[Letter to the Editor] Caution: choice of fixative can influence the visualization of the location of a transcription factor in mammalian cells

Arinjay Banerjee[1], Darryl Falzarano[1,2] & Vikram Misra*[,1]
[1]*Department of Veterinary Microbiology, Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, S7N 5B4, Canada;* [2]*Vaccine & Infectious Disease Organization-International Vaccine Centre (VIDO-Intervac), University of Saskatchewan, Saskatoon, Canada*

BioTechniques 65: 65–69 (August 2018) 10.2144/btn-2018-0060
Keywords: bat cells • fixation • human cells • IRF3

Fluorescent microscopy is frequently used to study protein location in cells. It provides us with a visual snapshot of the cellular location of a particular protein of interest. It also allows us to study physical interactions between different proteins. Generally, cells are grown on slides or coverslips and fixed to preserve the cellular location of proteins. These cells are then incubated with antibodies specific to proteins of interest. Secondary antibodies conjugated to dyes are used to microscopically detect these proteins. The secondary dyes are excited using an ultraviolet light source or lasers, which emit light of a specific wavelength. This emitted light is then detected using fluorescent microscopy.

Several fixatives are currently used. 10% Neutral buffered formalin (10% NBF) and methanol are regularly used as fixatives for immunofluorescence studies in cell culture (reviewed in [1]).

IRF3 is a transcription factor that is activated in response to recognition of pathogen-associated molecular patterns (PAMPs) by cellular receptors called pattern recognition receptors (PRRs). This recognition by PRR leads to downstream expression of antiviral genes such as interferons and inflammatory cytokines such as TNFα (reviewed in [2]). Several studies in rodent and primate cells have shown that the transcription factor IRF3 remains predominantly in the cytoplasm in the absence of PRR activation in cells. When PRRs are activated by PAMPs, IRF3 undergoes phosphorylation and dimerization. Dimerized IRF3 then migrates to the nucleus to initiate gene expression [3–5].

Bat species have been implicated as reservoirs for several emerging viruses that cause severe and often fatal disease in other species. These include viruses that cause diseases such as severe acute respiratory syndrome, Middle-East respiratory syndrome (MERS), Marburg hemorrhagic disease, and Hendra and Nipah respiratory and neurological syndromes. These viruses, or bat viruses closely related to them, do not appear to cause noticeable morbidity or pathology in their natural bat hosts (reviewed in [6,7]), possibly due to unique mechanisms to dampen potentially harmful inflammation in response to viral infections [8] and a constitutively active antiviral interferon response [9].

IRF3 drives the expression of interferons in response to viral infection [3]. Although IRF3 has been studied extensively in rodents and primates, not much is known about bat IRF3. We have previously detected big brown bat (*Eptesicus fuscus*) IRF3 transcripts and studied the upregulation of IRF3 transcripts in response to mimic viral challenge in both human and bat cells (see supplementary information in [8]). While optimizing the detection of bat IRF3 protein by immunofluorescence, we discovered that the location of this transcription factor varied depending on whether we used 100% methanol or 10% NBF as fixative. For us, the use of 10% NBF was imperative because we needed to inactivate MERS-coronavirus (MERS-CoV) in these cells before taking the samples out of bio-containment level 3. Methanol alone has not been tested for MERS-CoV inactivation [10].

We seeded human lung fibroblast (MRC5; ATCC CCL-171 [VA, USA]) and bat kidney (Efk3) cells [11] in chamber slides (Nunc, Thermofisher, MA, USA; Catalogue number: 177445) at a concentration of 3×10^4 cells/chamber. Media used for these cell types have been previously described [8]. The chambers were incubated at 37°C in a humidified incubator overnight. When the cells were 80% confluent, cells in each chamber were washed twice with phosphate-buffered saline (PBS). The cells were then fixed with either ice-cold 100% methanol (Fisher chemical, PA, USA; Catalogue number: A452SK-4), 10% NBF (Sigma, MI, USA; Catalogue number: HT501128) or 4% paraformaldehyde (PFA; prepared as previously described [12]). Methanol-fixed slides were kept at –20°C, while 10% NBF- and 4% PFA-fixed slides were kept at +4°C for the duration of the fixation. Following incubation, 10% NBF- and 4% PFA-fixed slides were permeabilized using 0.2% TritonX-100 (VWR, PA, USA; Catalogue number: VW3929–2) diluted in PBS for 5 min. Slides that were simultaneously fixed in methanol followed by 10% NBF were also permeabilized using 0.2% TritonX-100 for consistency. All cells were washed twice and then stained for IRF3, Lamin B1, GAPDH and nuclear DNA using a protocol previously described [8]. 150 µl of solution was used for staining and subsequent washes in each chamber. Primary antibodies used were 1/100 dilution of rabbit anti-IRF3 (Abcam, MA, USA; Catalogue number: ab68481; RRID: AB_11155653), 1/100 dilution of rabbit Lamin B1 (Abcam, USA; Catalogue number: ab16048; RRID: AB_10107828) and 1/100 dilution of mouse anti-GAPDH (EMD Millipore, MA, USA; Catalogue number: AB2302; RRID:

