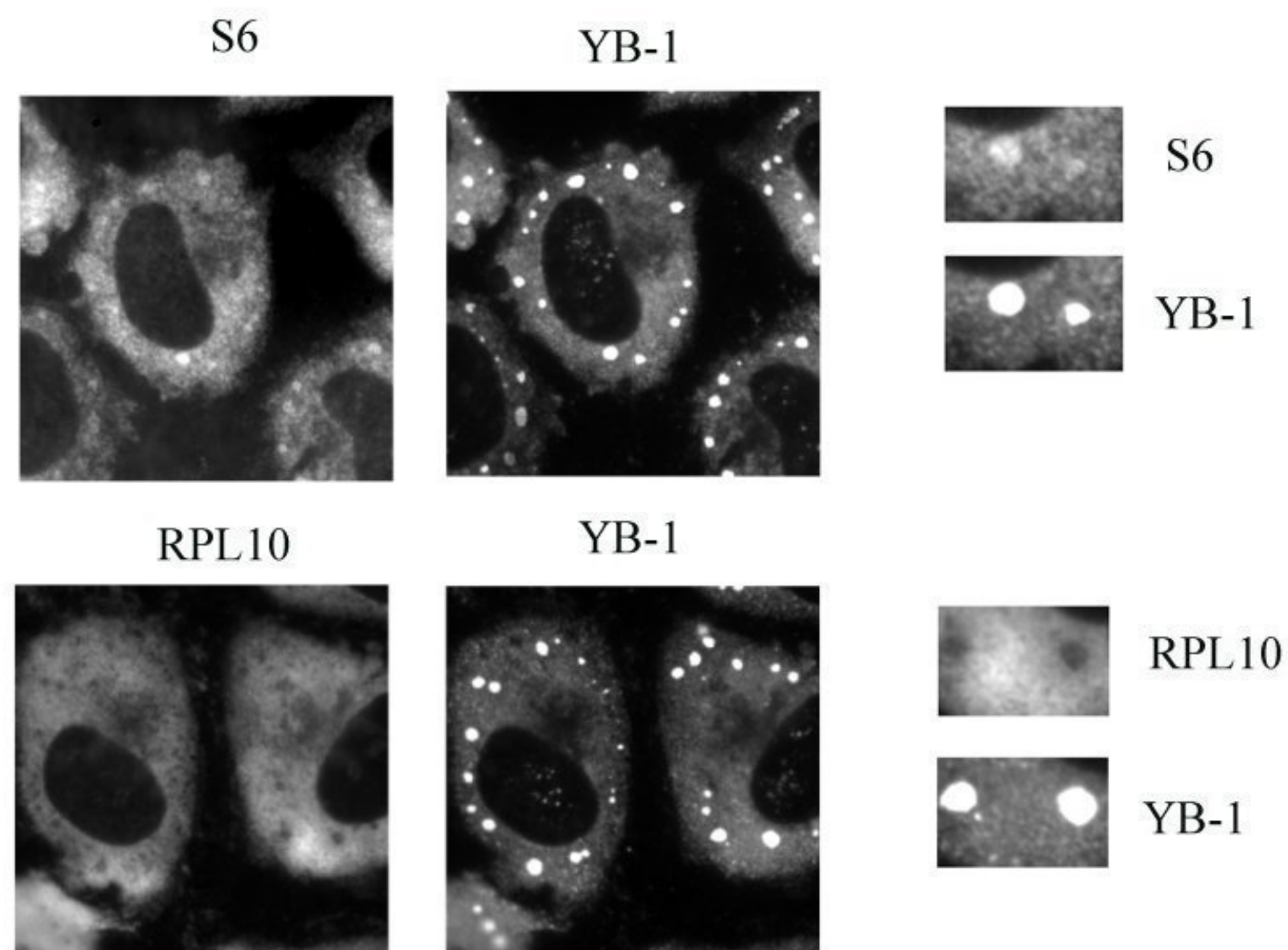
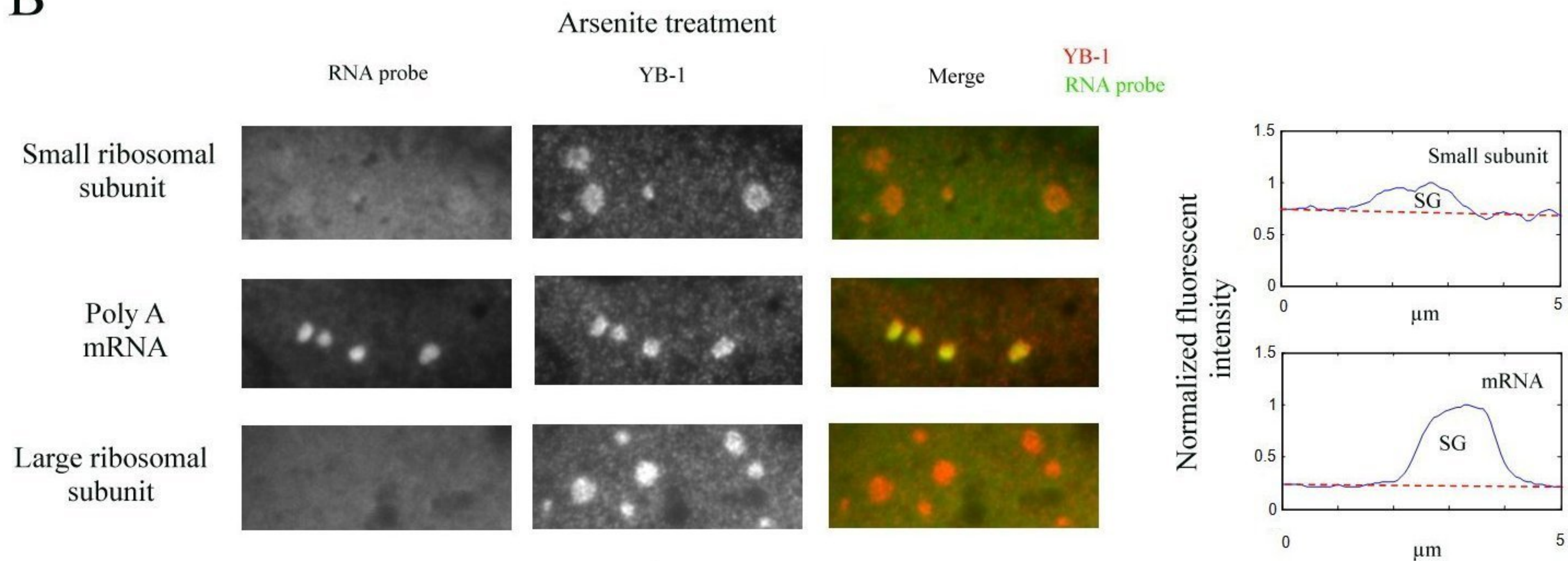
NRK cells were transfected with a GFP-YB-1 encoding plasmid (0.5 μ g/mL) and exposed to 10 μ M VER-155008 plus 2.5 μ g/mL puromycin. Fluorescence images were collected at 1 min intervals for 70 min beginning 10 min after the addition of VER-155008 plus puromycin. We selected only cells displaying low levels of GFP-YB-1 expression since higher expression levels inhibit stress granule assembly (see figure 4).

Video 2: Time-Lapse Videomicroscopy of stress granule disassembly after cycloheximide exposure.

NRK cells were transfected with a GFP-YB-1 encoding plasmid (0.5 μ g/mL) and exposed to 10 μ M VER-155008 plus 2.5 μ g/mL puromycin for 2 h. Cycloheximide (20 μ g/mL) was then added to the incubation buffer. Fluorescence images were collected at 3 min intervals for 63 min.

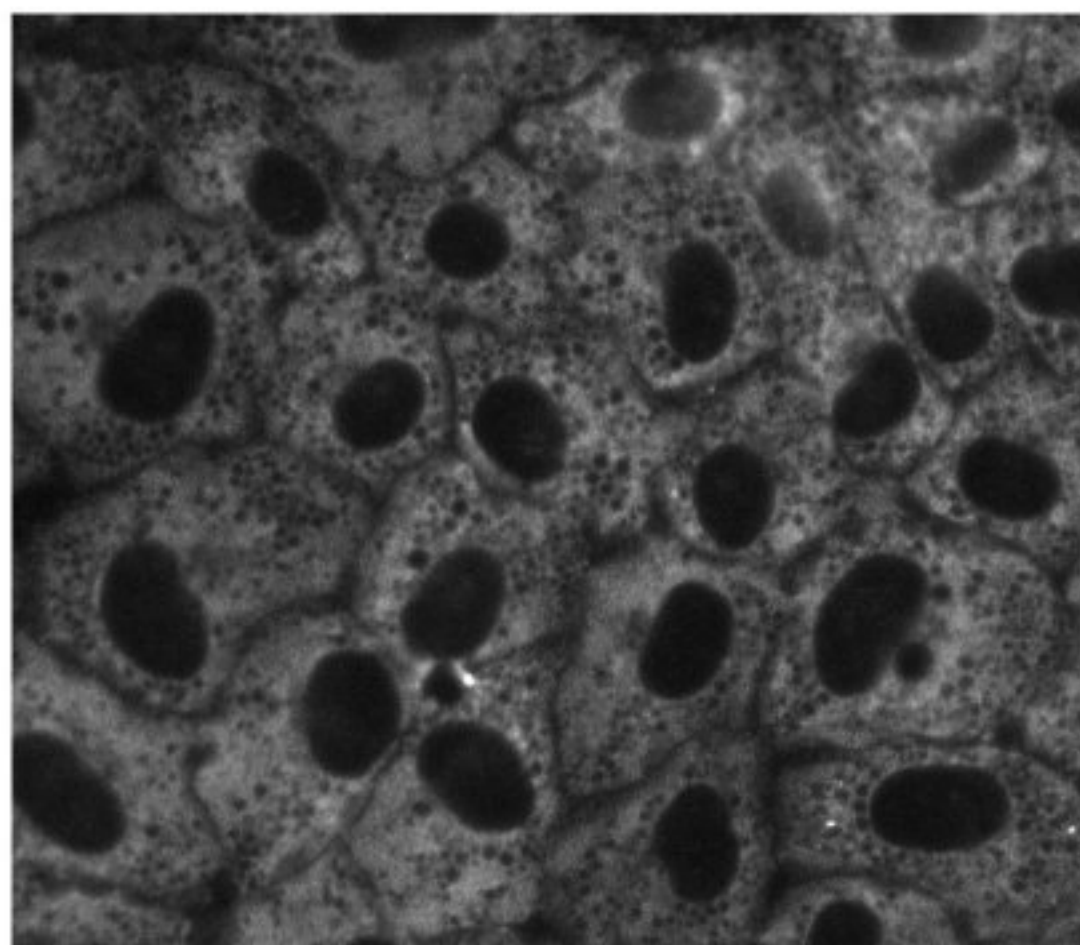
References of supplementary files:

1. Kedersha N, *et al.* (2002) Evidence that ternary complex (eIF2-GTP-tRNA(i)(Met))-deficient preinitiation complexes are core constituents of mammalian stress granules. (Translated from eng) *Mol Biol Cell* 13(1):195-210 (in eng).
2. Mazroui R, Di Marco S, Kaufman RJ, & Gallouzi IE (2007) Inhibition of the ubiquitin-proteasome system induces stress granule formation. (Translated from eng) *Mol Biol Cell* 18(7):2603-2618 (in eng).
3. Johnston JA, Ward CL, & Kopito RR (1998) Aggresomes: a cellular response to misfolded proteins. (Translated from eng) *J Cell Biol* 143(7):1883-1898 (in eng).
4. Kawai T, *et al.* (2006) Translational control of cytochrome c by RNA-binding proteins TIA-1 and HuR. (Translated from eng) *Mol Cell Biol* 26(8):3295-3307 (in eng).
5. Schwanhausser B, *et al.* (2011) Global quantification of mammalian gene expression control. (Translated from eng) *Nature* 473(7347):337-342 (in eng).

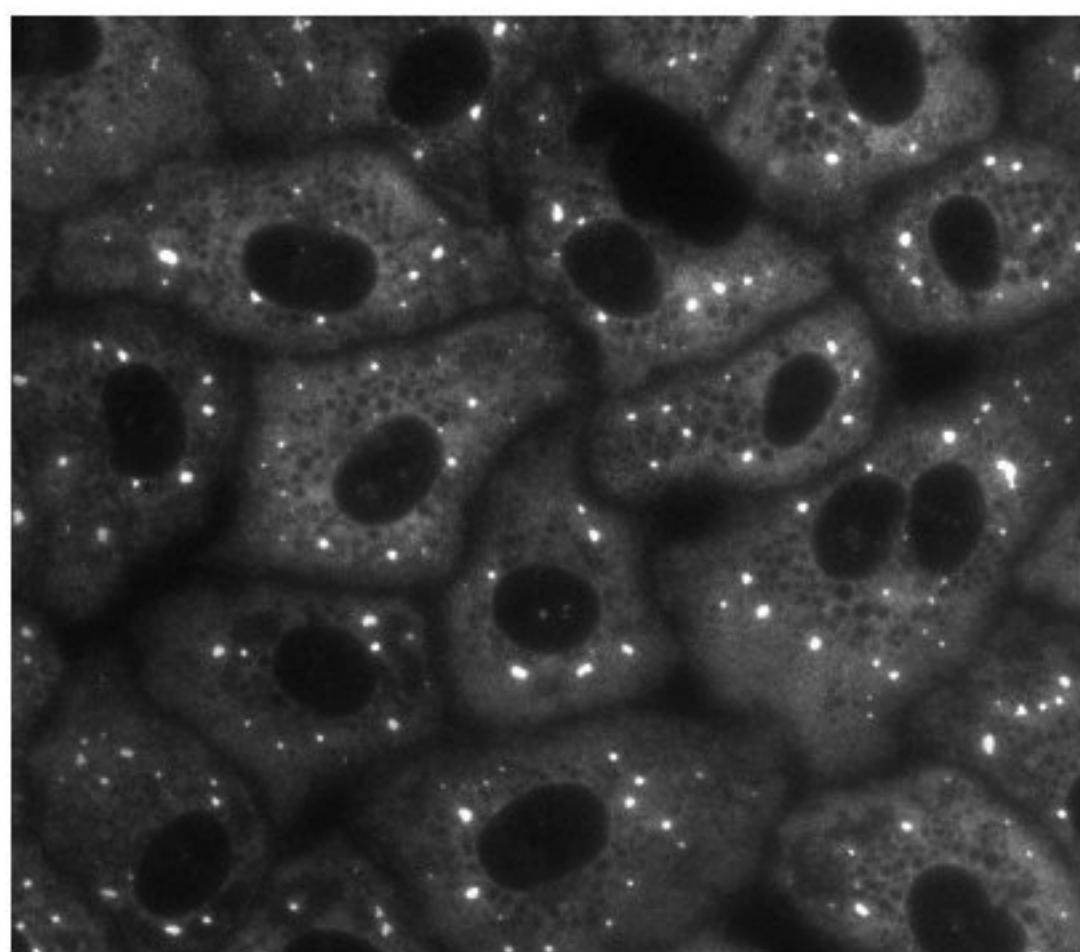
A**B****Figure S1**

A

Quercetin

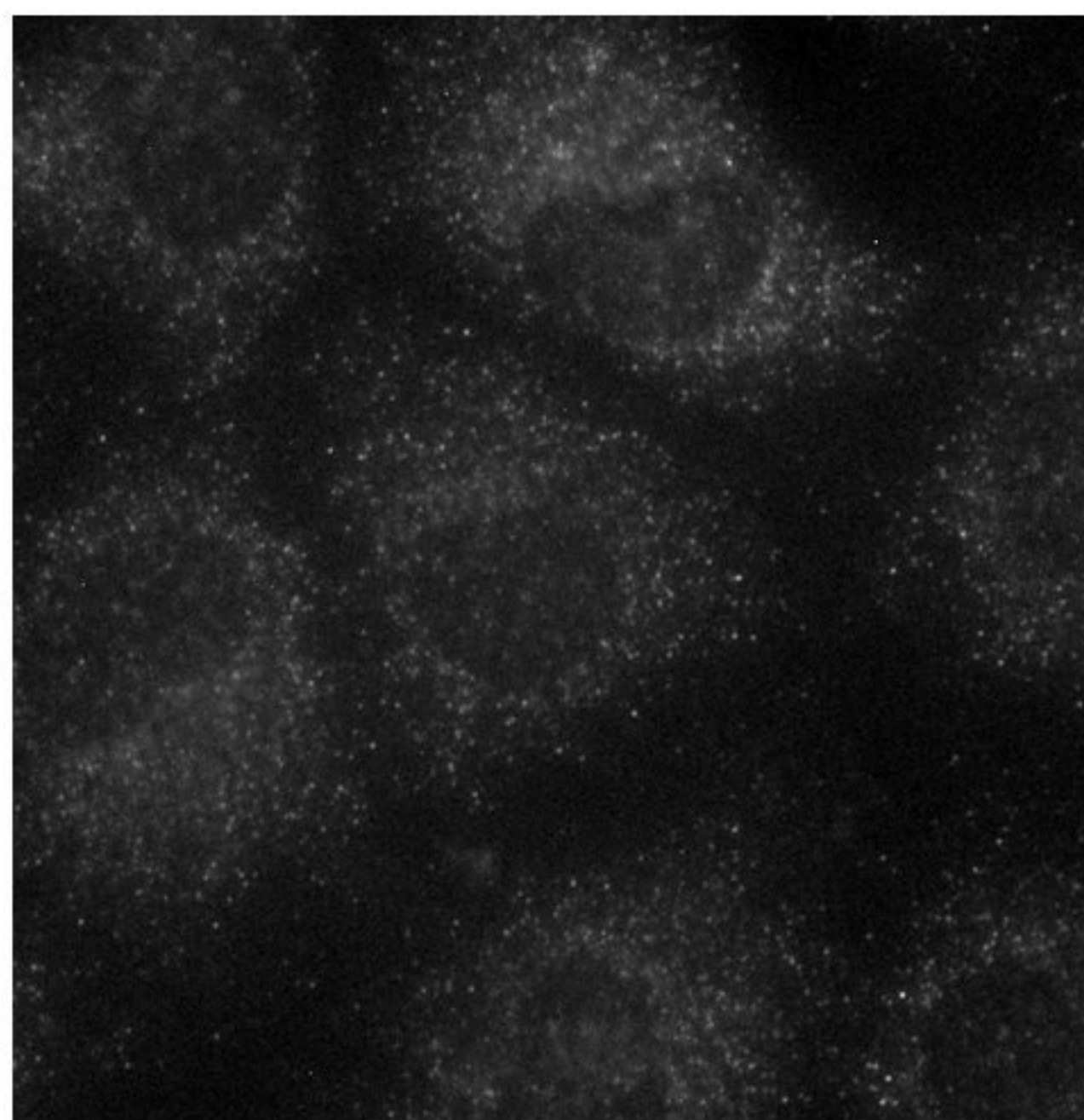


Quercetin
puromycin

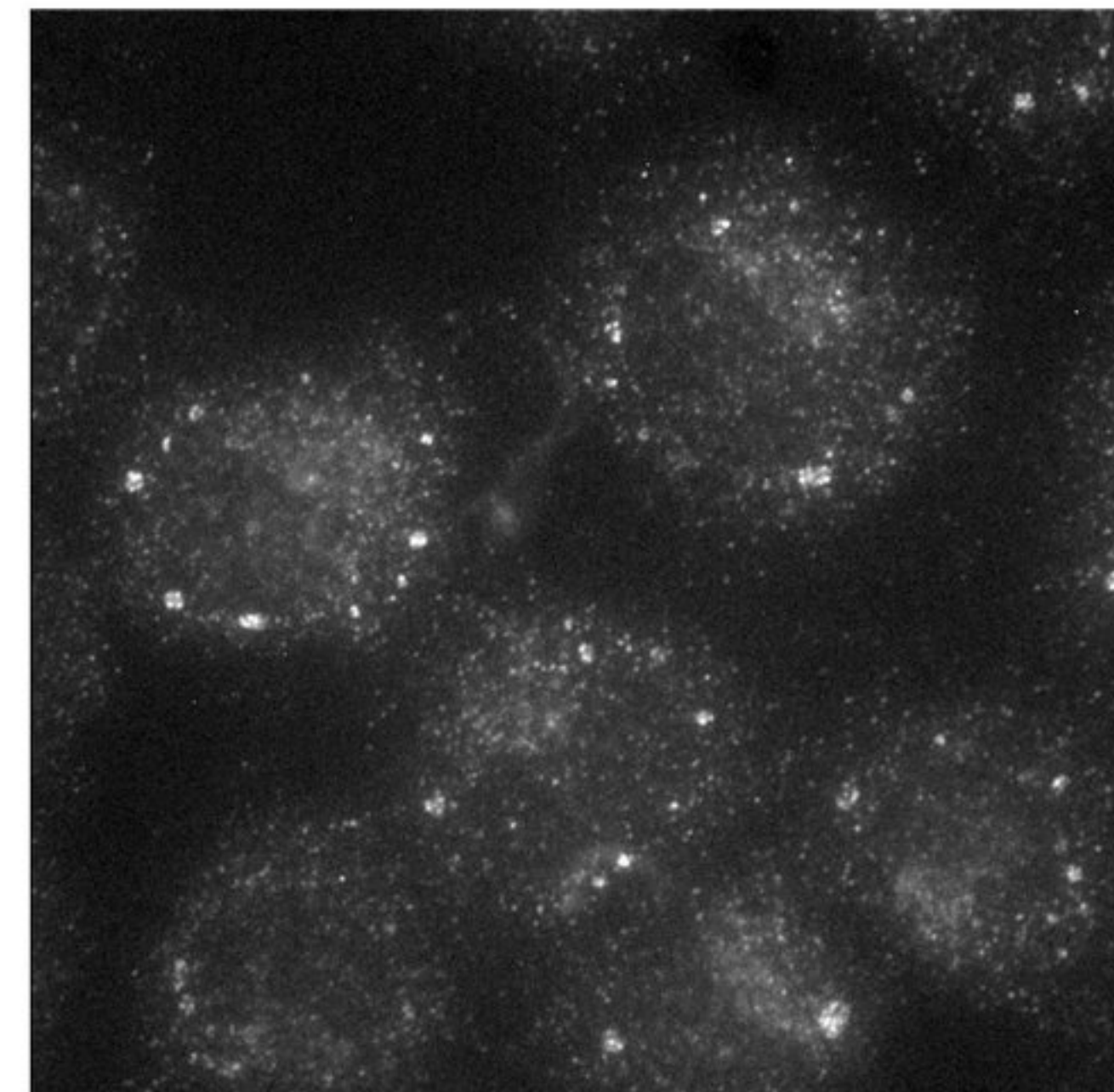


B

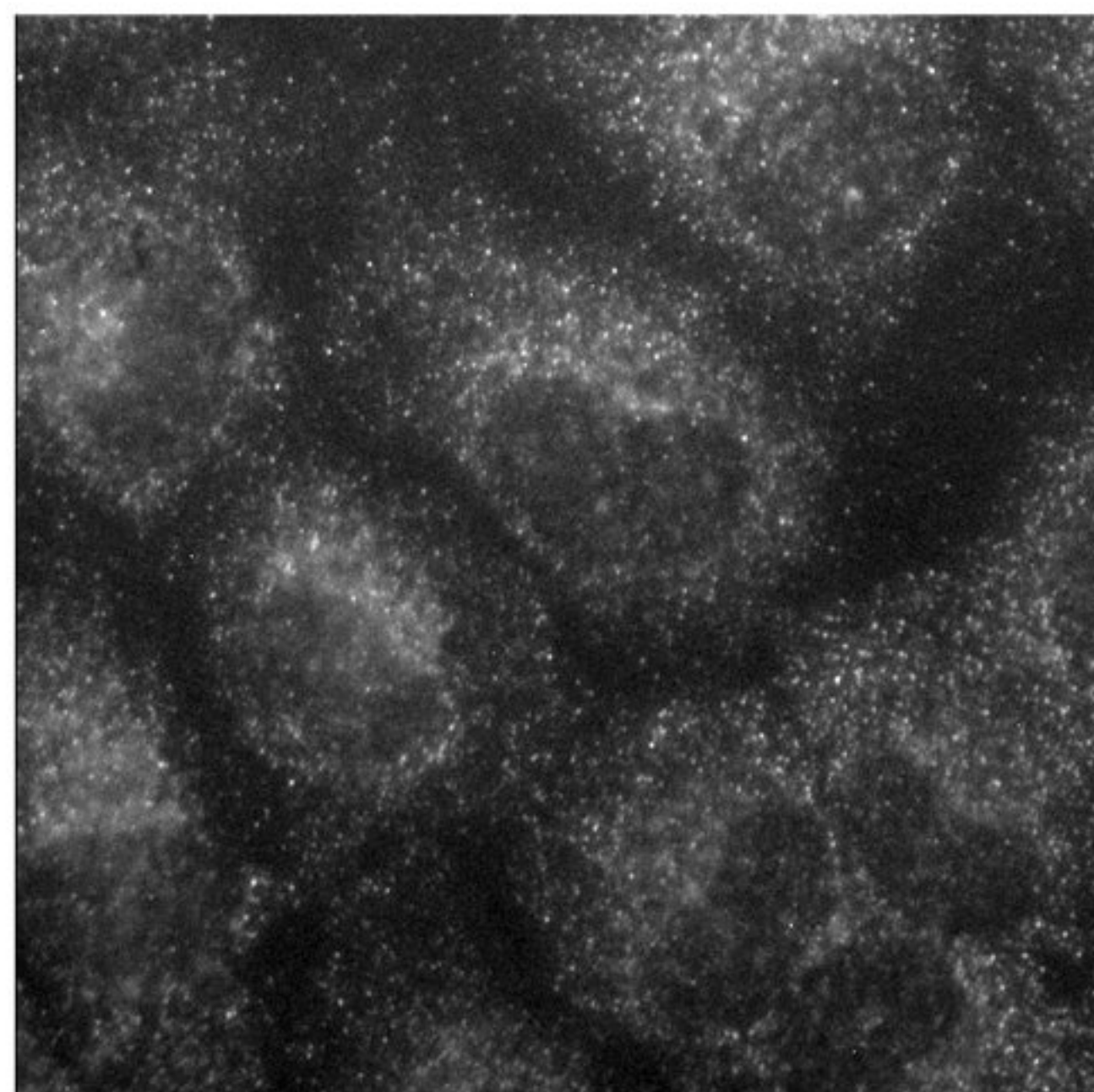
Control



Arsenite



MG132



MG132 + puromycin

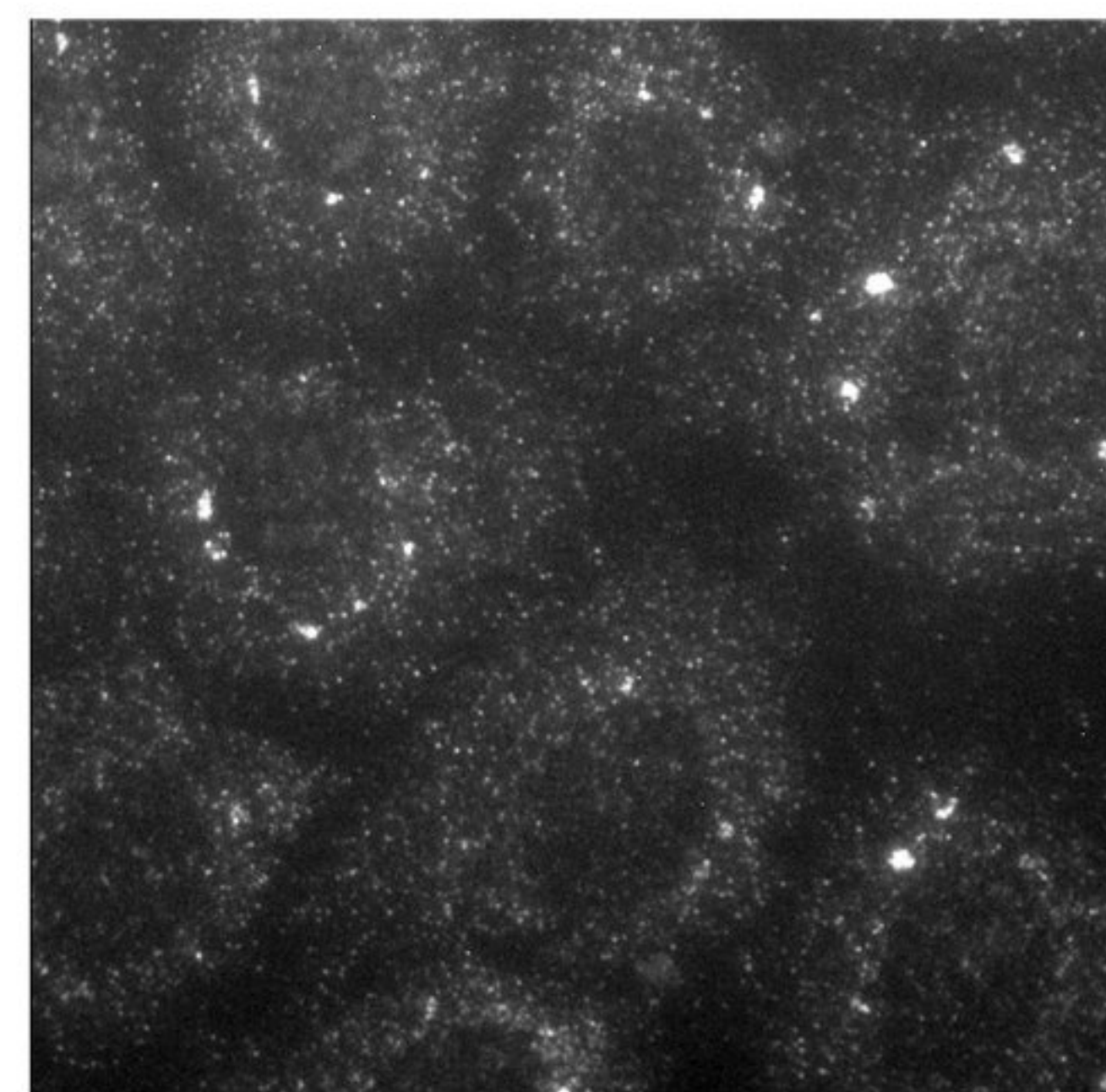
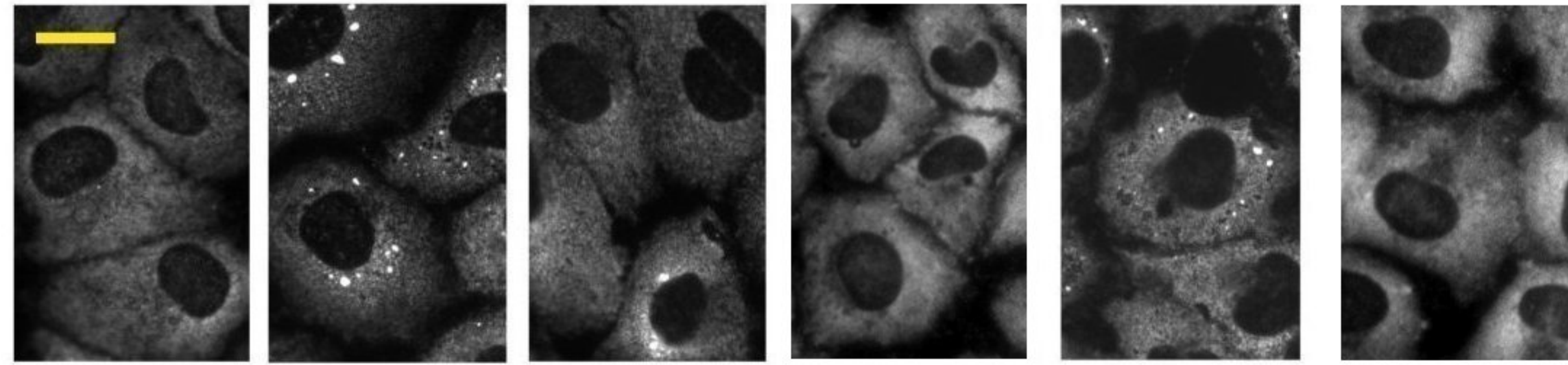
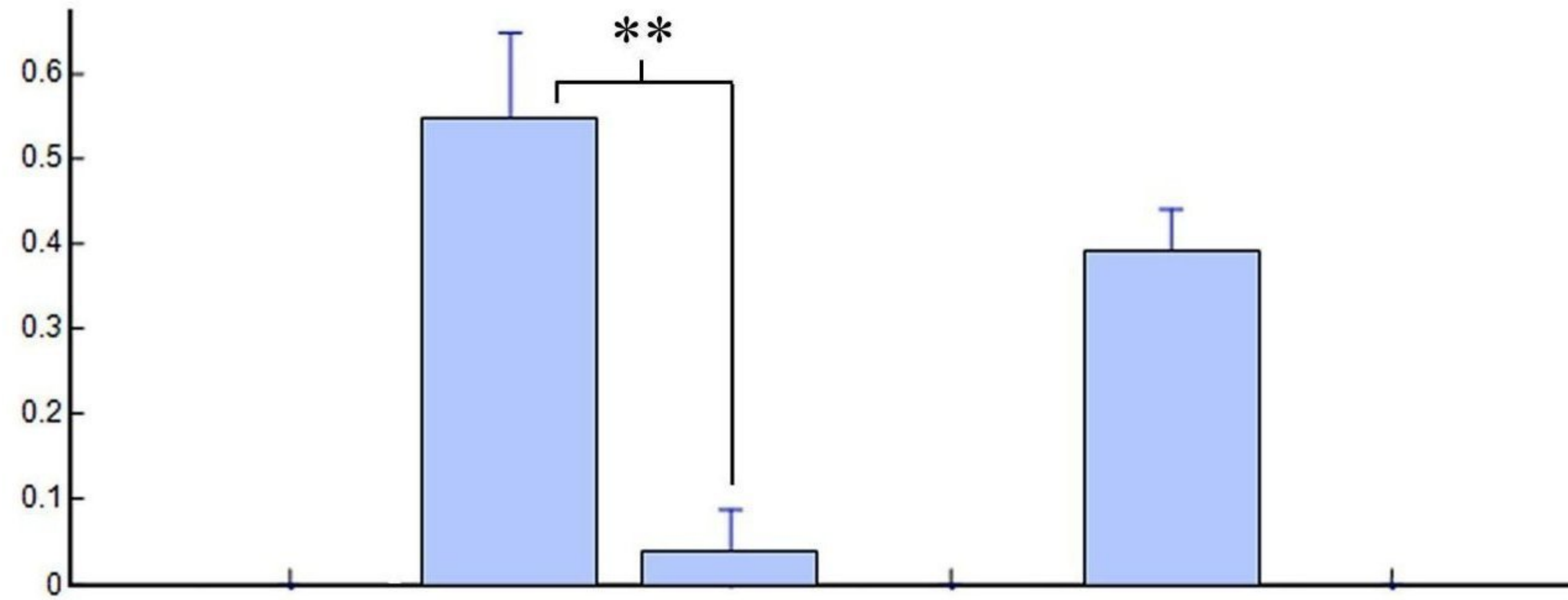


Figure S2

A

MG132

Cells with stress granules
(number ratio)

T= 0 to 3 h: - Puro Puro Cyclo Cyclo Puro

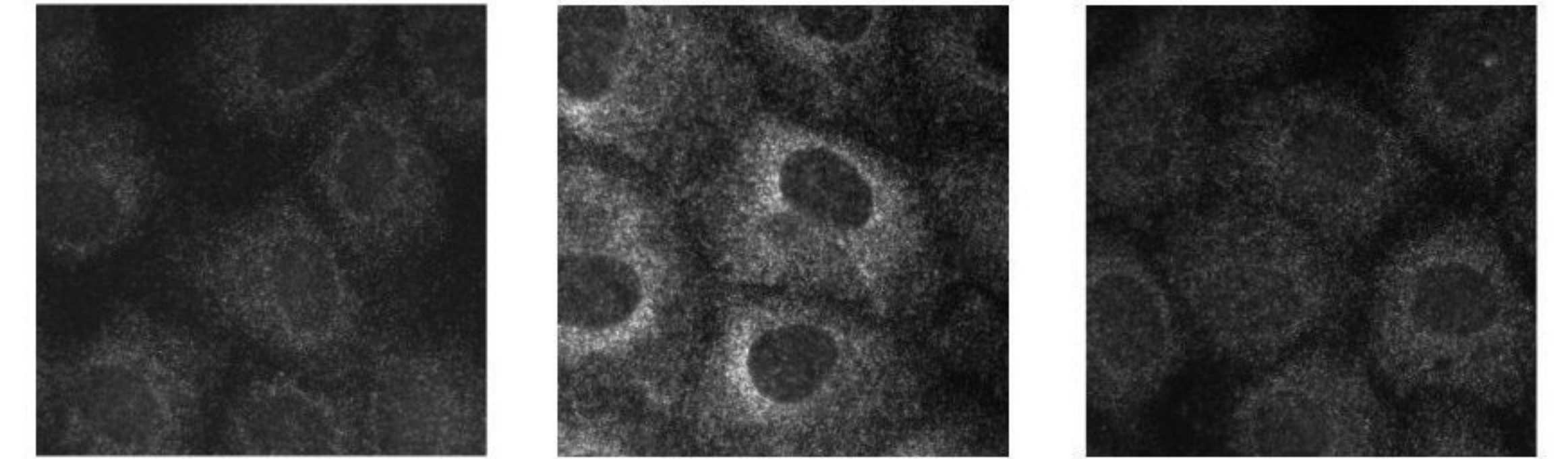
T= 3 to 4 h: - Puro Wash Cyclo Puro Cyclo

B

Control

Arsenite

MG132

p-eIF2 α 

C

MG132 (10 μ M)

3 h

10 h

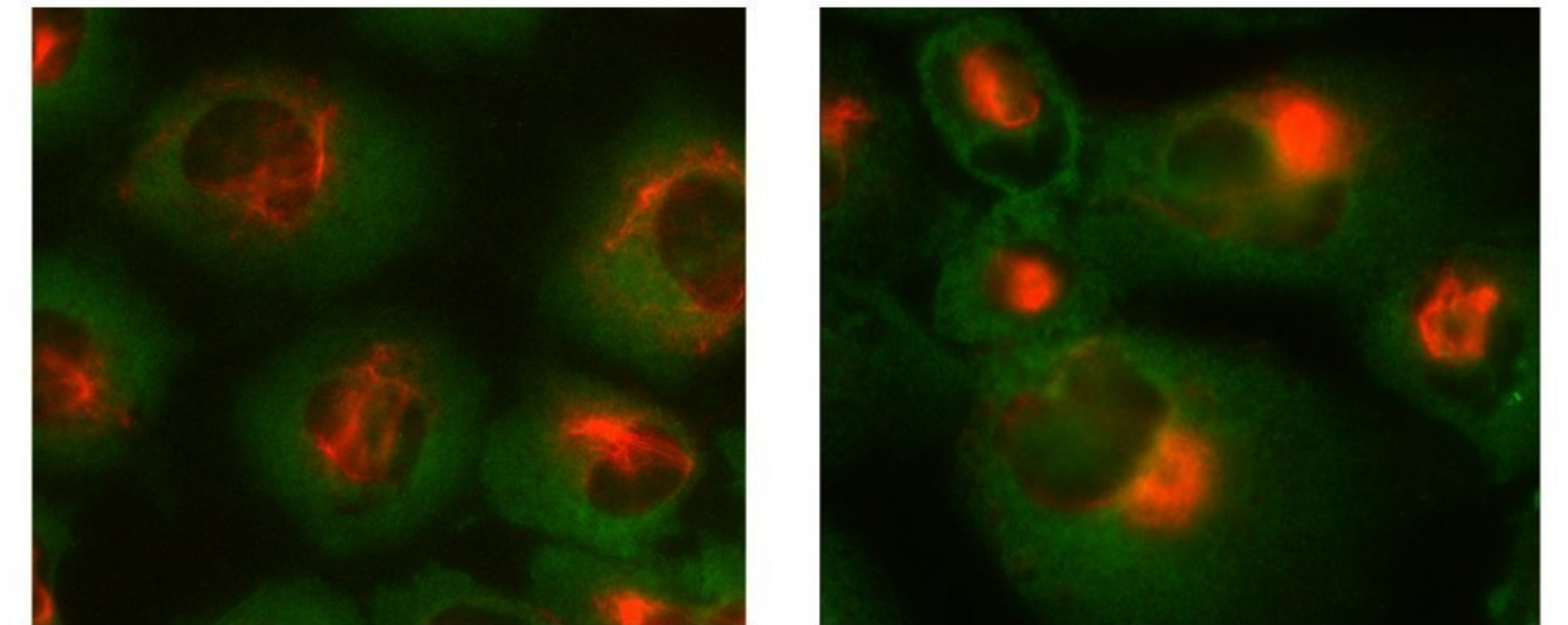


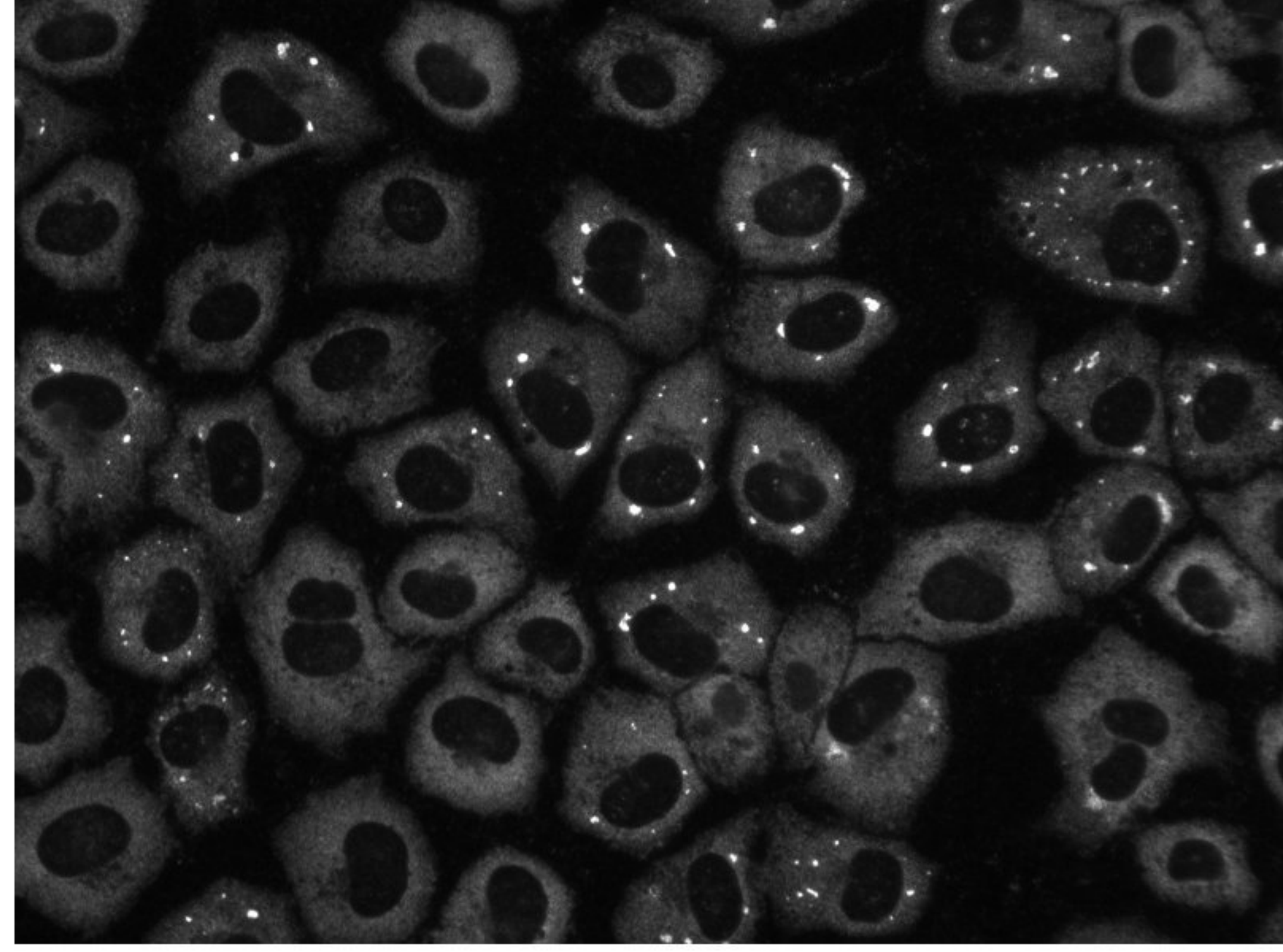
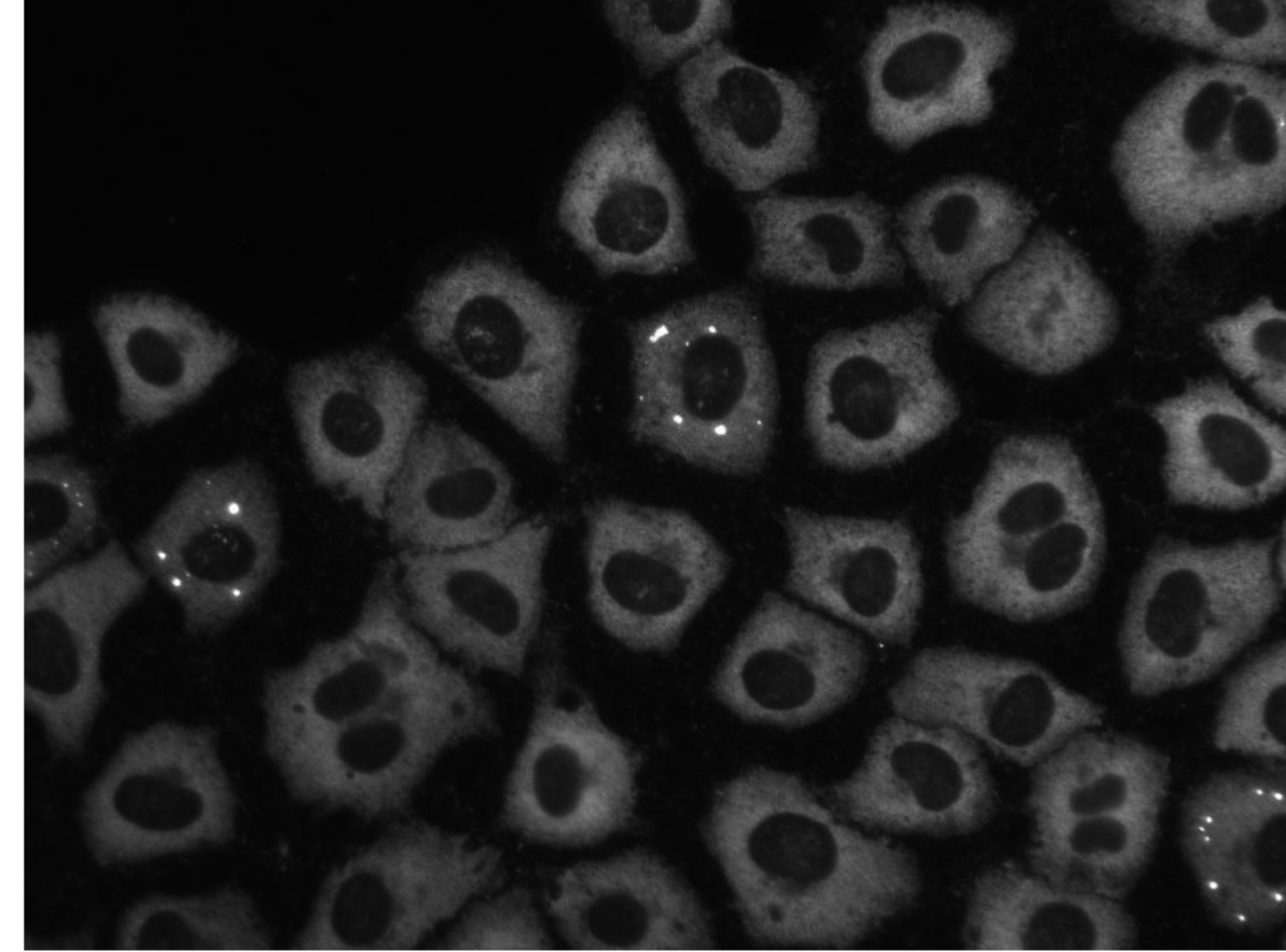
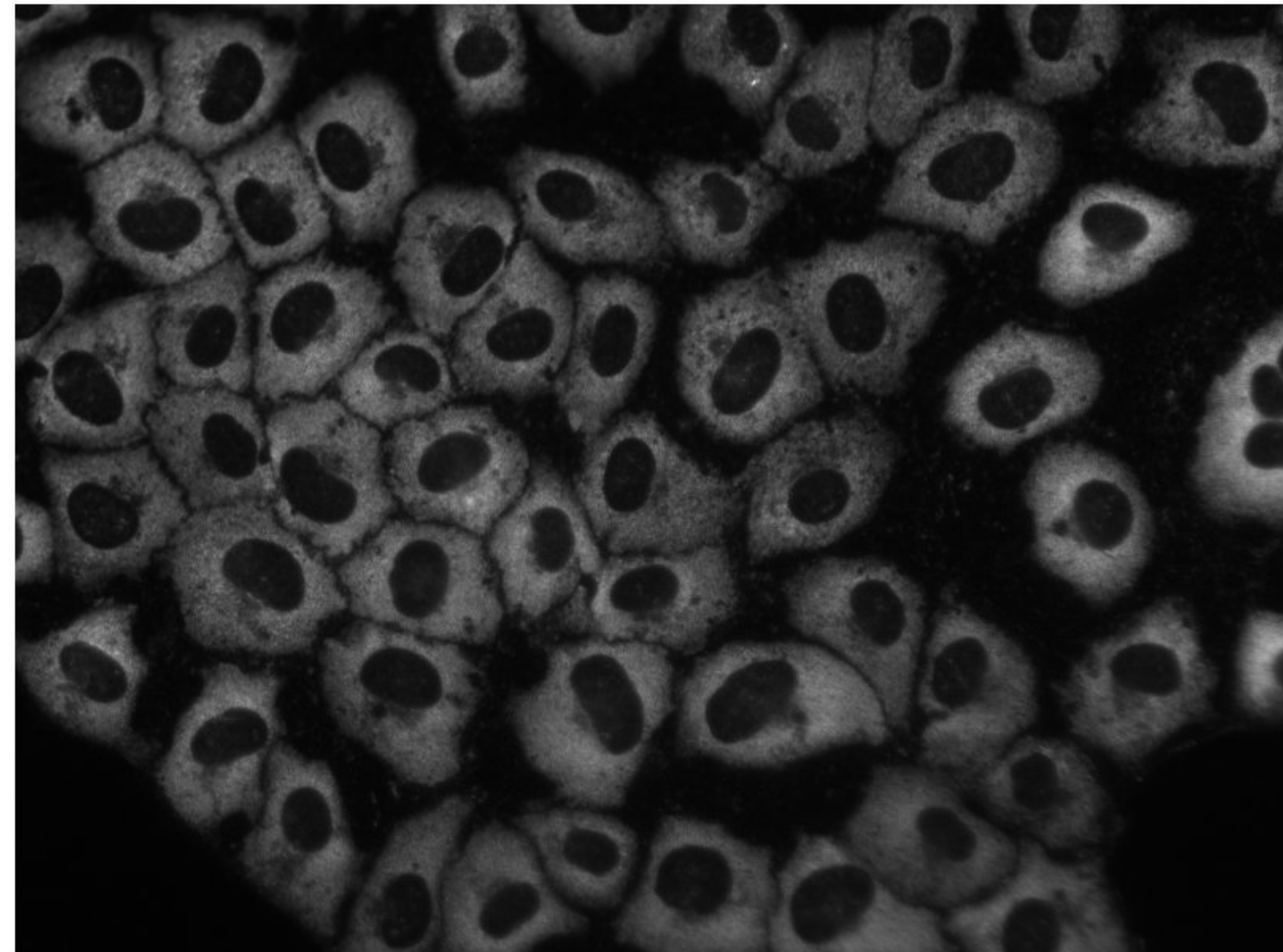
Figure S3

A

0.1 $\mu\text{g/mL}$

0.3 $\mu\text{g/mL}$

1 $\mu\text{g/mL}$



globin mRNA

B

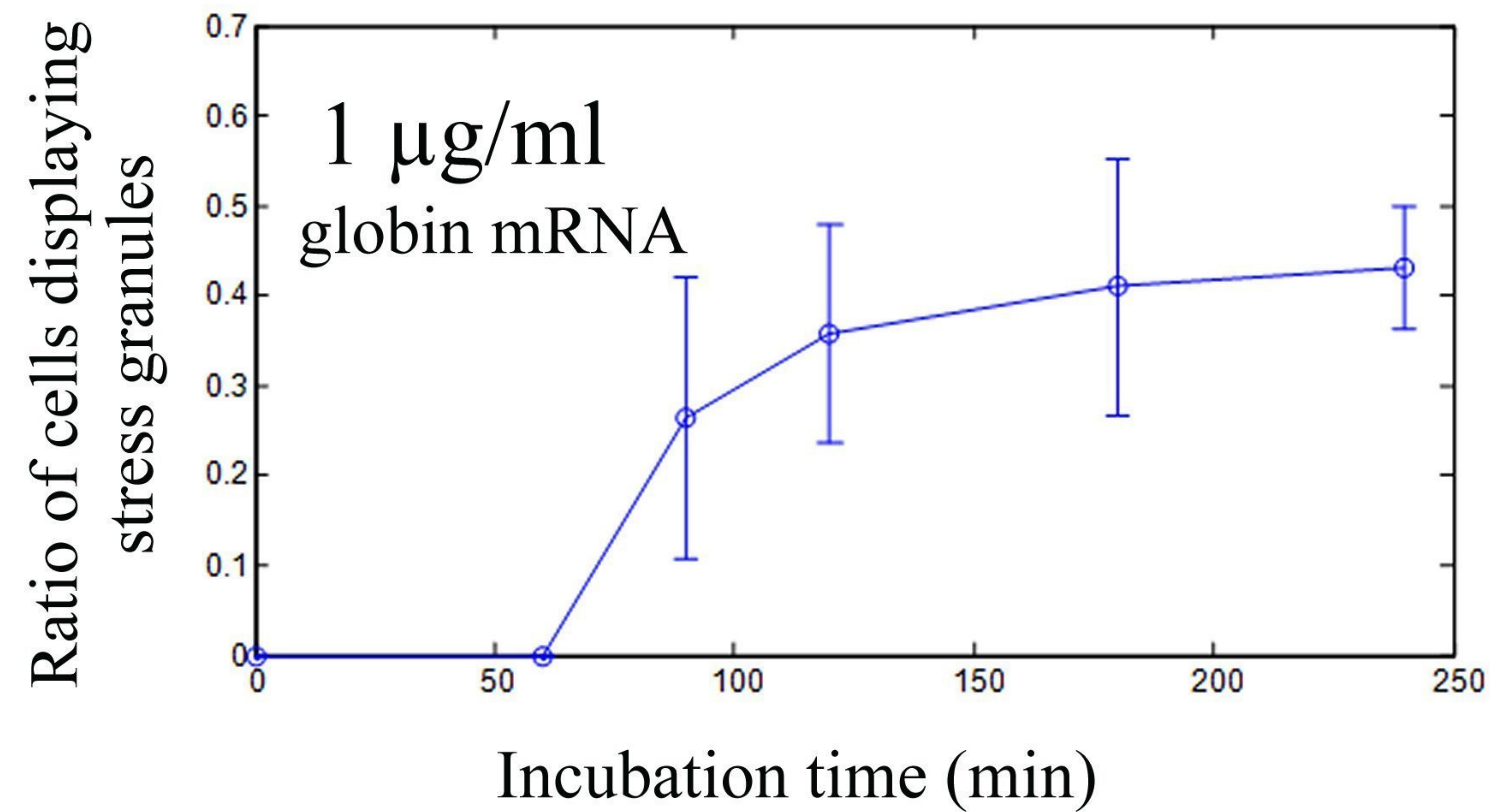


Figure S4

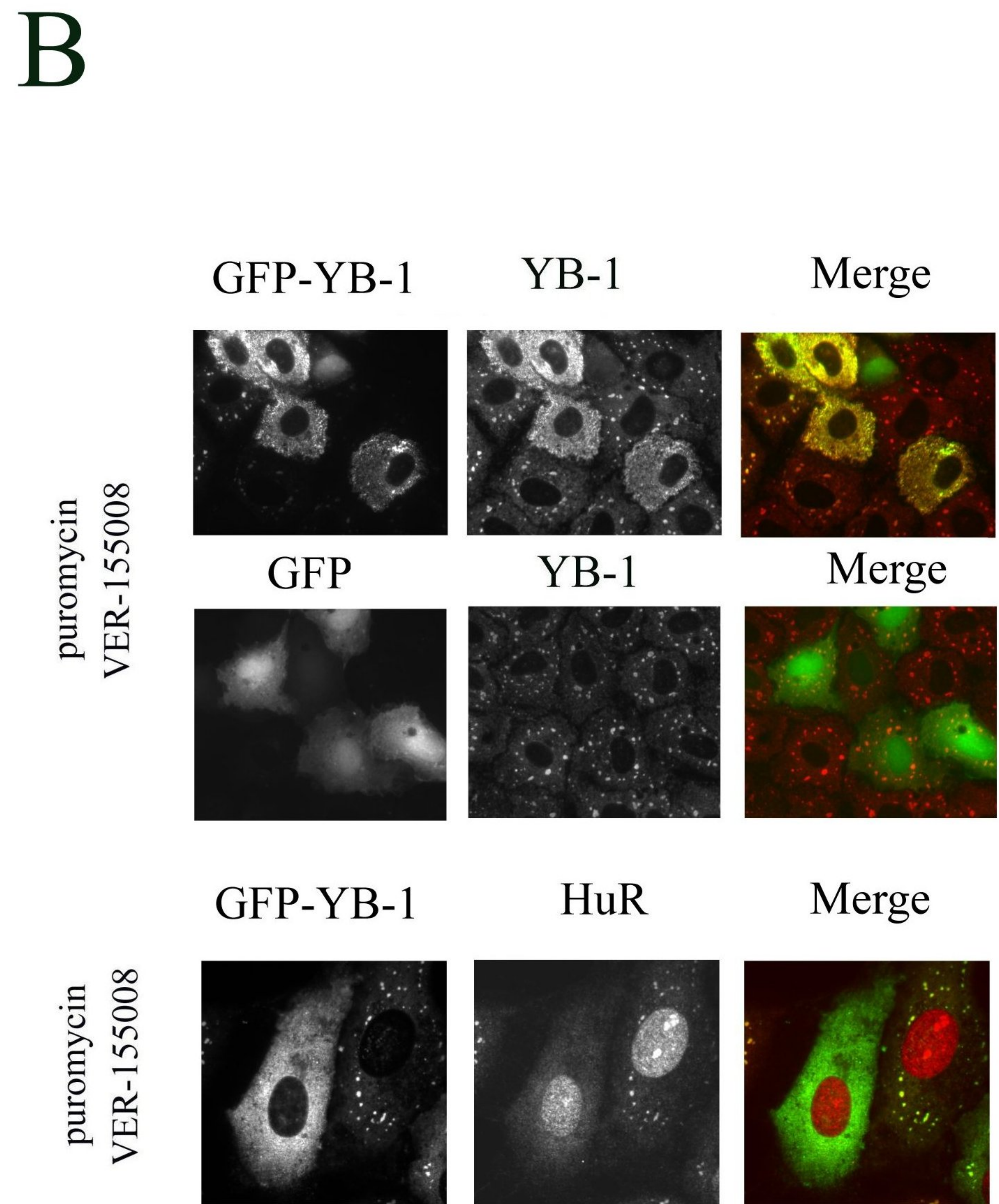
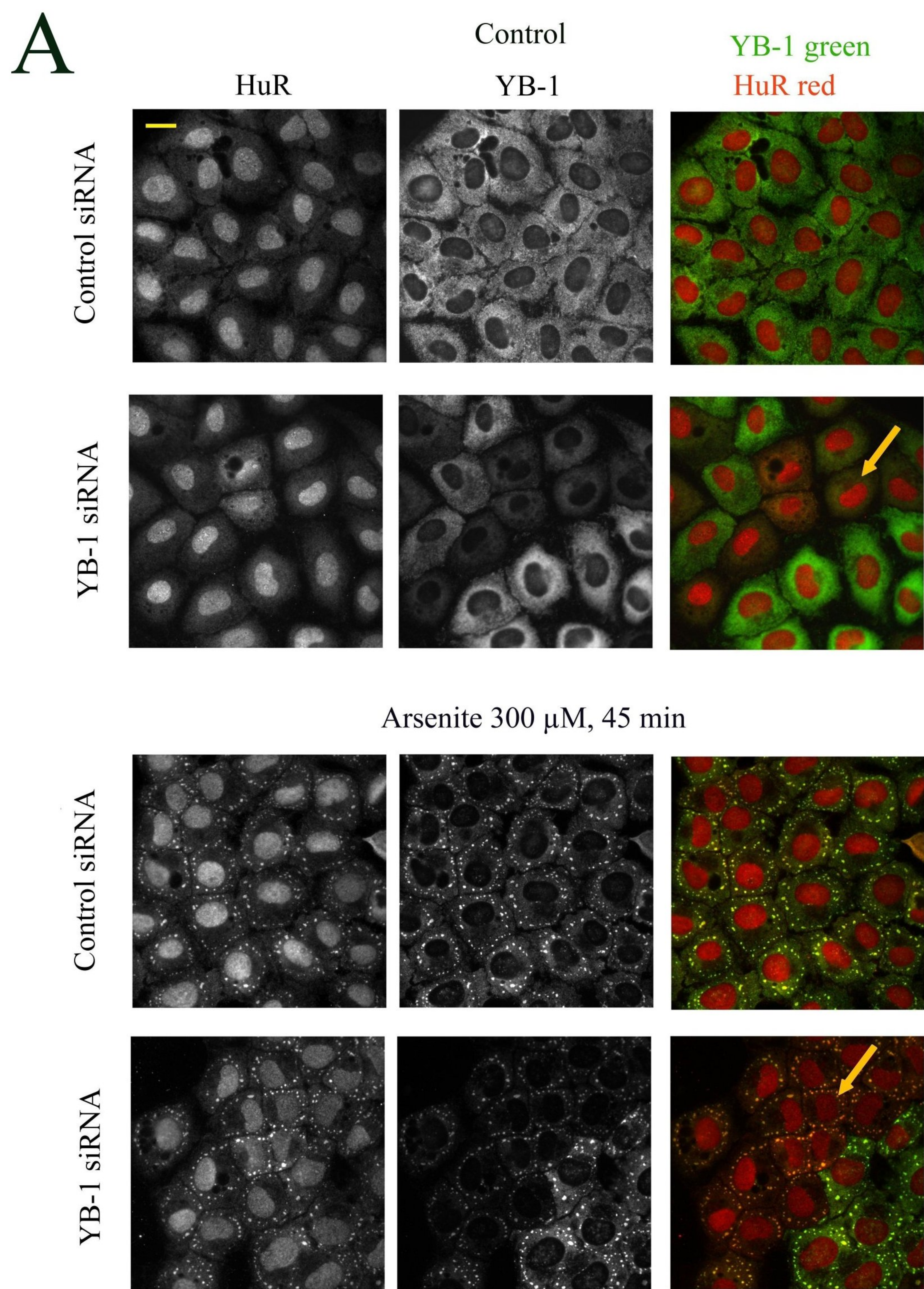


Figure S5

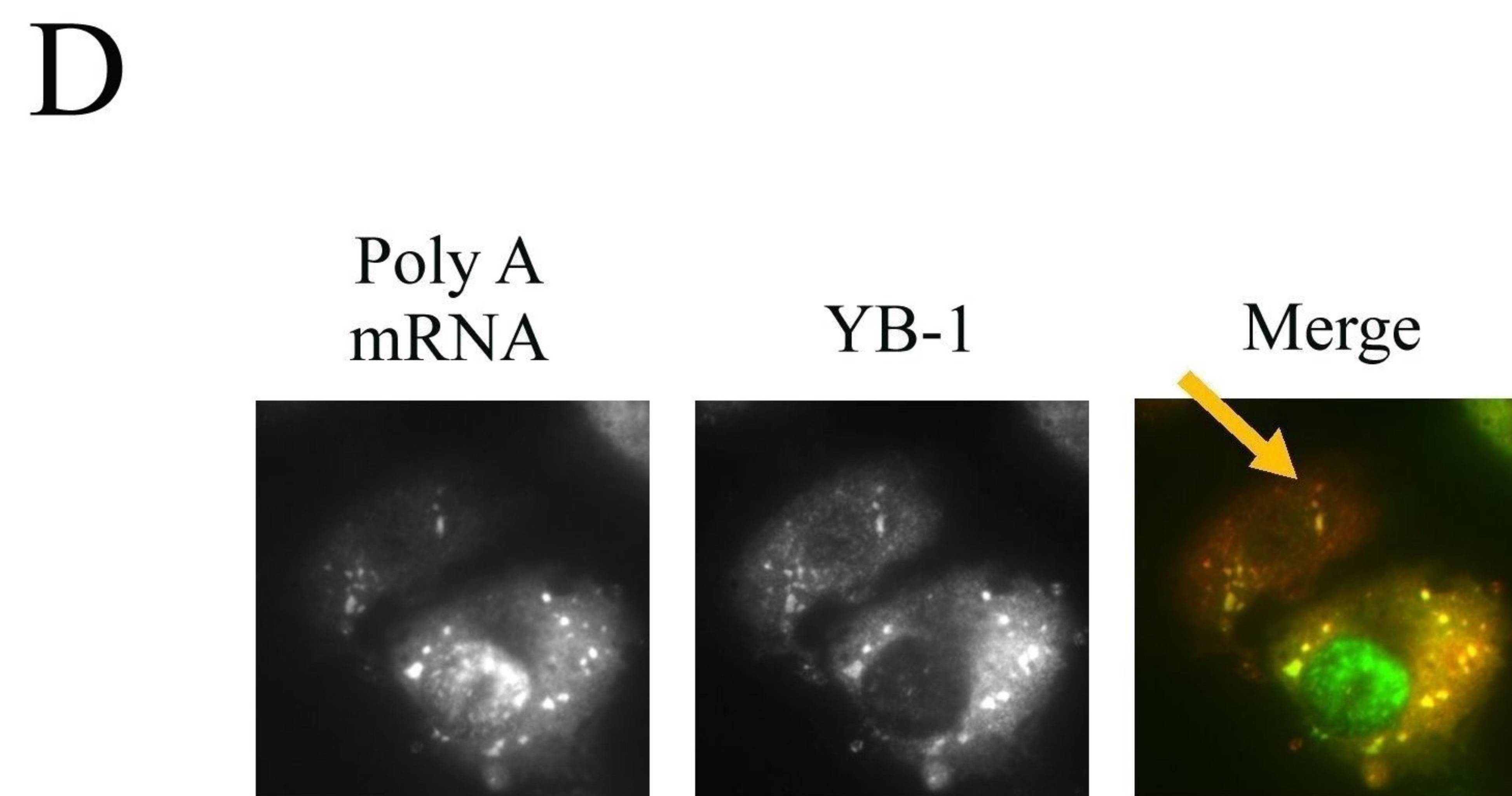
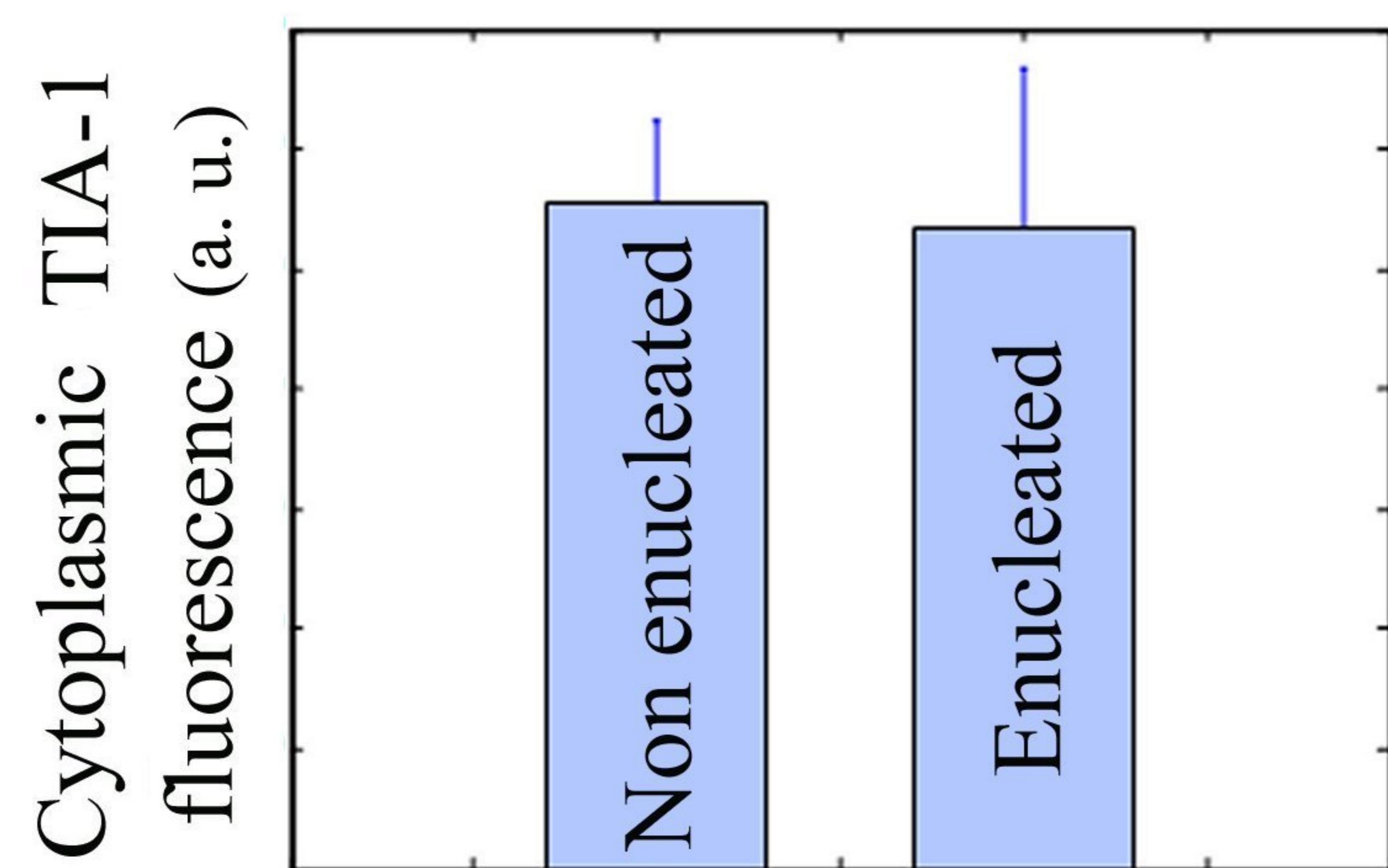
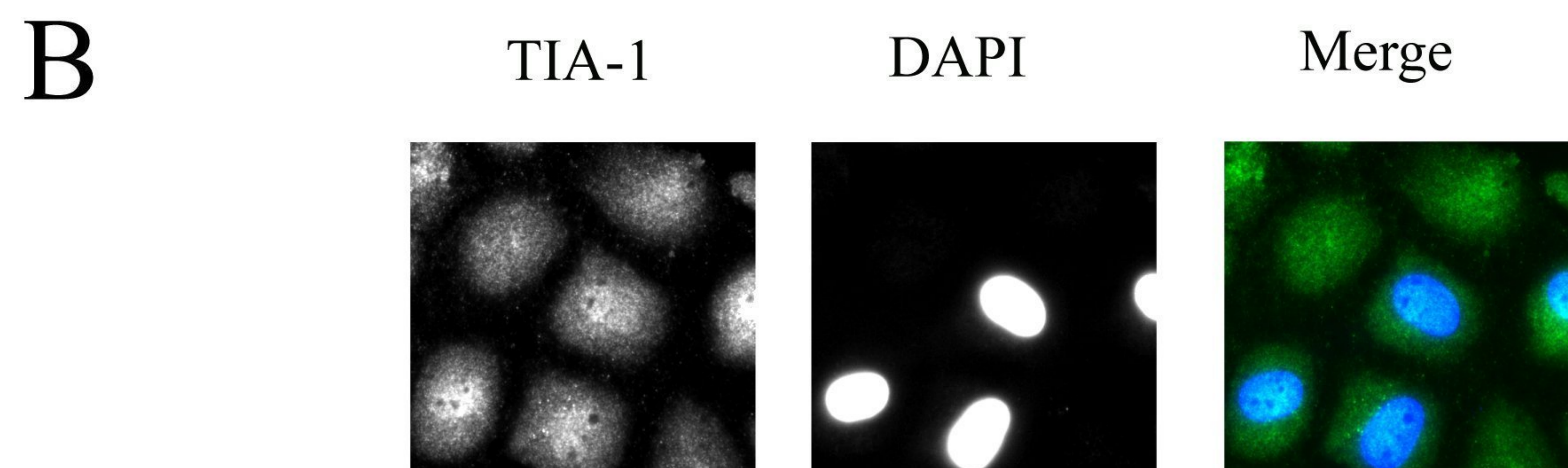
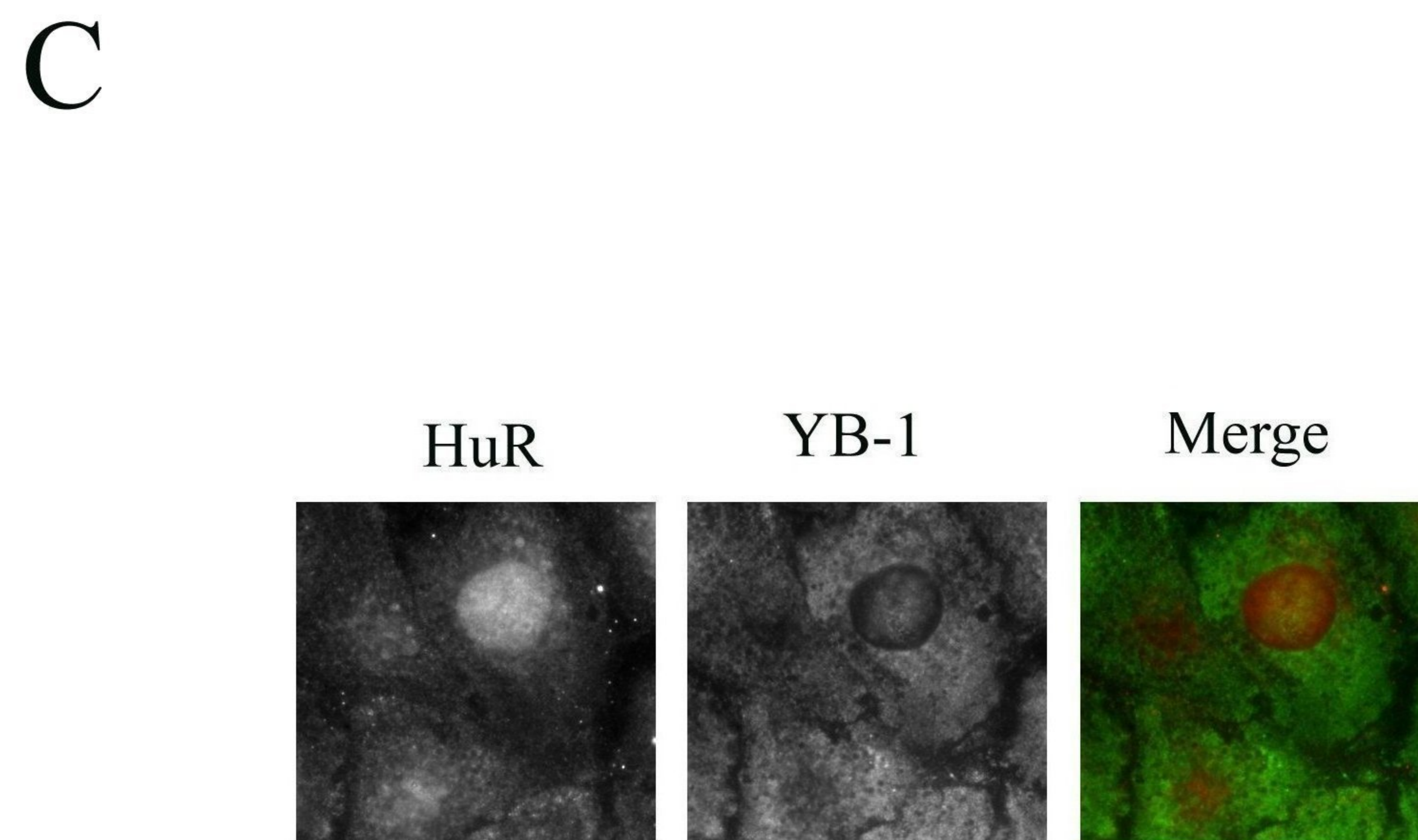
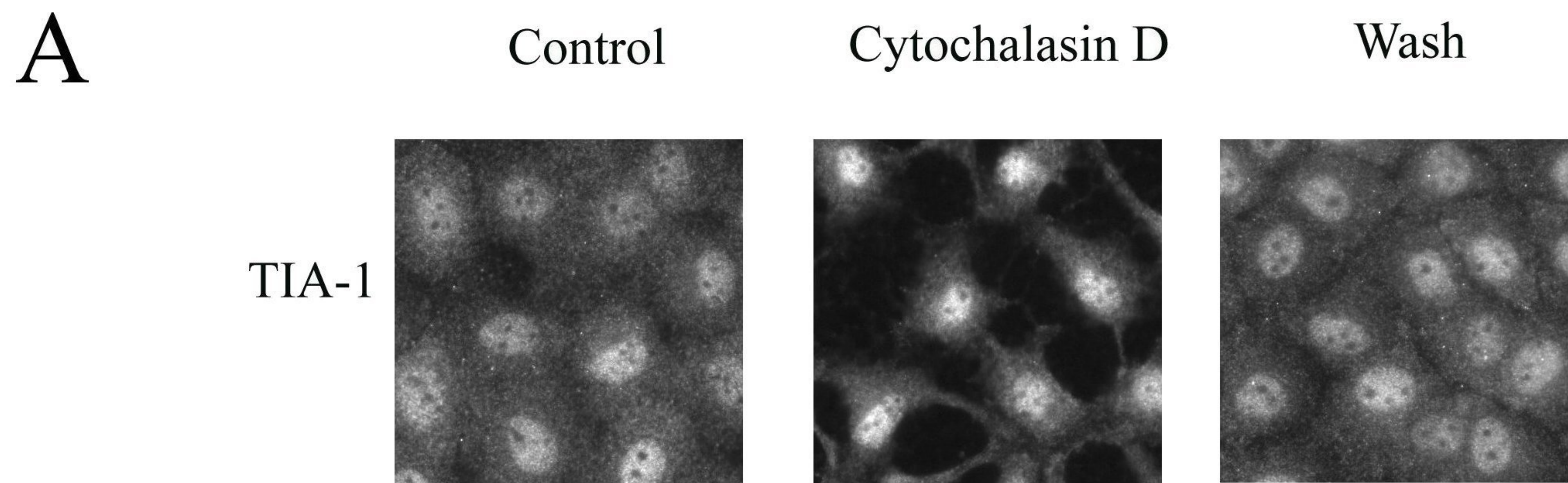


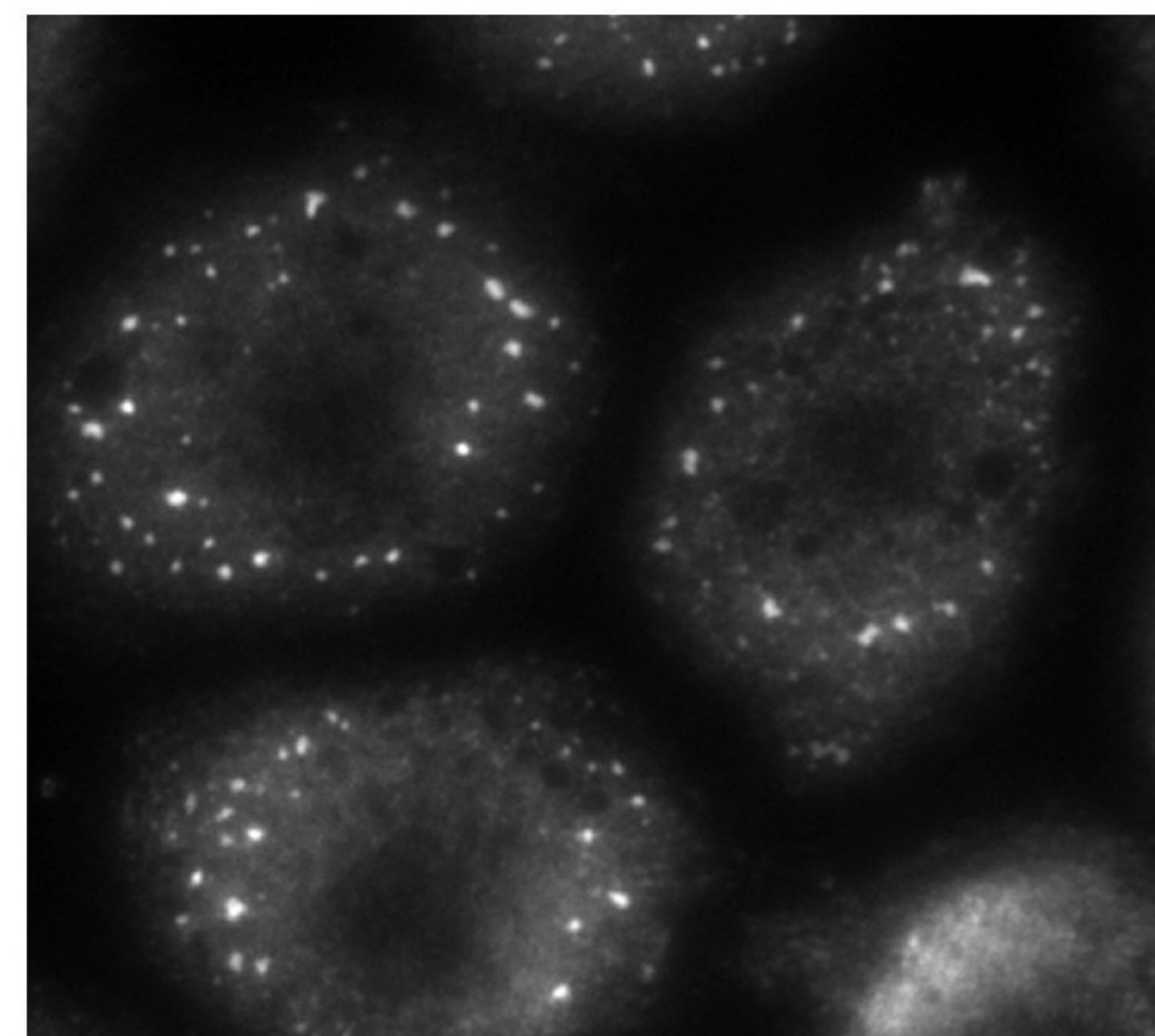
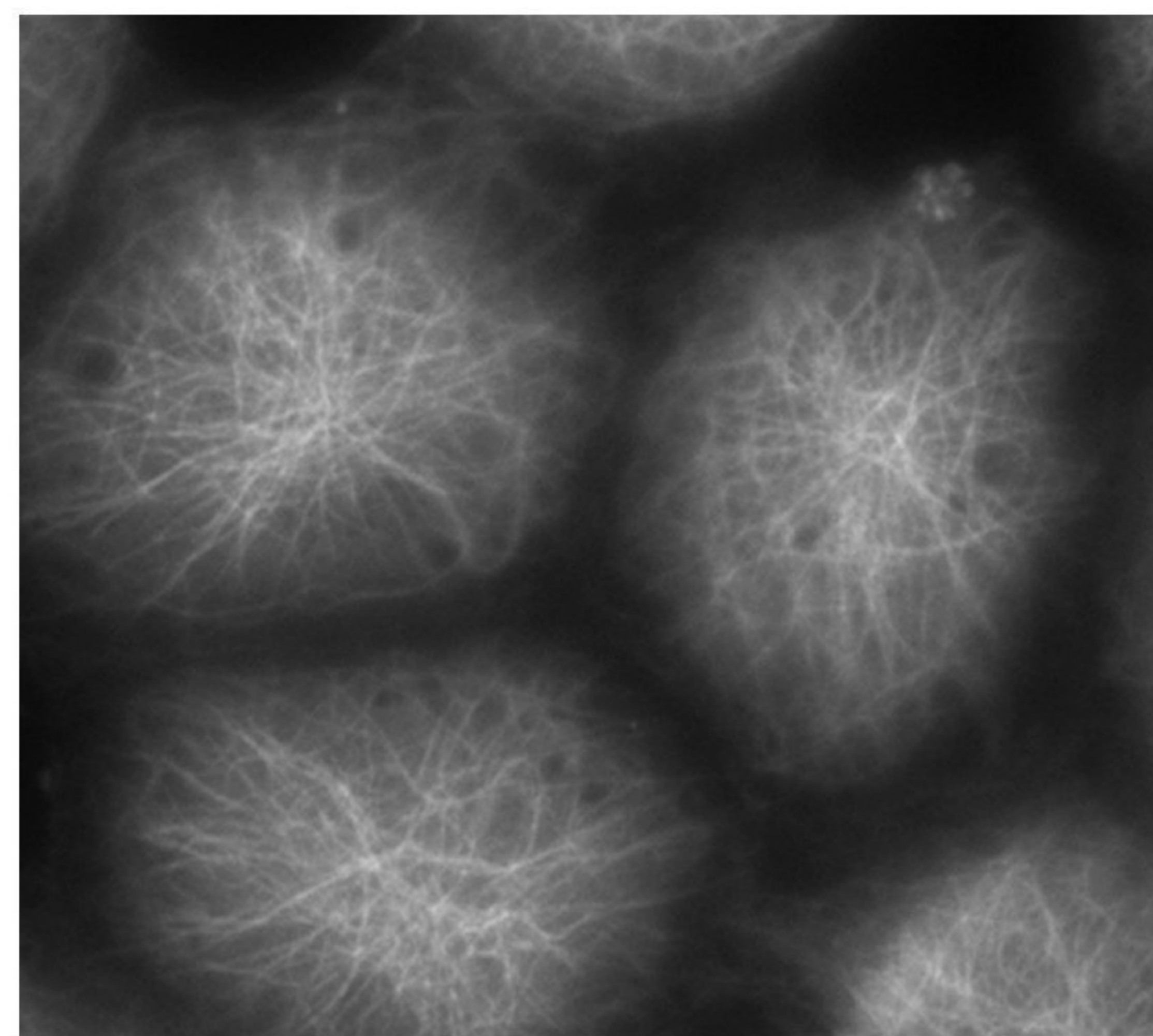
Figure S6

A

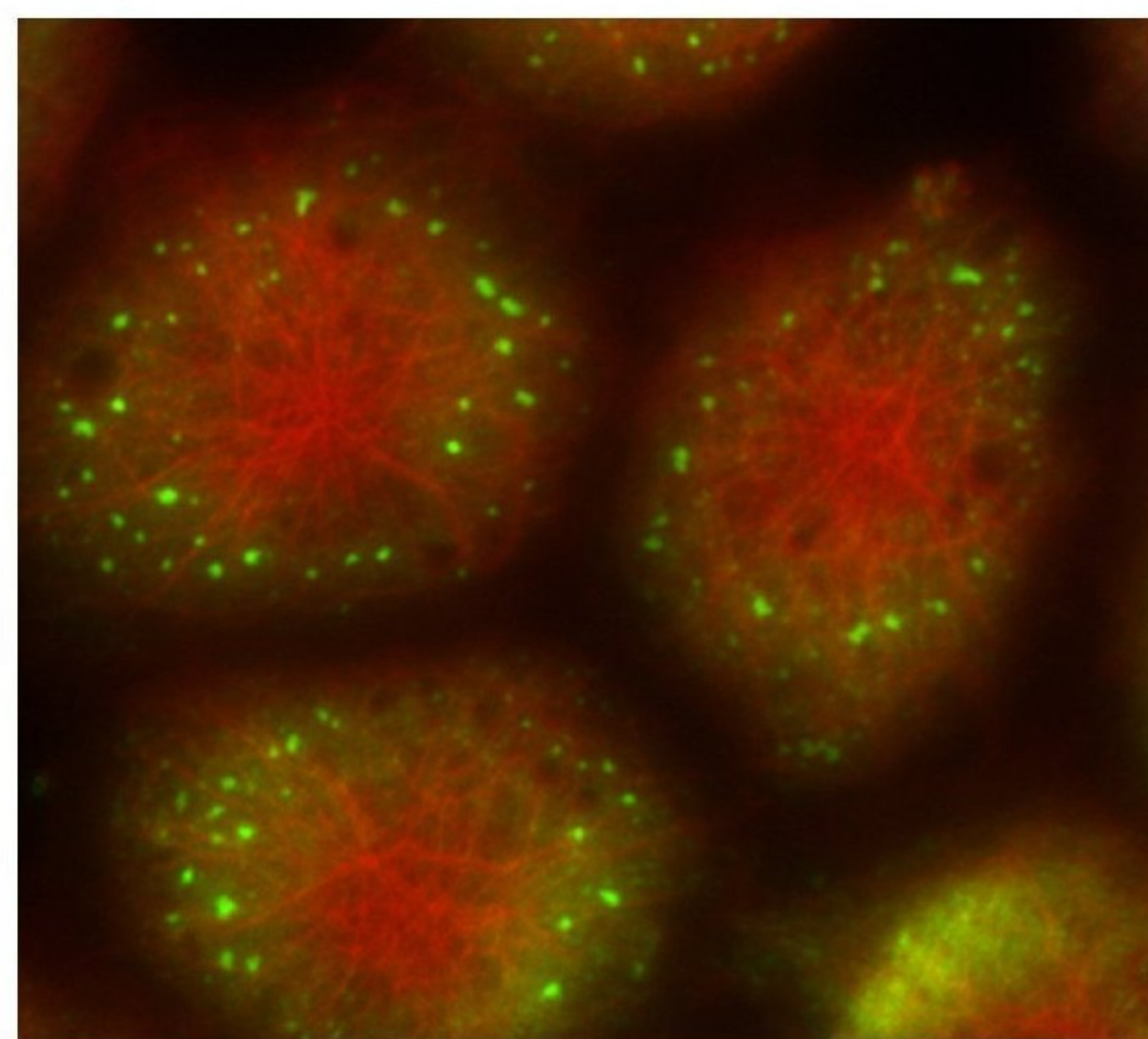
VER-15508 plus puro

Tubulin

YB-1

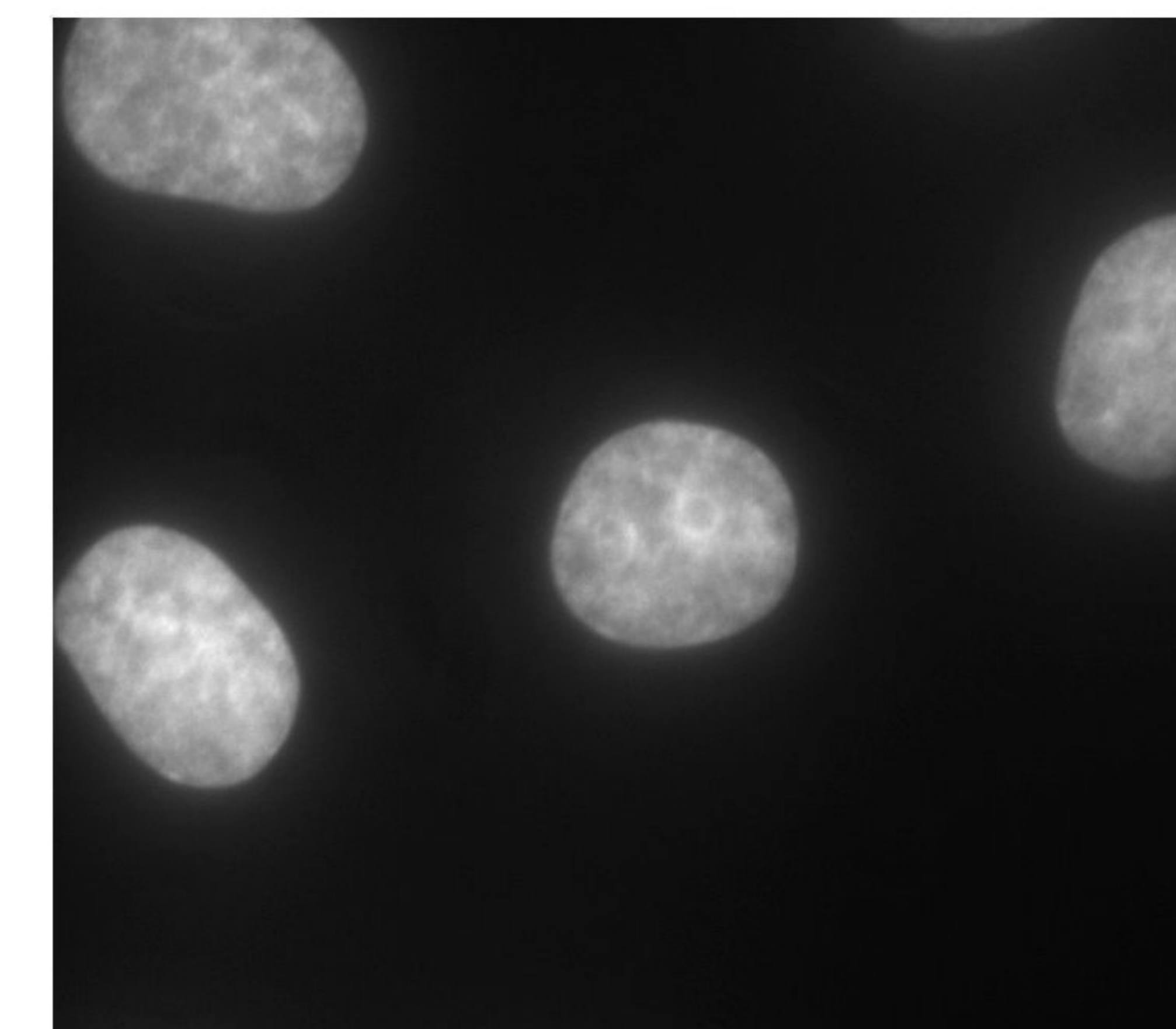
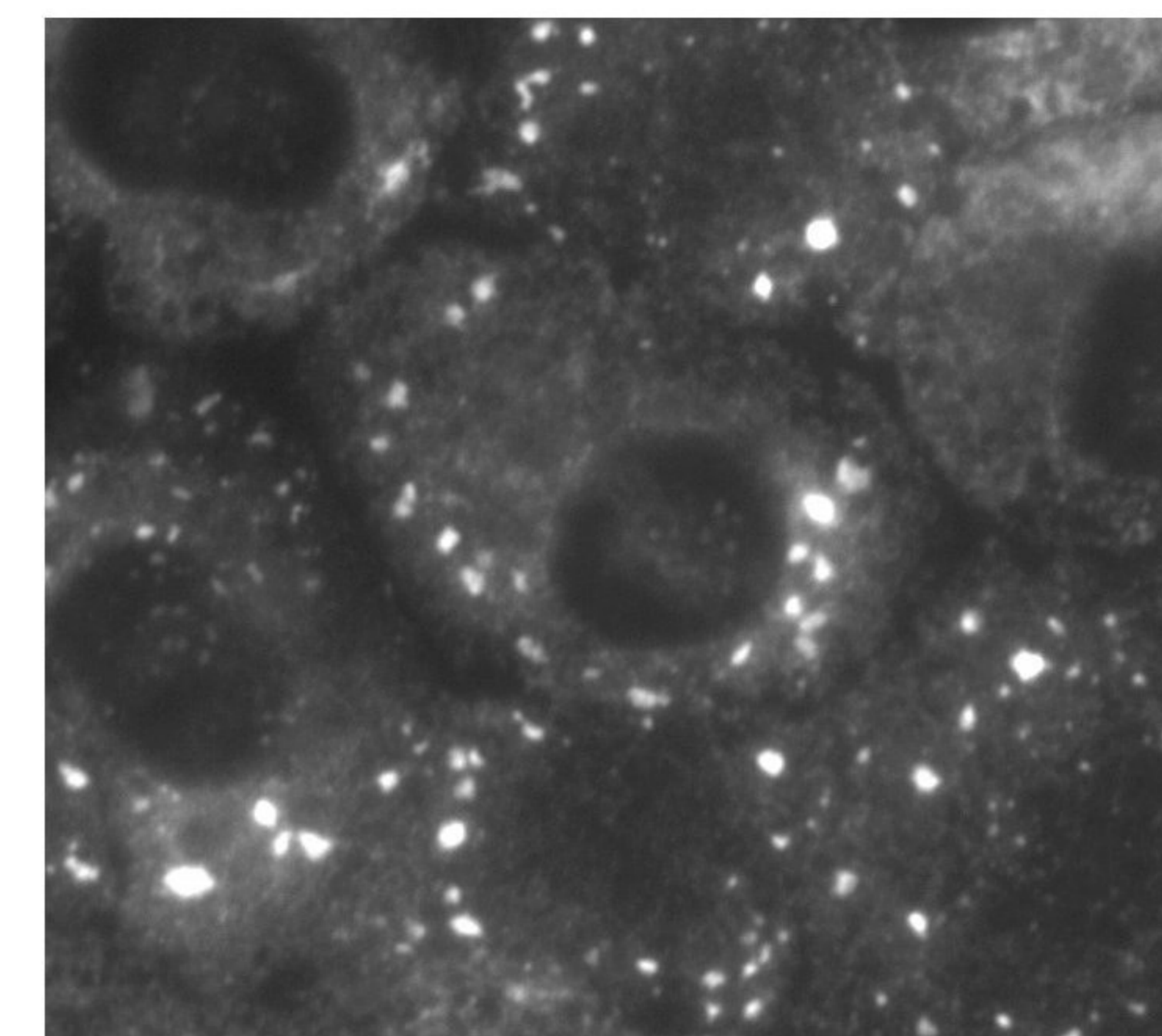


Merge

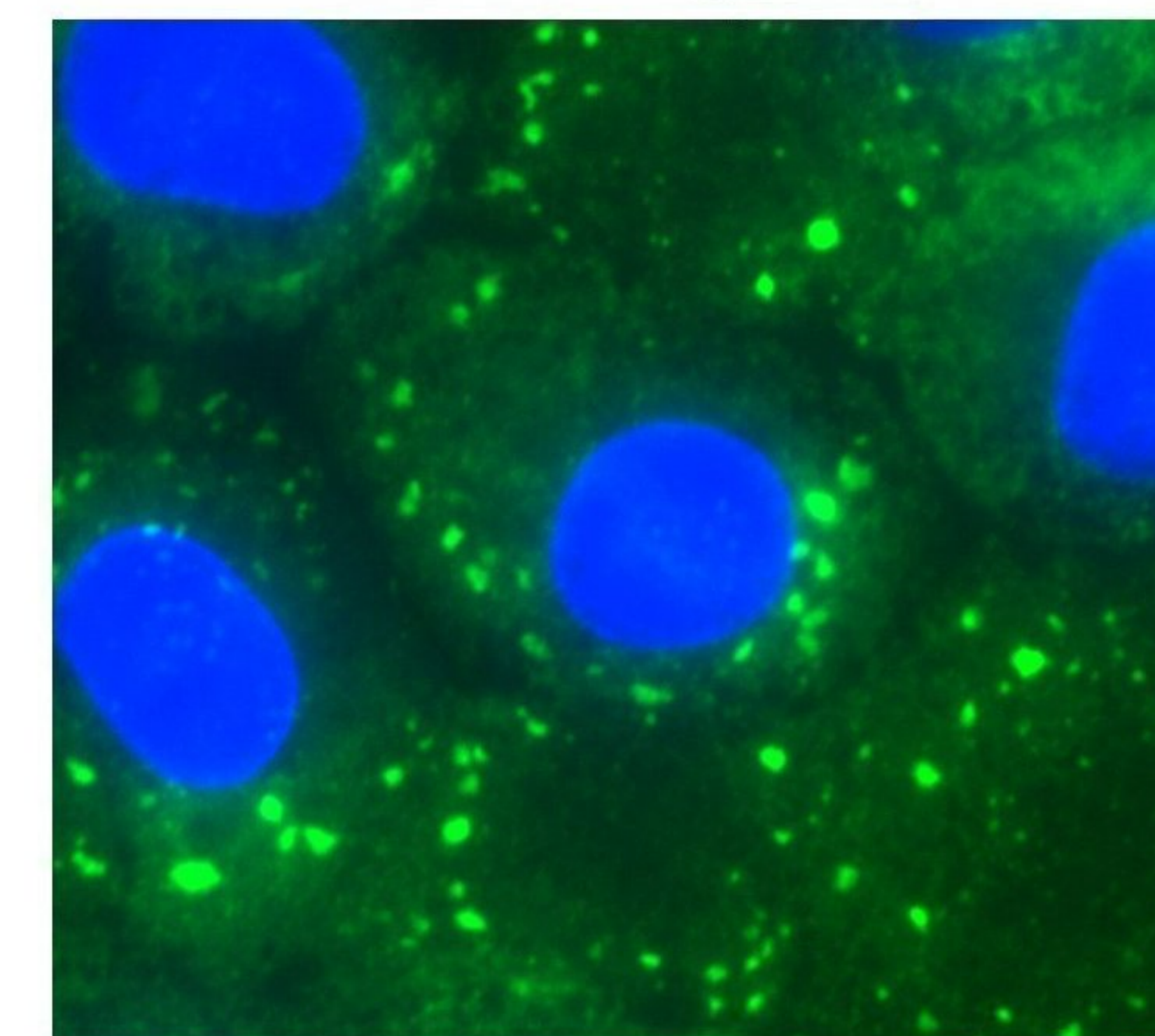
**B**VER-15508 plus puro
prior to and after
enucleation

YB-1

DAPI



Merge

**Figure S7**

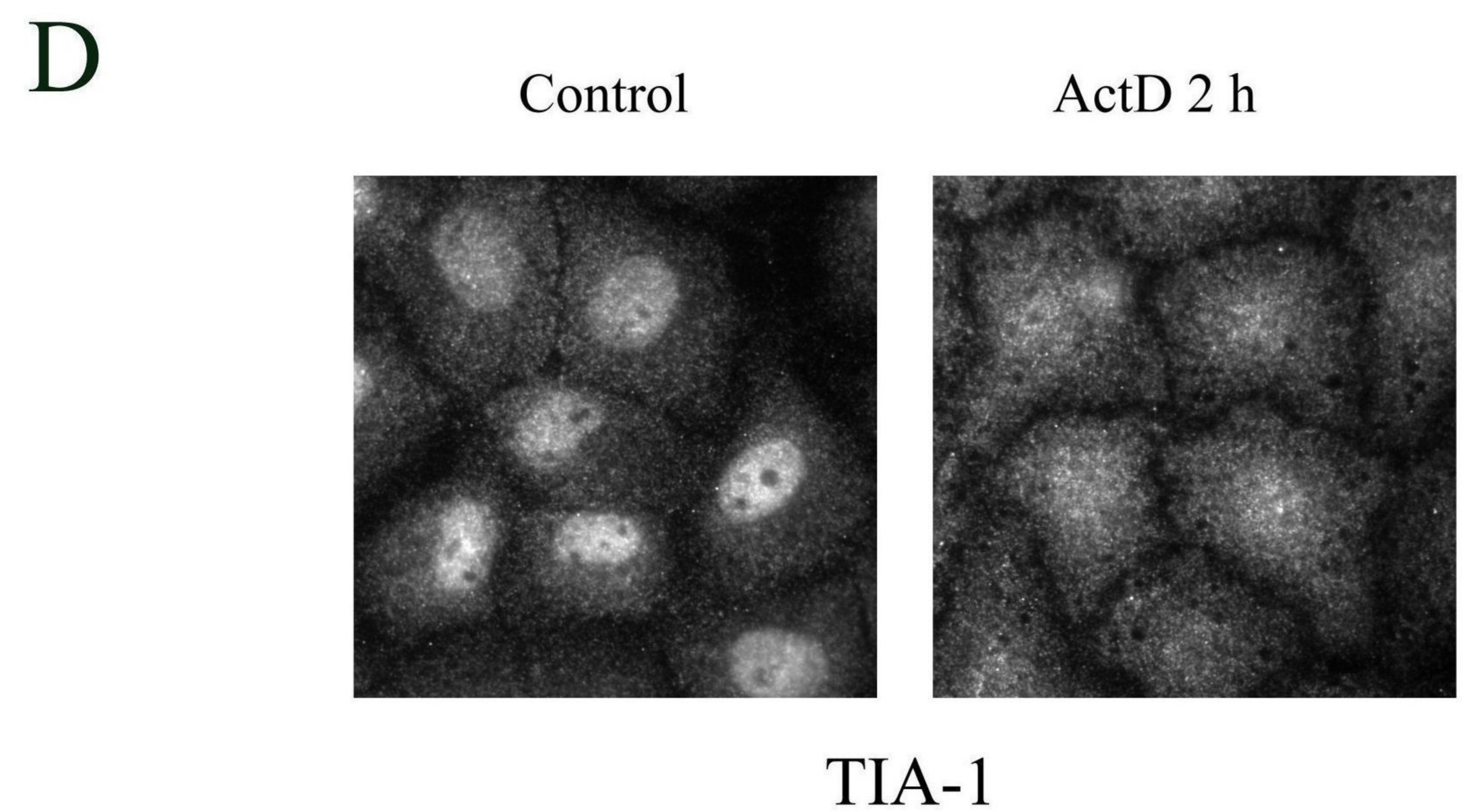
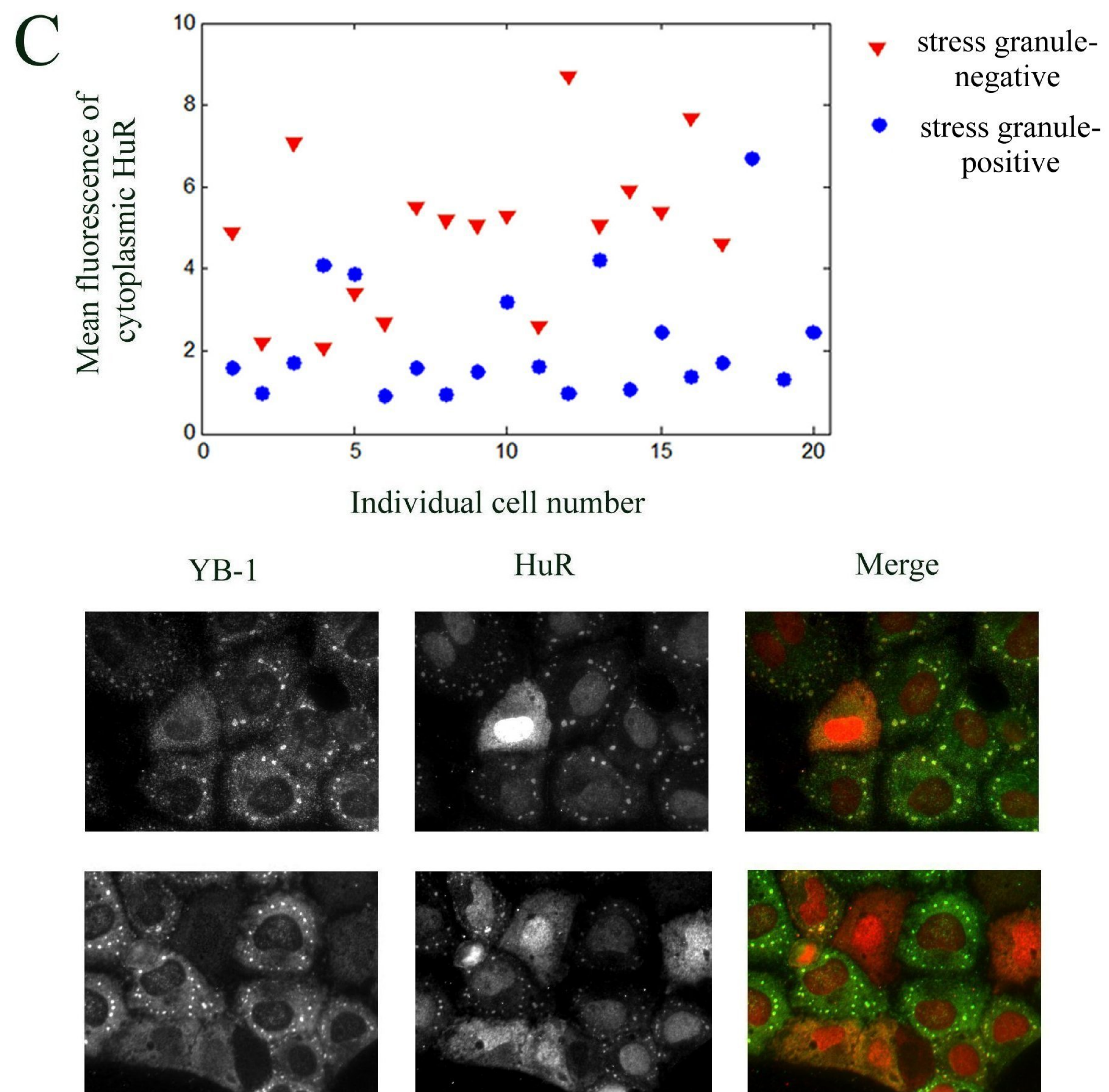
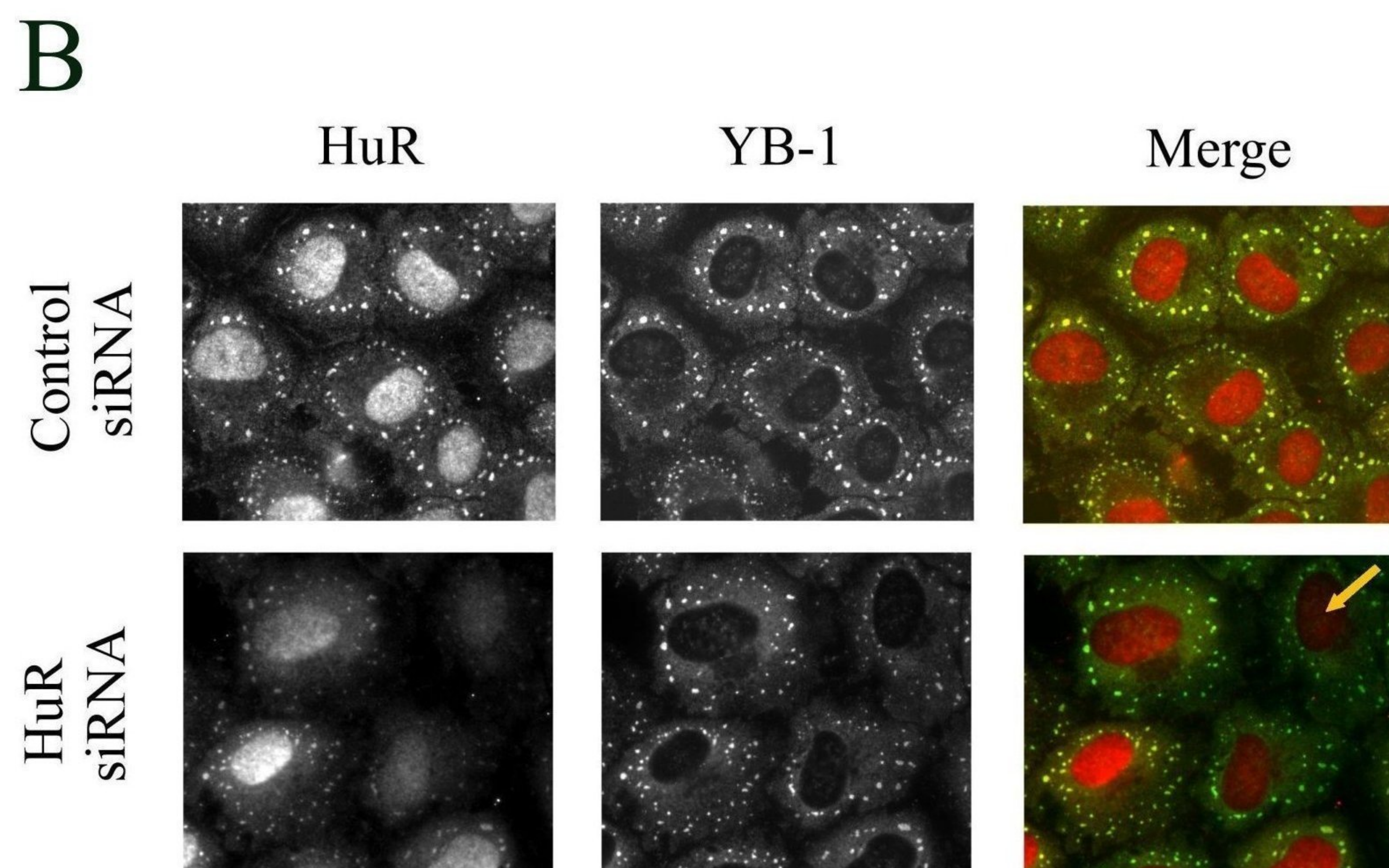
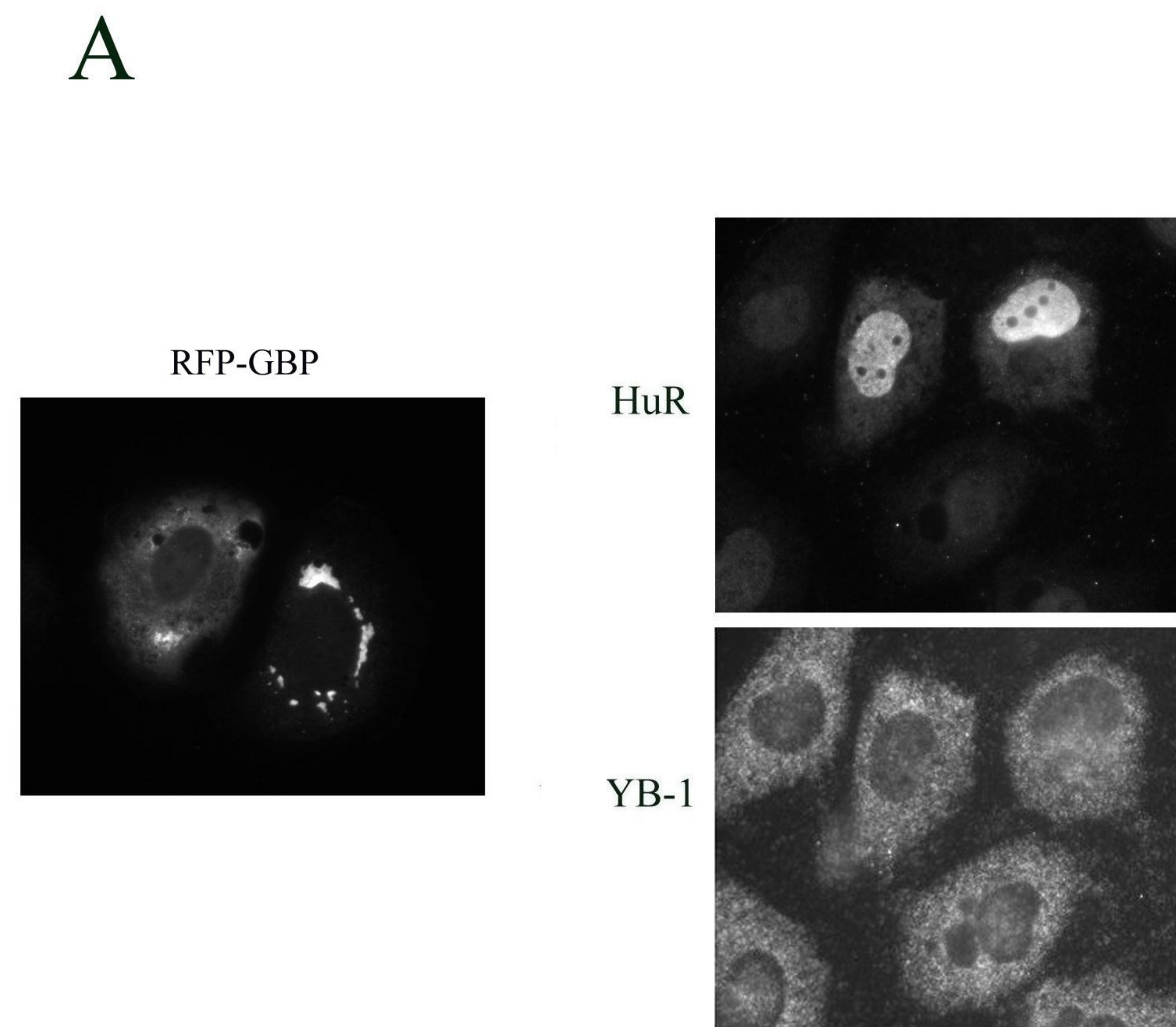


Figure S8

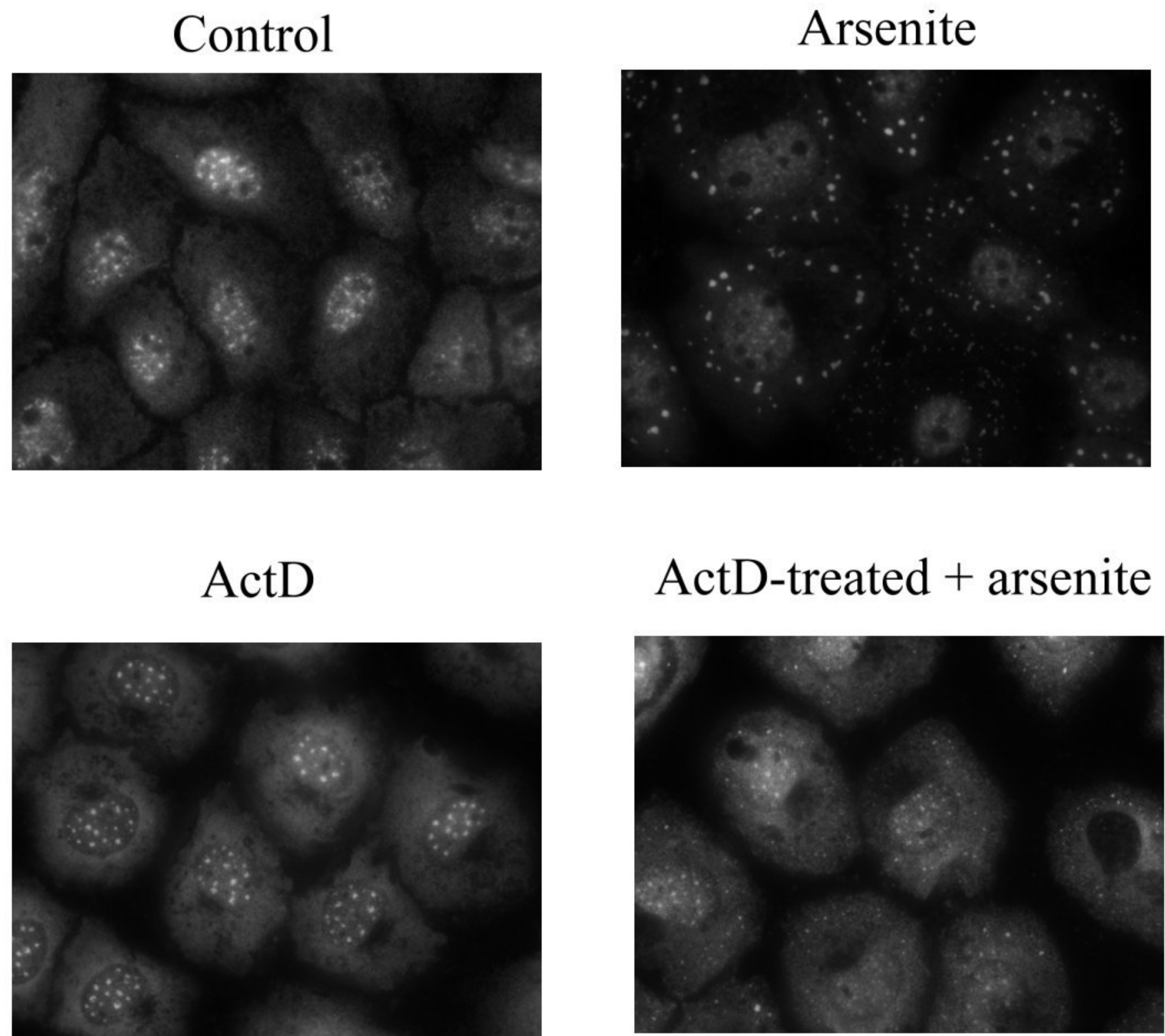
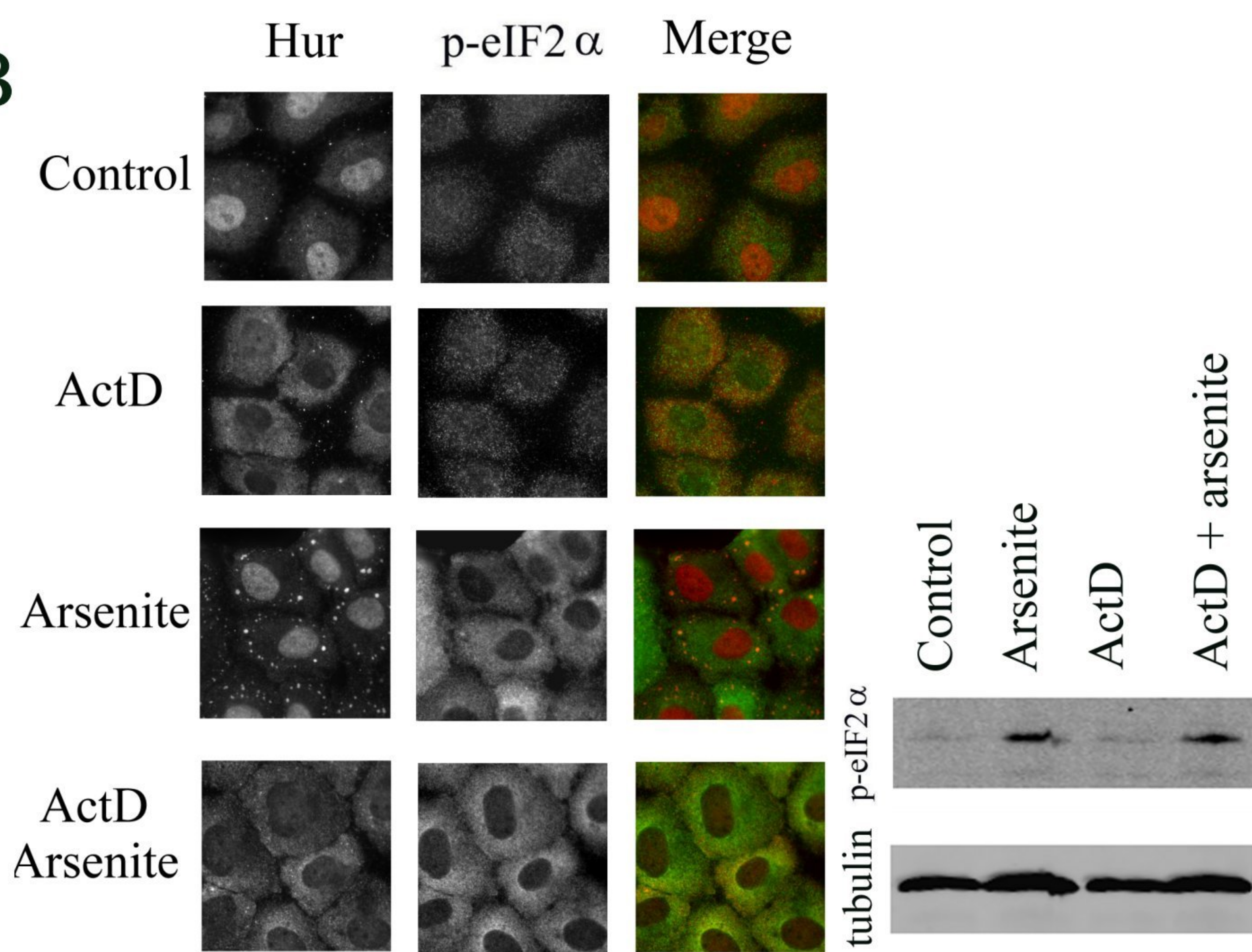
A**B**

Figure S9

HuR

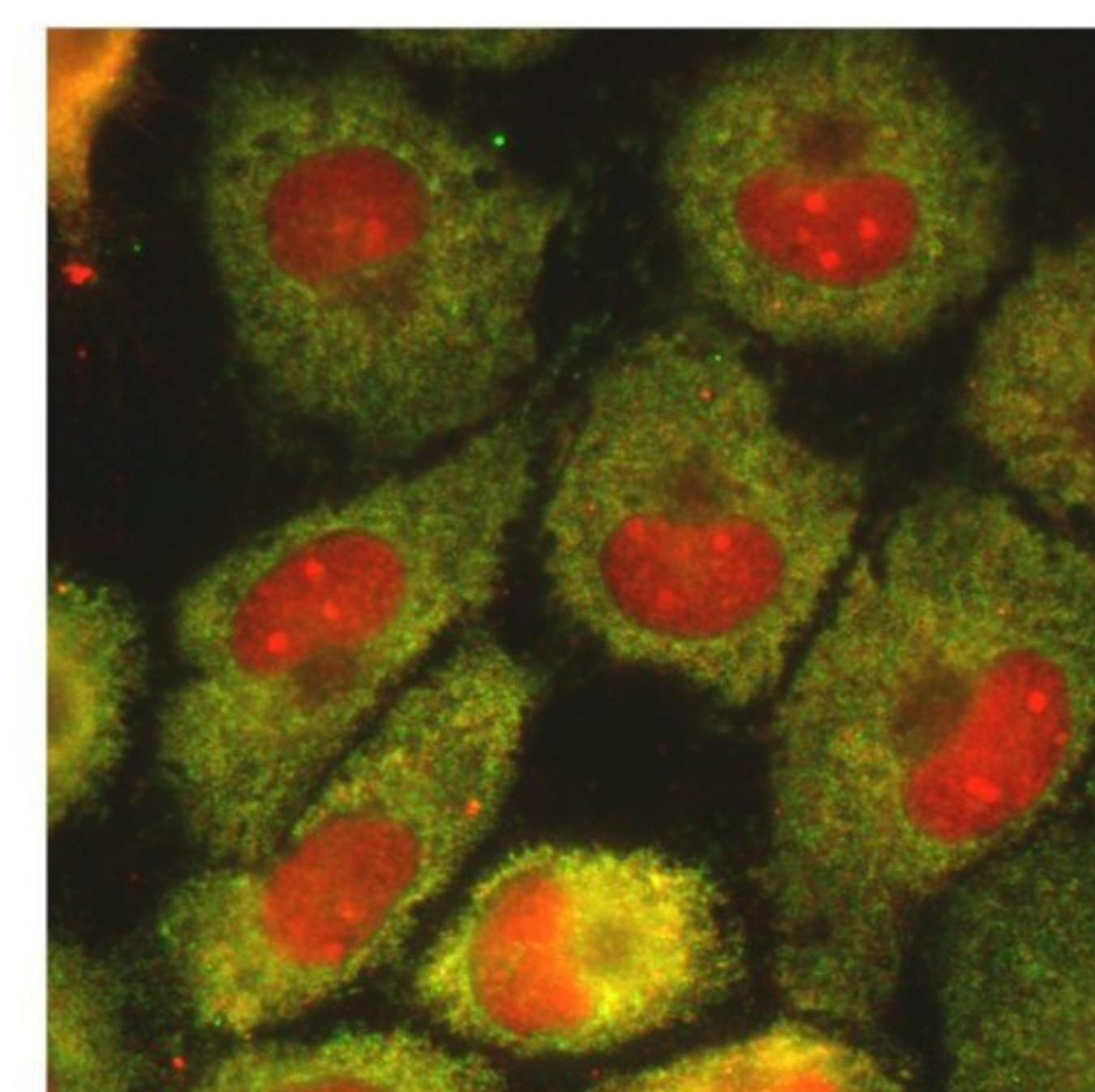
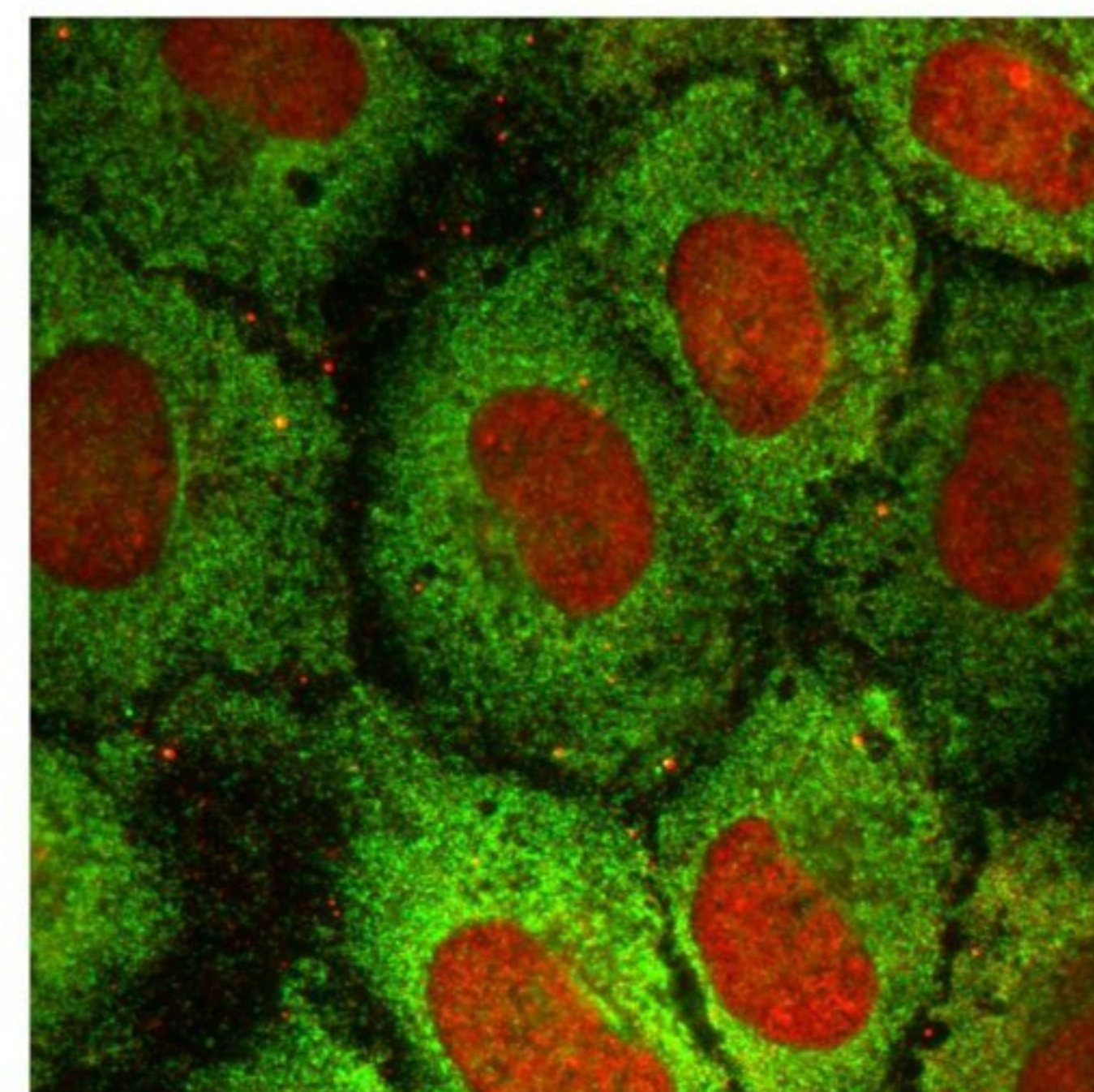
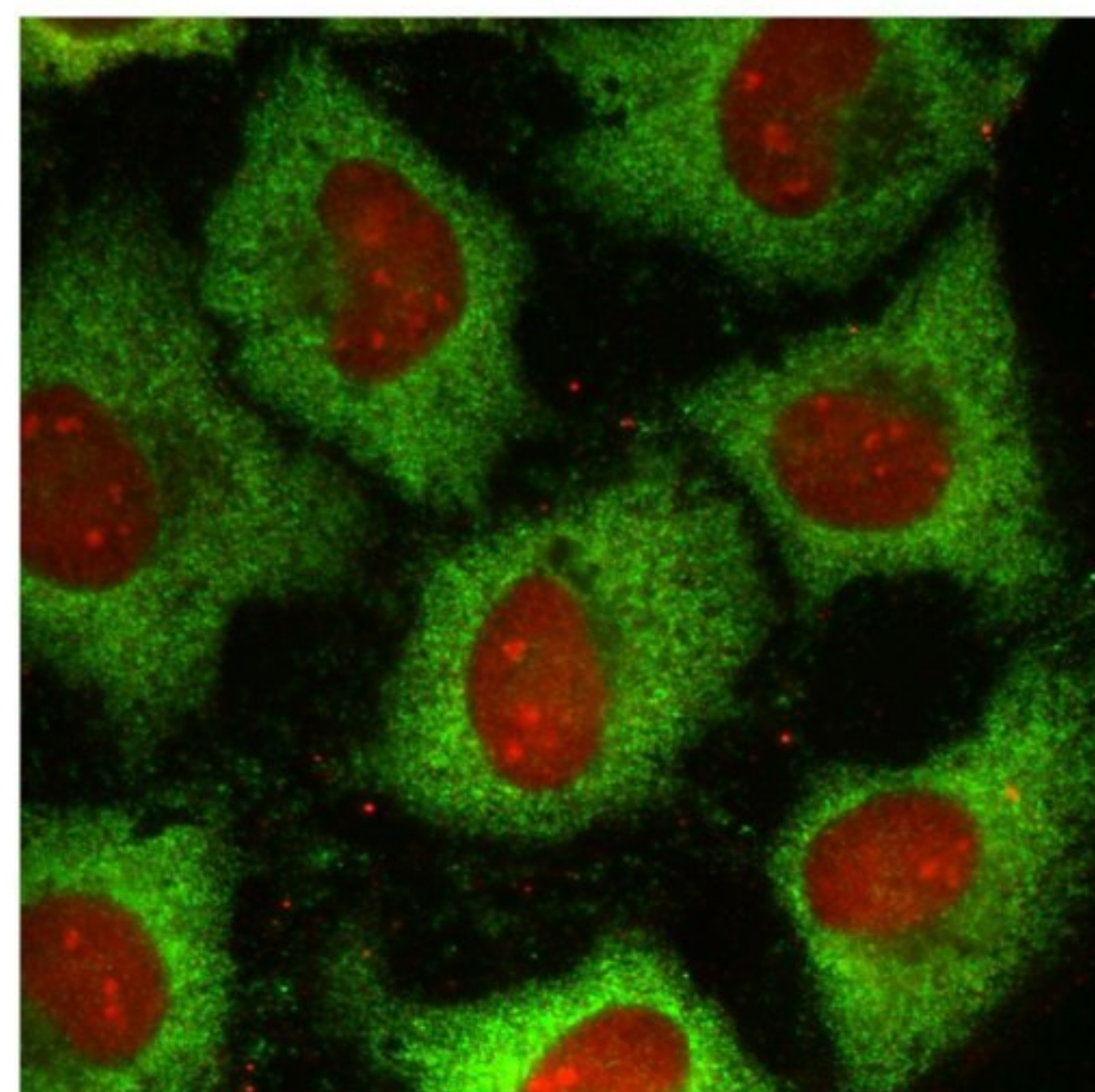
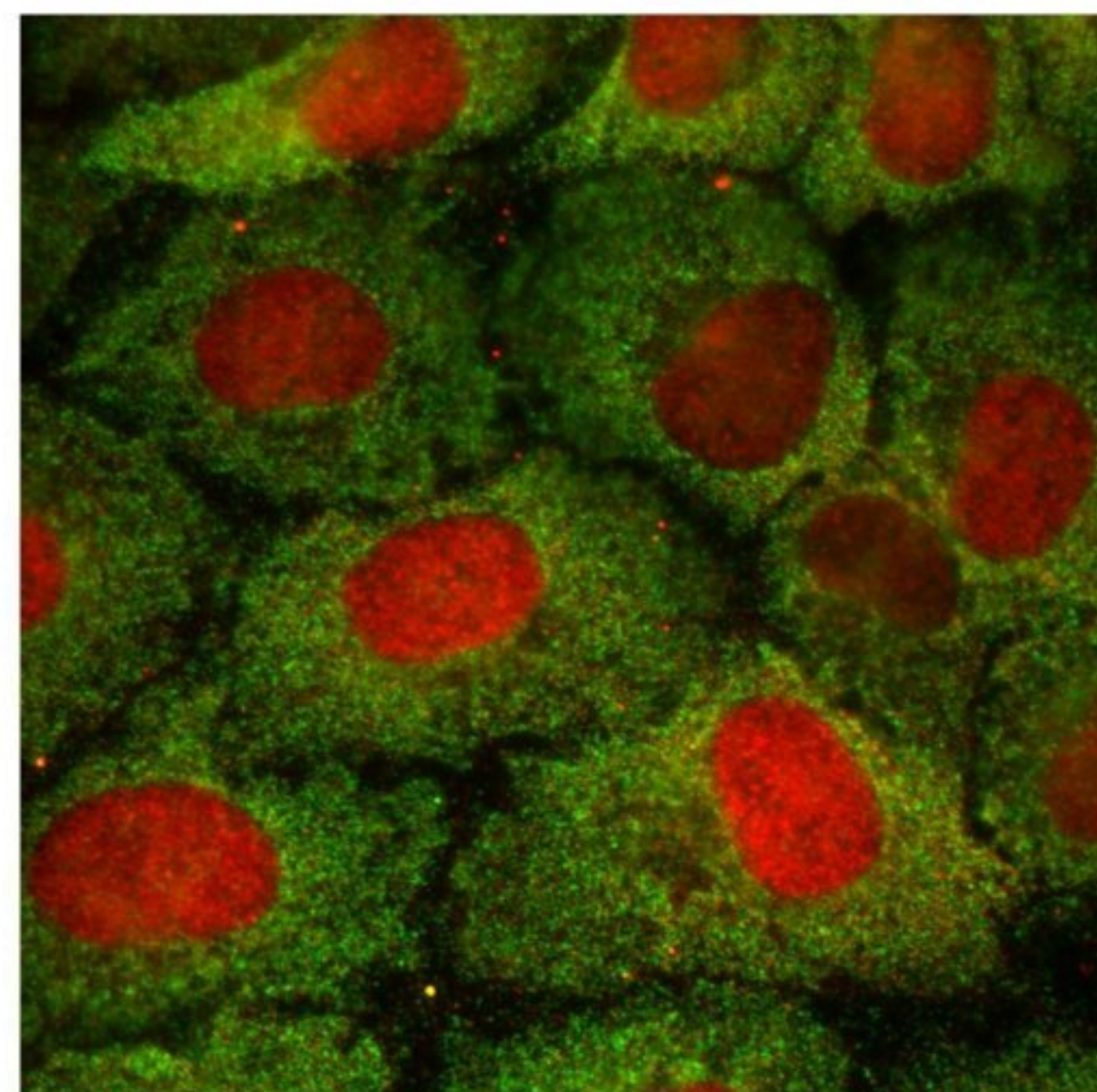
YB-1

Control

Vanadate

U1026

NaF

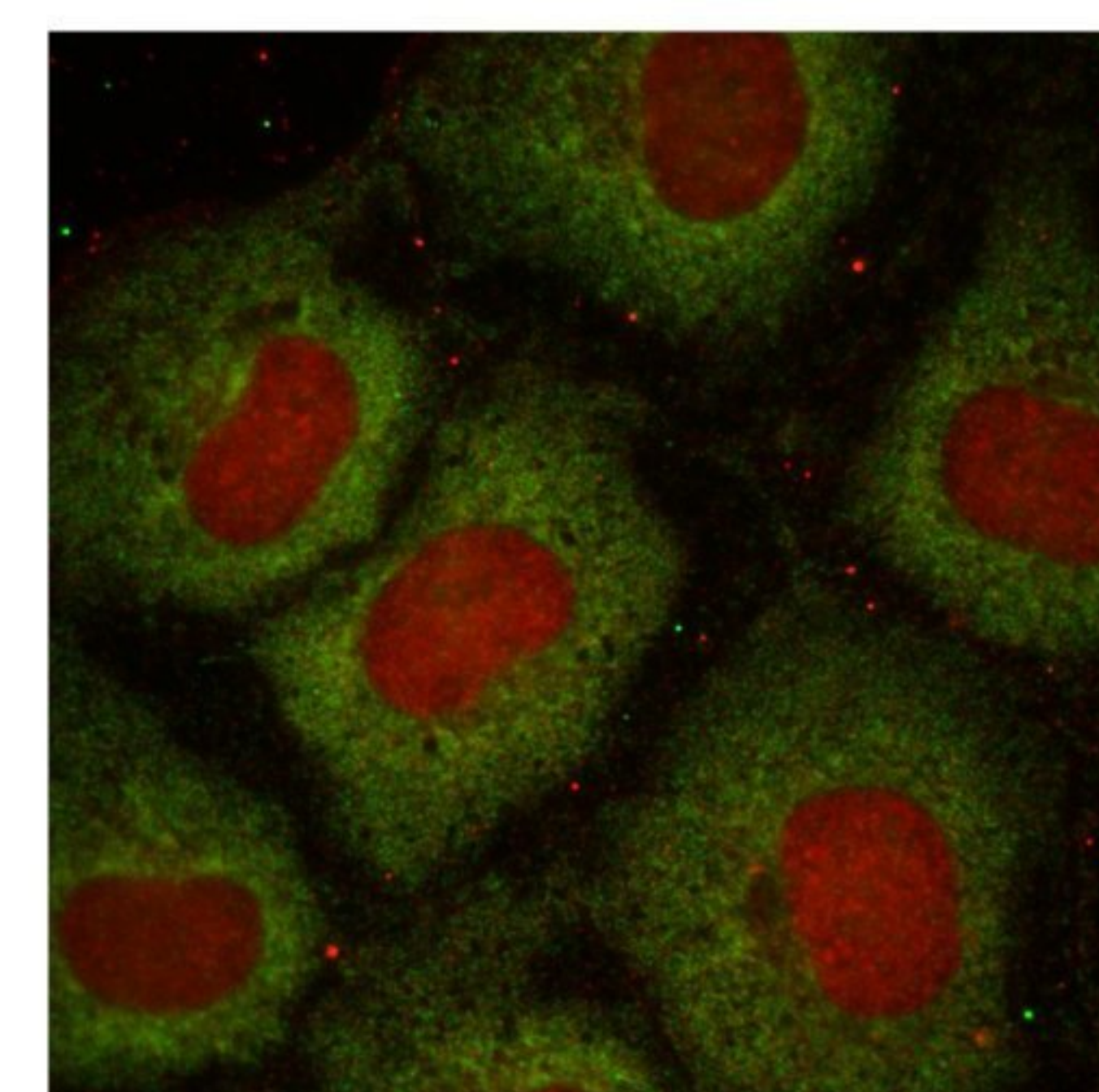
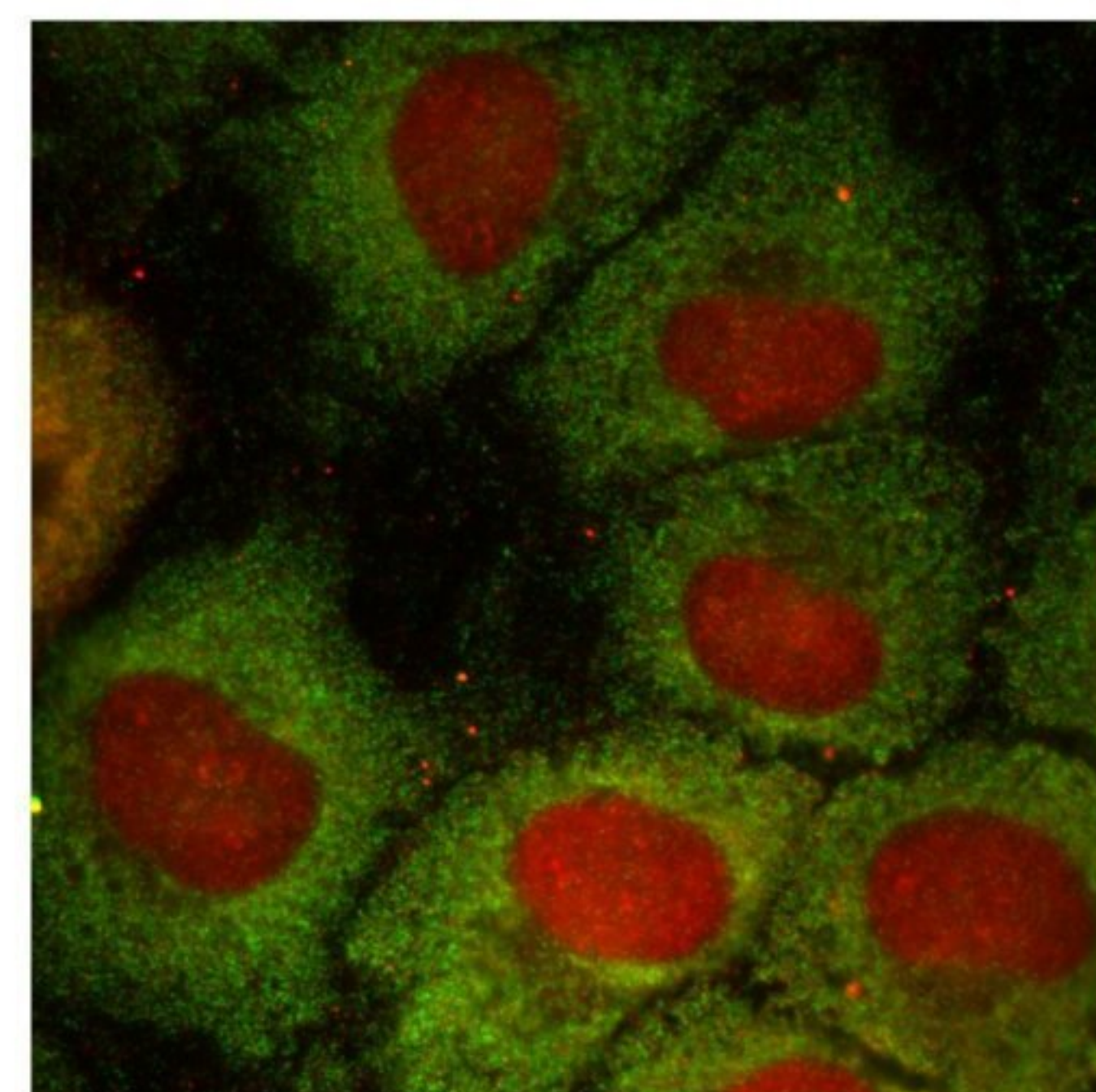
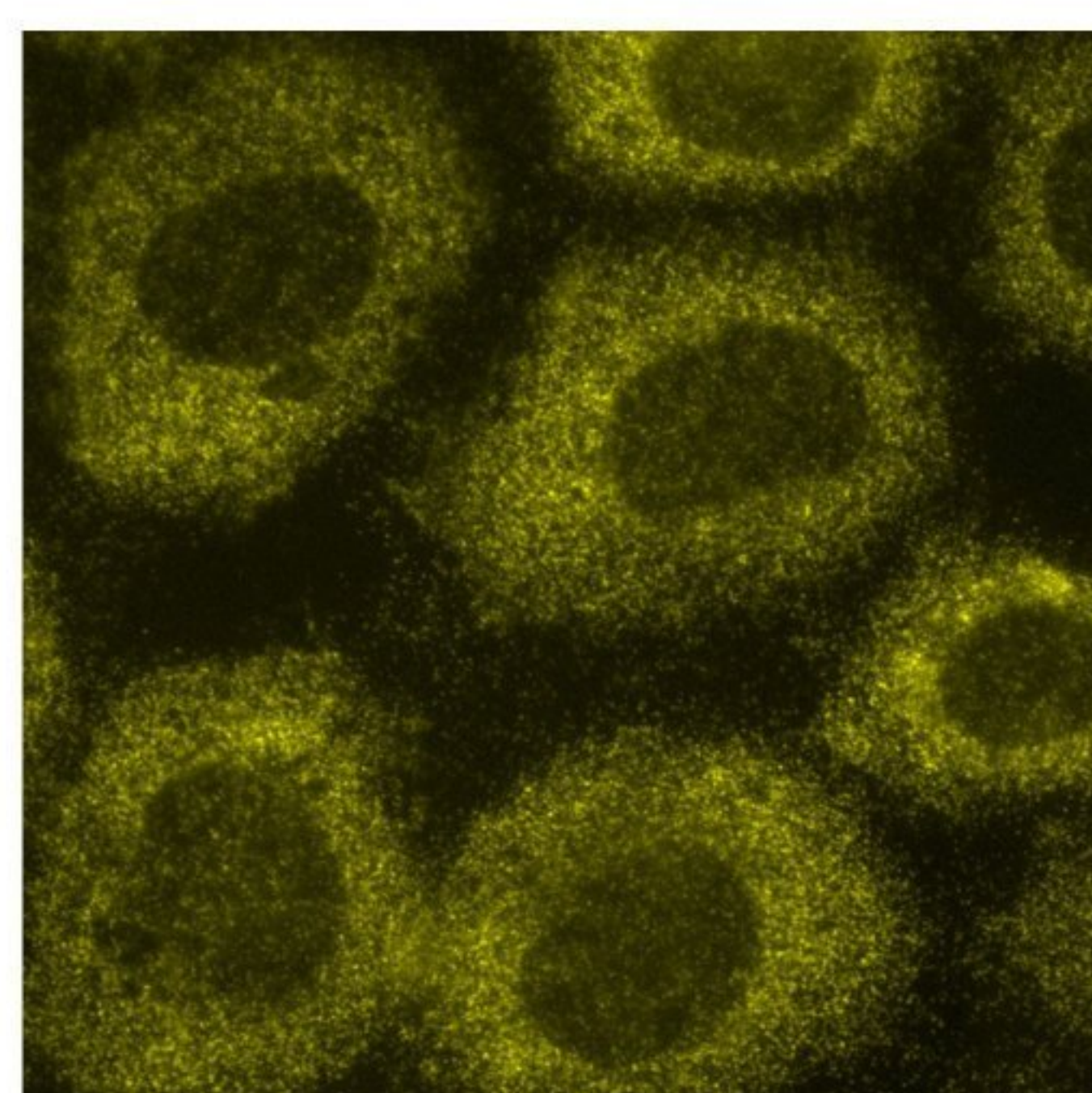
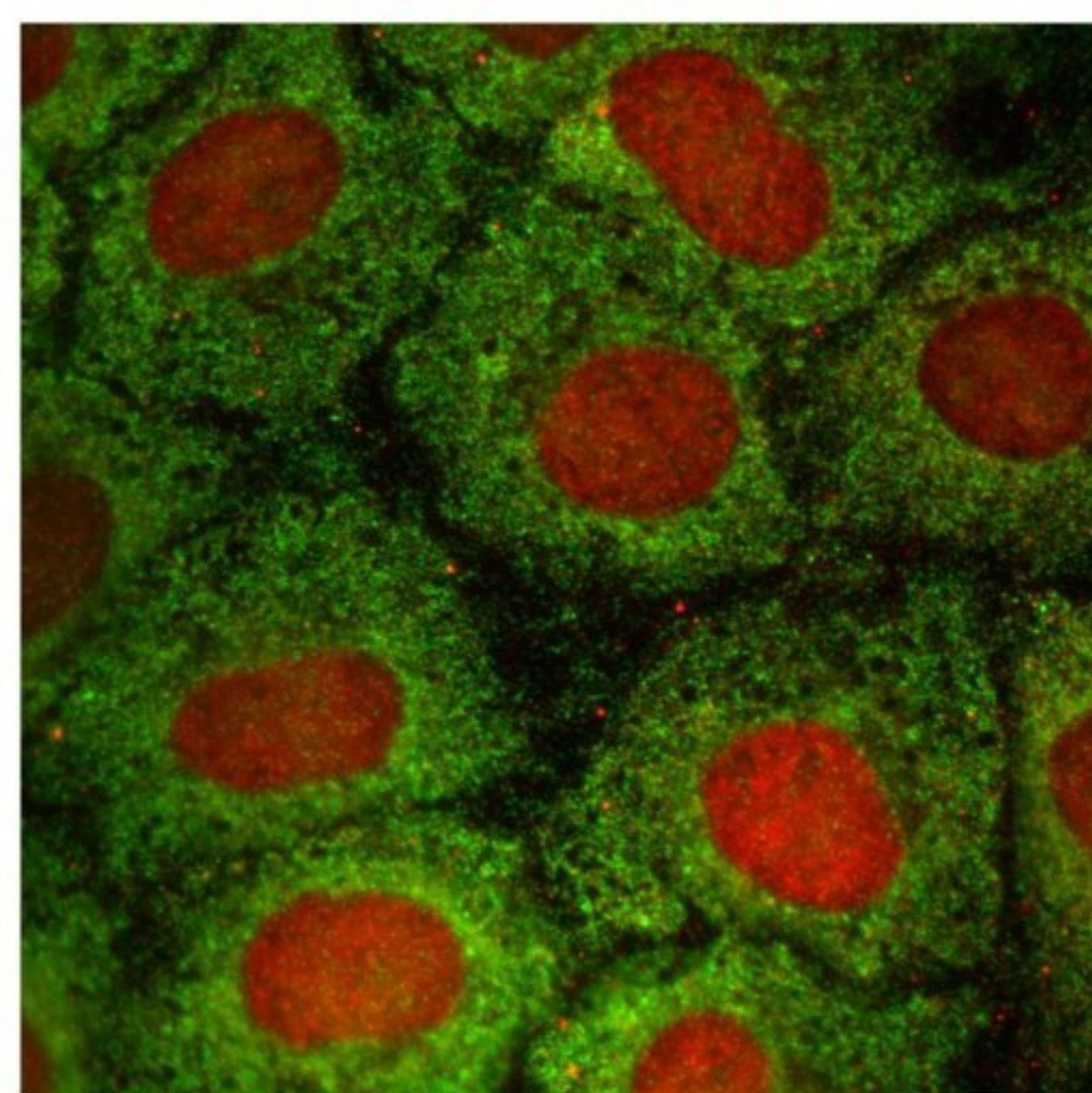


SB203580

PKRi

p80359

SP600125



OA

CDKi

Hemin

H89

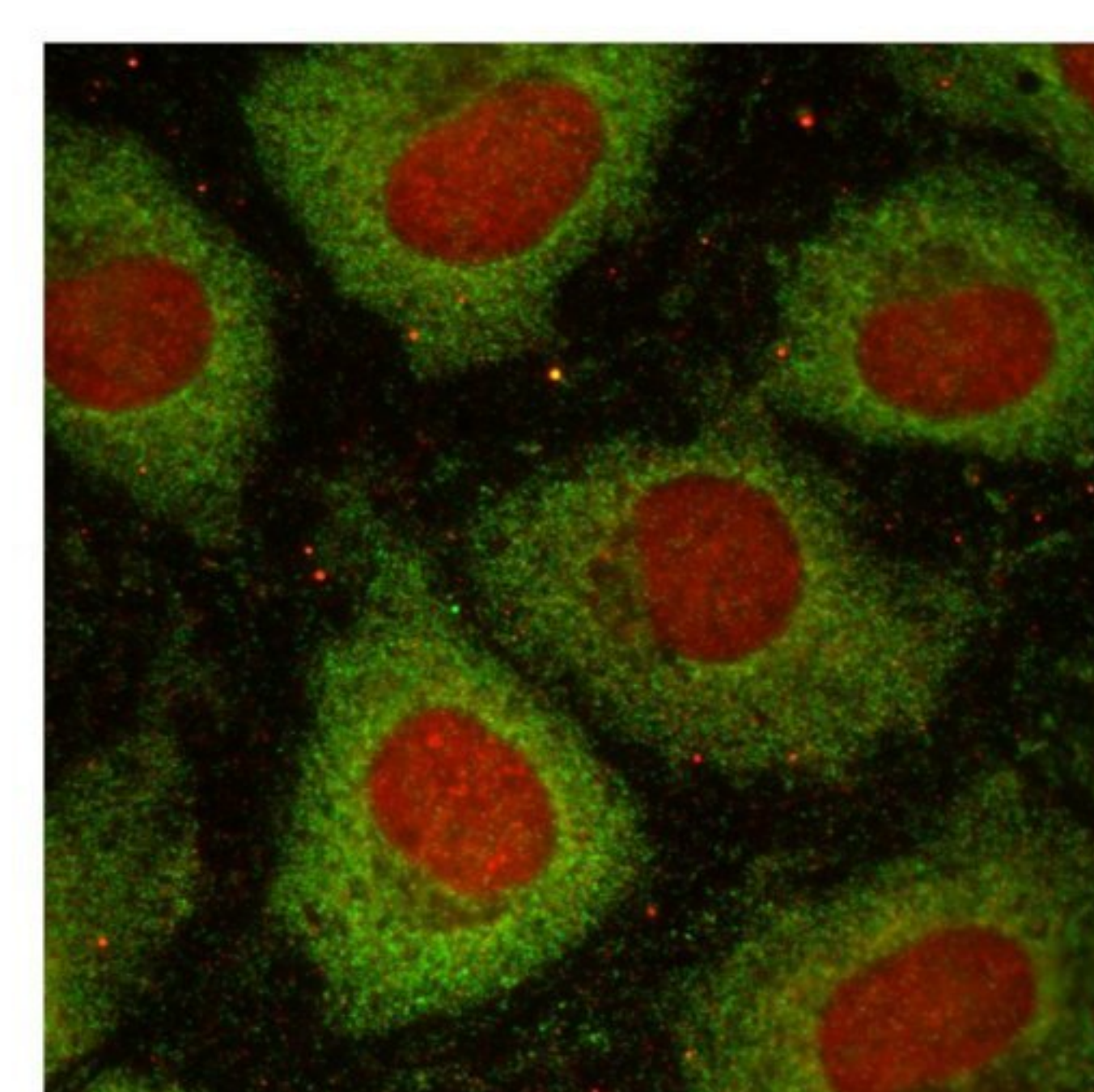
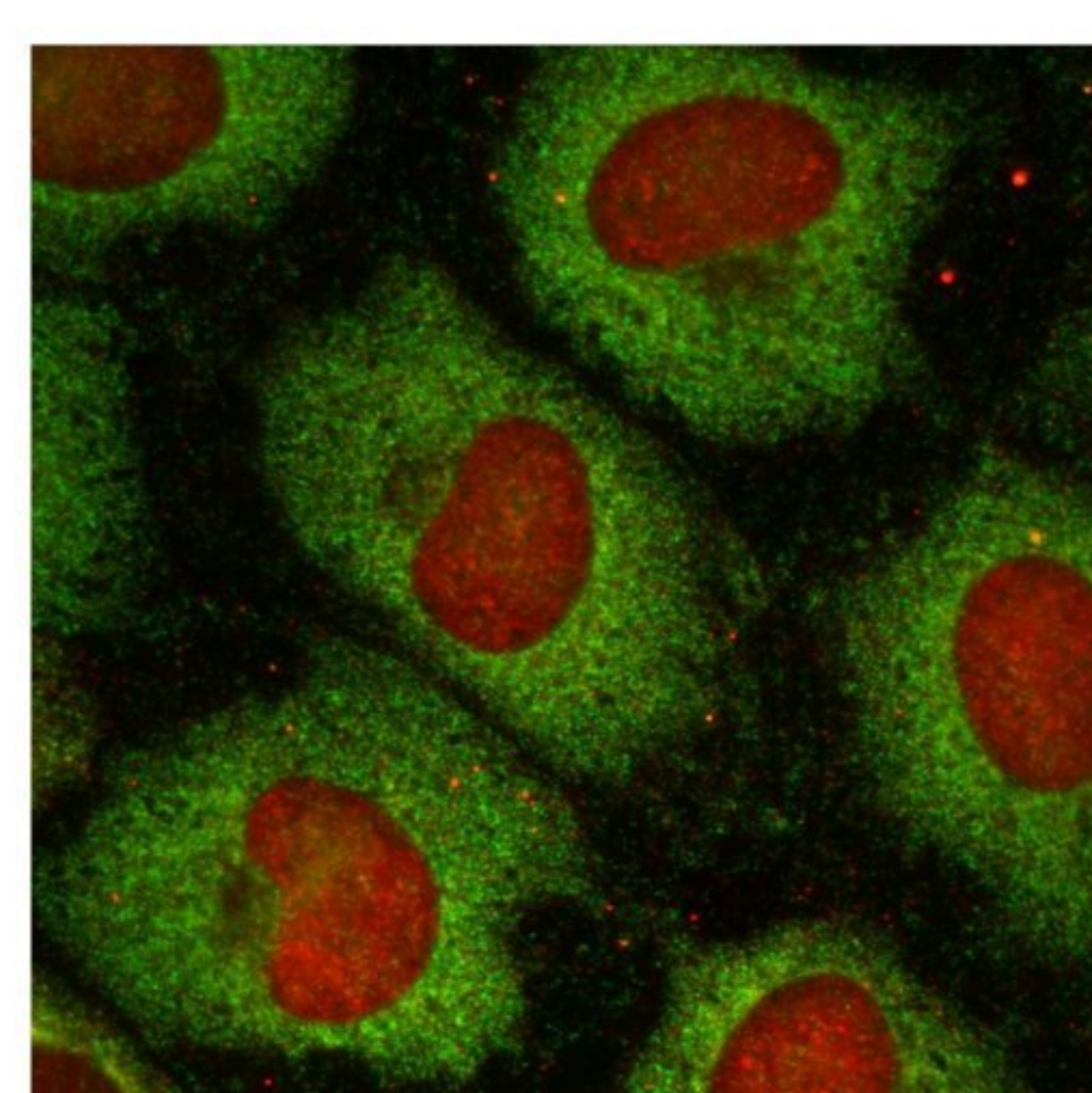
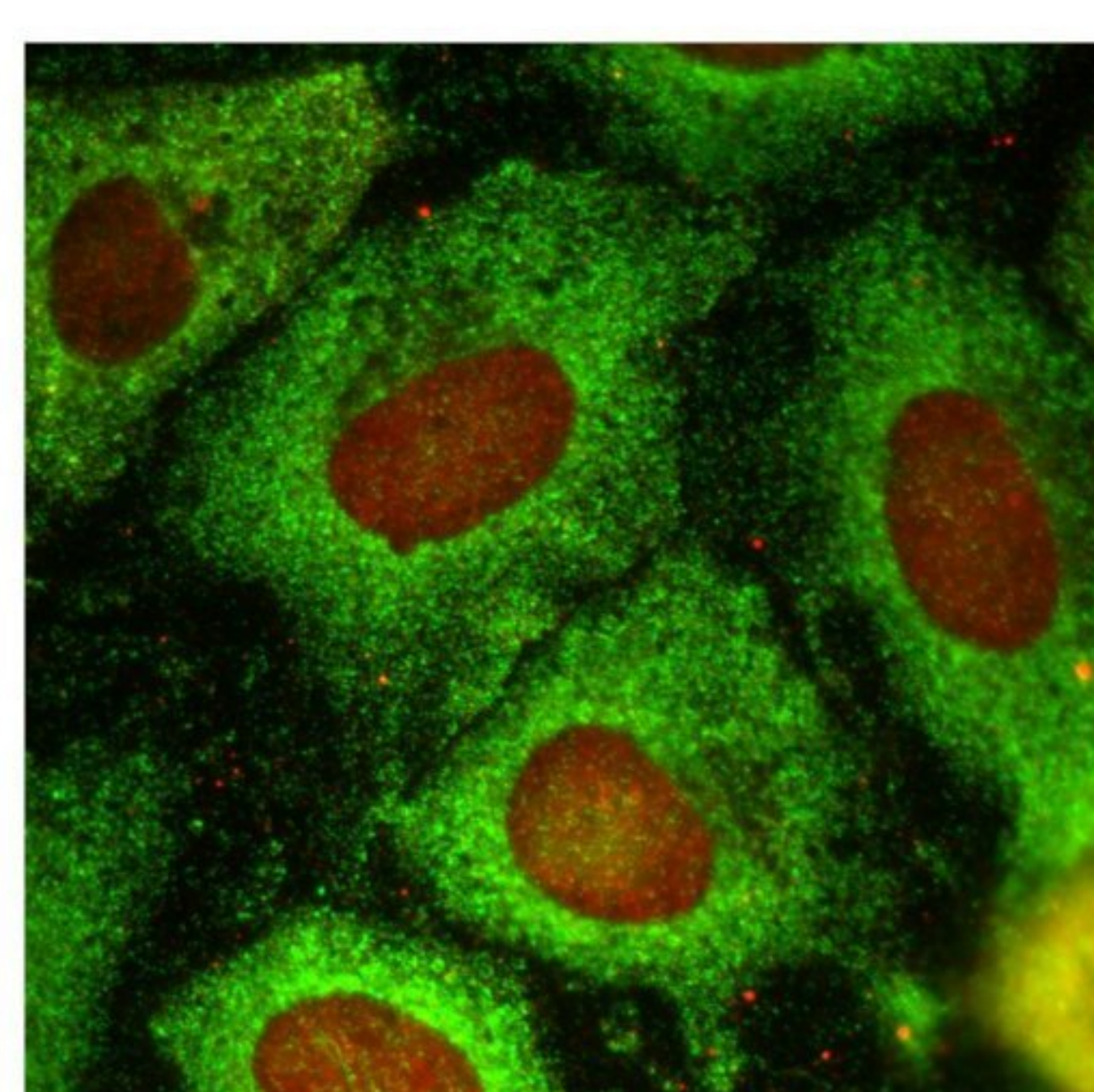
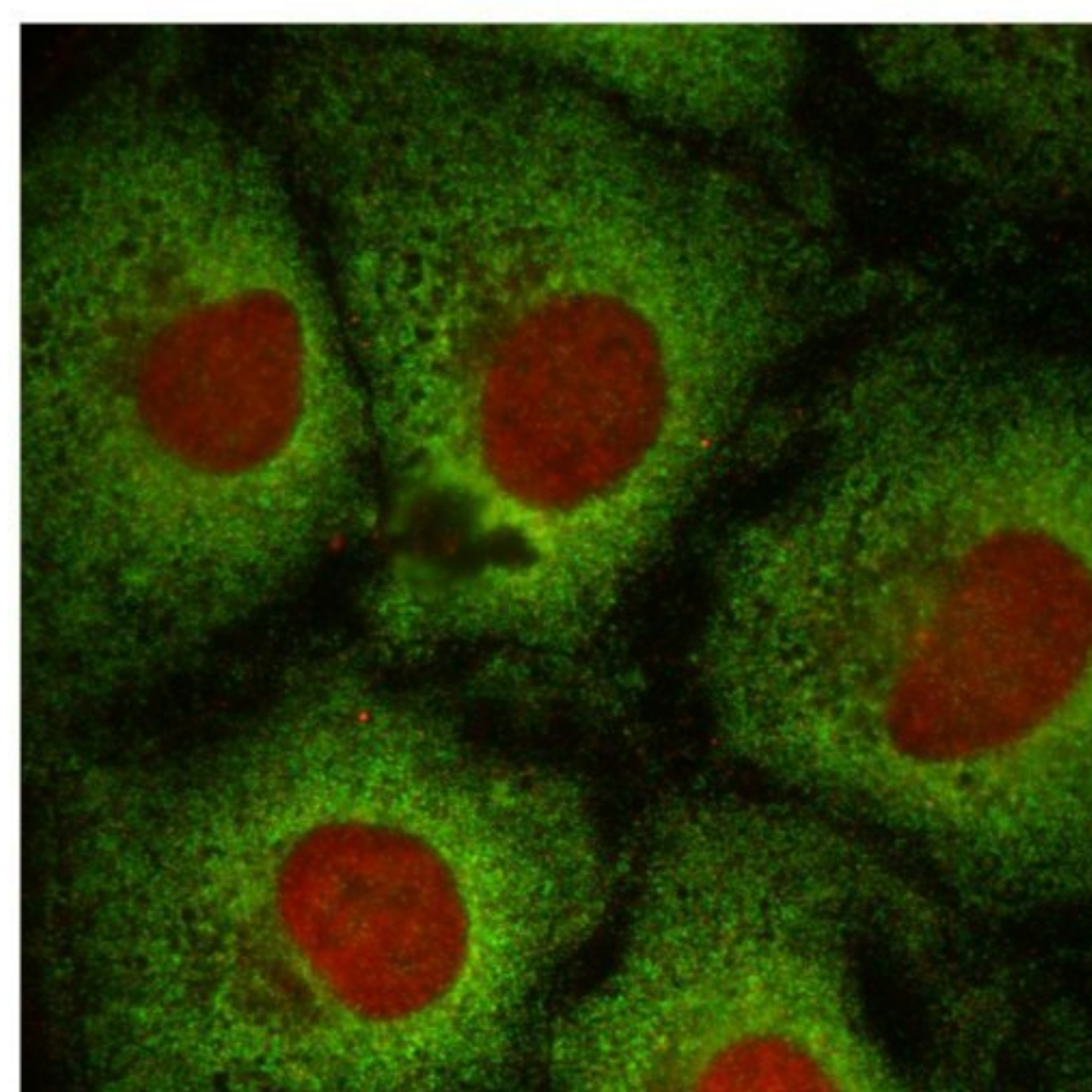


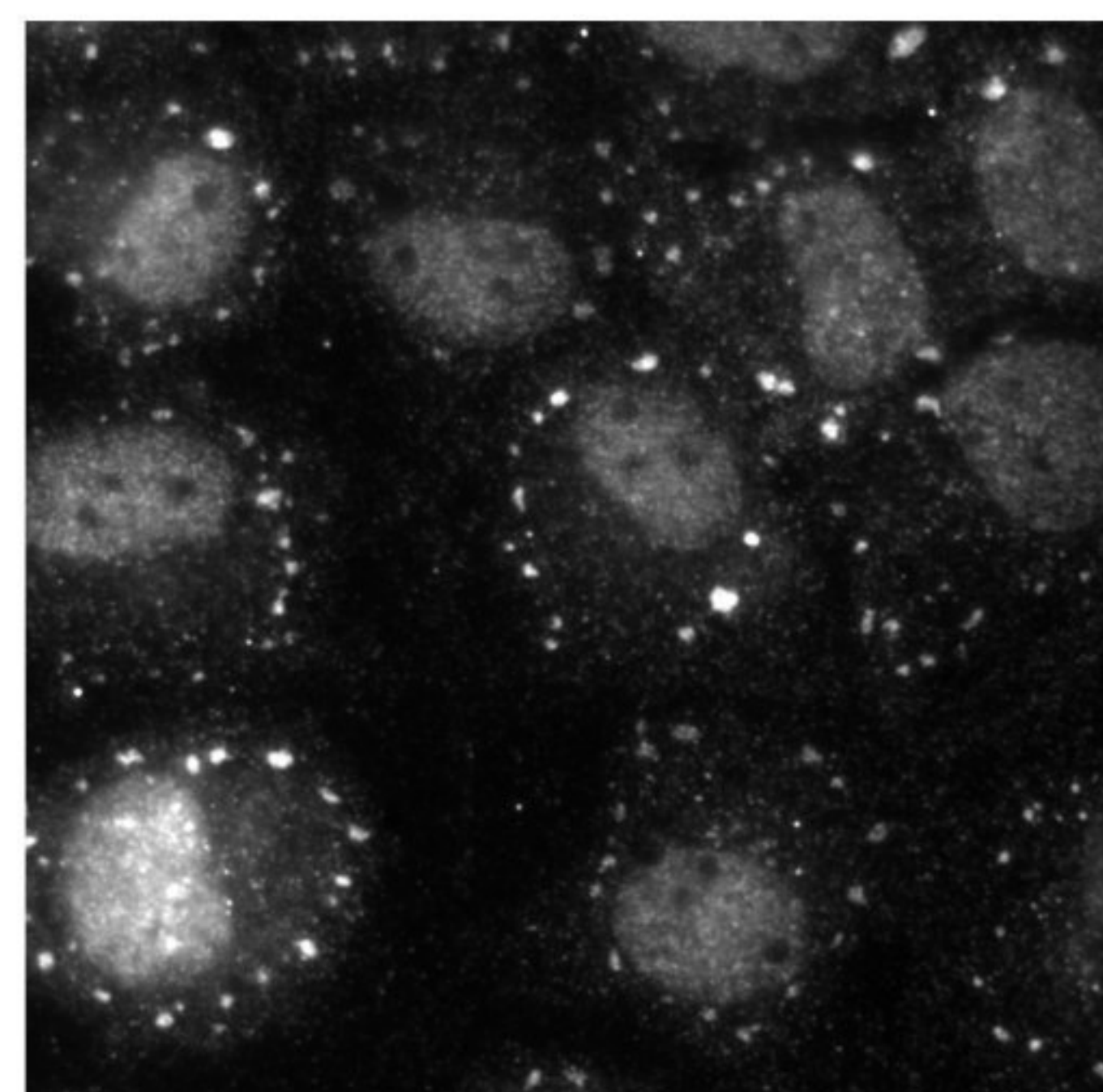
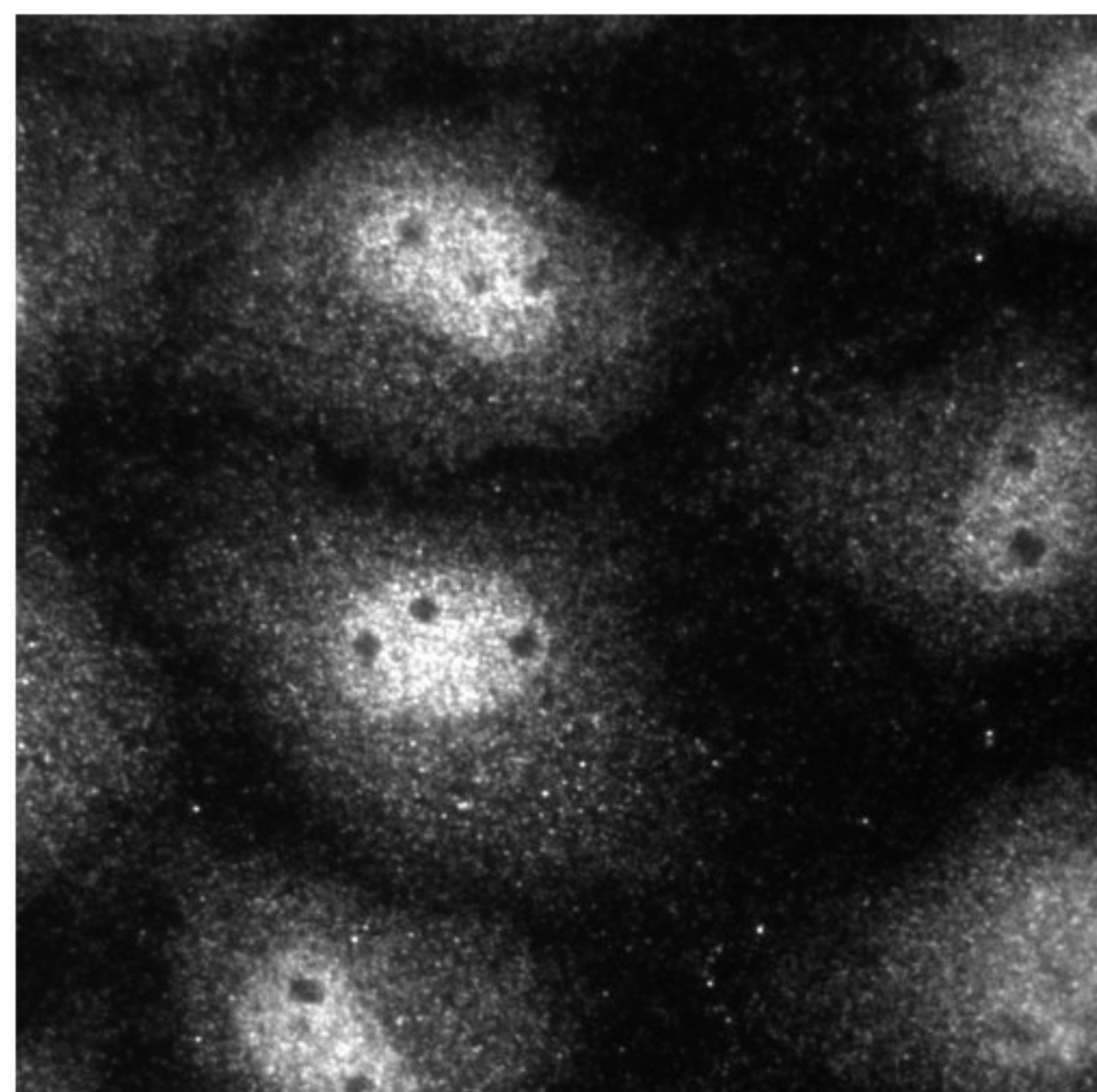
Figure S10

A

Control

Arsenite

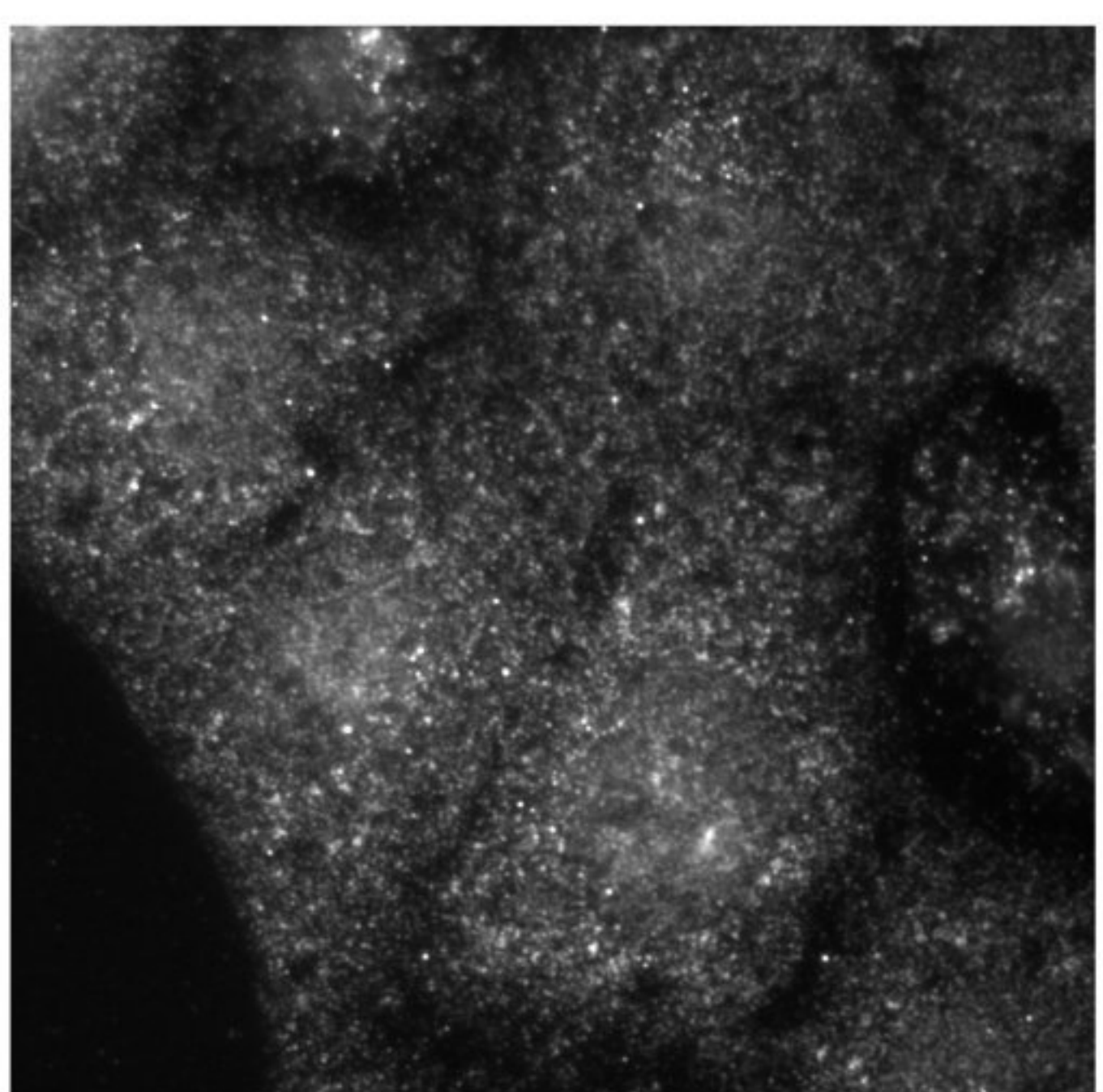
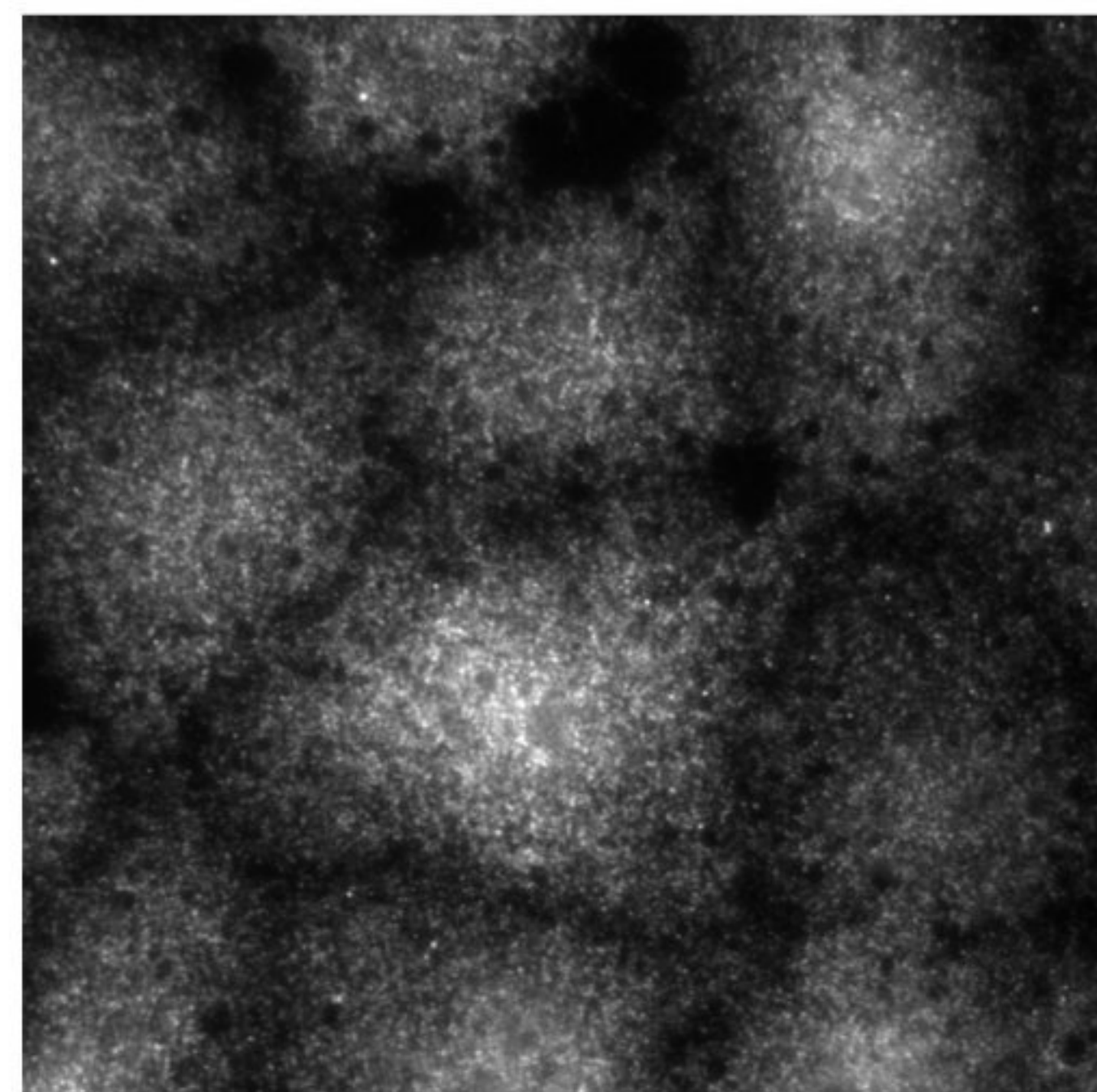
TIA-1



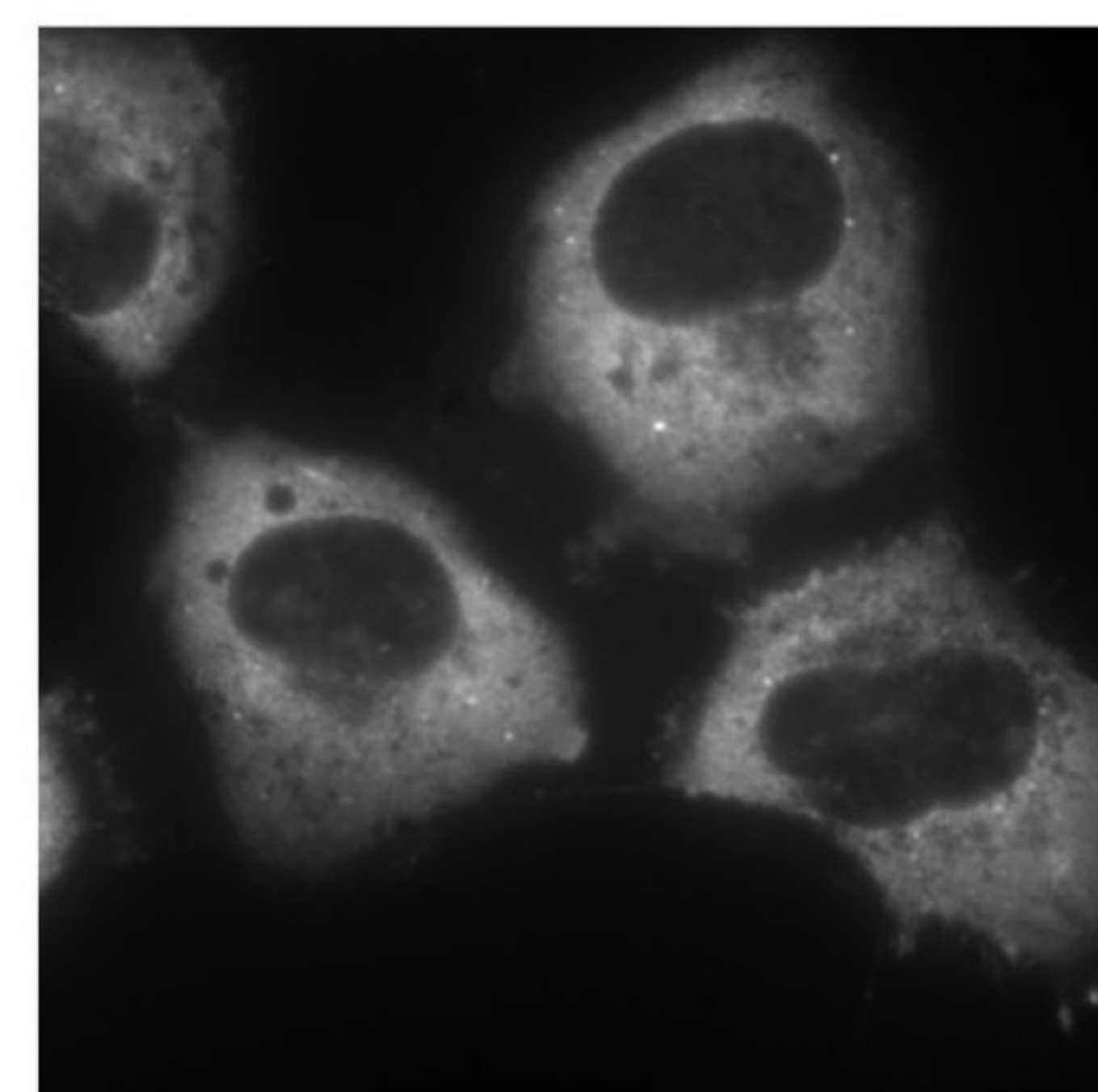
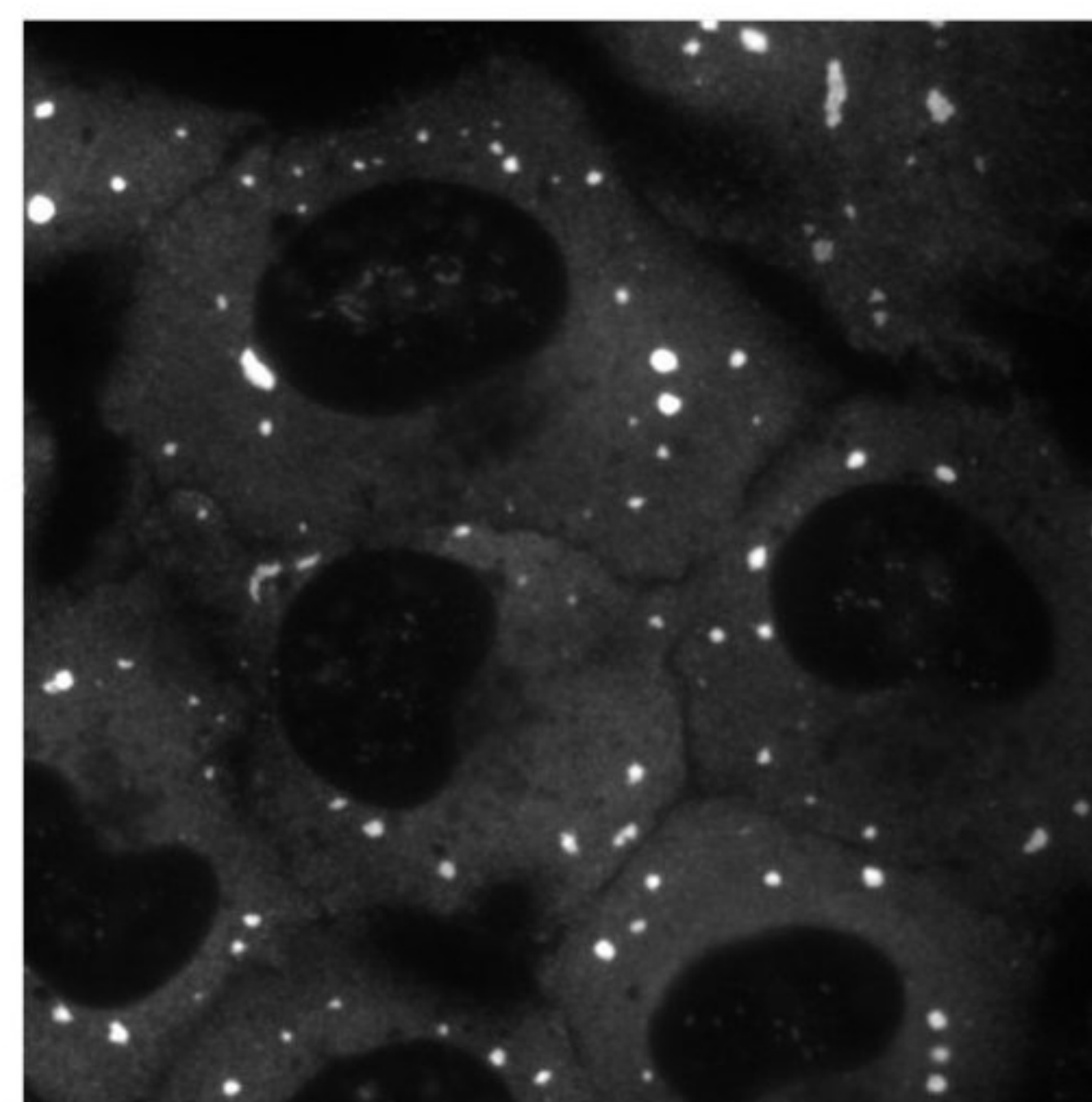
PKRi

PKRi + Arsenite

TIA-1

**B**Arsenite (300 μ M, 45 min)

YB-1

Non pretreated
cellsArsenite-pretreated
cells**Figure S11**