AB_10615768). To stain the nucleus, 1/50,000 dilution of Hoechst 33258 (Molecular probes, MA, USA; Catalogue number: H3570) was used. Secondary antibodies used were 1/500 dilution of goat anti-mouse Alexa 488 (Molecular probes; Catalogue number: A-11001; RRID: AB_2534069), 1/500 dilution of goat anti-rabbit Cy5 (GE Healthcare, Little Chalfont, UK; Catalogue number: PA45012; RRID: AB_772204), 1/250 dilution of goat anti-mouse Cy5 (GE Healthcare; Catalogue number: PA45009; RRID: AB_772199) and goat anti-rabbit Alexa 488 (Molecular Probes; Catalogue number: A-11008; RRID: AB_143165). The chambers and gaskets were removed after staining. Slides were dipped in ultrapure water and air dried. Cover slips were mounted on slides using Prolong gold anti-fade mounting fluid (Life Technologies, USA; Catalogue number: P36930). Slides were observed under a TCS SP5 confocal microscope (Leica) and Olympus IX83 fluorescence microscope.

To compare the location of IRF3 in human and bat cells, we fixed the cells with methanol or 10% NBF (a treatment required for inactivating hazardous viruses) and prepared them for fluorescent microscopy. Methanol-fixed slides showed the presence of IRF3 in the cytoplasm of both human and bat cells (Figure 1A & 1B). However, we observed that in 10% NBF-fixed cells, IRF3 was present in both the cytoplasm and the nucleus in bat cells (Figure 1A), while in human cells IRF3 was predominantly present in the nucleus (Figure 1B). The no-antibody controls were negative (Figure 1C) and there was no background staining observed in the secondary antibody control (Figure 1D). Since we observed nuclear localization of IRF3 only in 10% NBF-fixed cells, we tested the ability of methanol to permeabilize the cells. By staining for an inner nuclear membrane protein, Lamin B1, we could demonstrate that methanol alone can permeabilize both human and bat cells (Figure 1E). To further rule out the possibility of partial permeabilization by methanol, we permeabilized methanol-fixed bat and human cells with Triton-X 100 (Tx-100) and also fixed both cell types using a 50:50 methanol:acetone fixative in a separate experiment. IRF3 could still be detected mostly in the cytoplasm of both cell types (Figure 1F). Thus, permeabilization was not a factor in methanol- and 10% NBF-fixed cells that were stained for IRF3.

We also noticed that cells that were fixed in methanol for 48 h retained their cellular integrity better than cells that were fixed with 10% NBF for the same duration of time. The protocol that we use for MERS-CoV inactivation involves fixing the cells for 24 h, followed by decontamination of the surface of the slides for another 24 h in 10% NBF. To rule out the role of extended fixation (48 h) with 10% NBF in the aberrant nuclear localization of IRF3, we fixed human and bat cells in 10% NBF for 30 min and stained both cell types for GAPDH and IRF3. IRF3 was detected in the nucleus of cells that were fixed with 10% NBF for 30 min (Figure 1G). Our data strongly indicates that fixing MRC5 and Efk3 cells with 10% NBF alters the localization of IRF3 form the cytoplasm to the nucleus.

To rule out the possibility of secondary antibody-mediated differences in methanol and 10% NBF-fixed cells, we used alternative secondary antibodies to stain IRF3. We used goat anti-rabbit Alexa488 and goat anti-mouse Cy5 to stain IRF3 and GAPDH that had been labelled with primary rabbit anti-IRF3 and mouse anti-GAPDH, respectively. IRF3 was still visible mostly in the cytoplasm of methanol-fixed bat and human cells (arrow; Figure 1H and 1I), whereas IRF3 was present in the nucleus of 10% NBF-fixed bat and human cells (arrow head; Figure 1H and 1I).

To compare the effects of other fixatives and fixation strategies on the cellular location of IRF3, we fixed human and bat cells with either 4% PFA or a simultaneous fixation with methanol, followed by 10% NBF. 4% PFA fixation did not completely prevent nuclear localization of IRF3 in bat and human cells (data not shown), but a simultaneous fixation with methanol followed by 10% NBF reduced the amount of nuclear IRF3 that was detected in these cells (Figure 2A and Figure 2C).

We performed immunoblots to determine the cross-reactivity of anti-IRF3 antibody in human and bat cells. All samples were treated and analyzed by immunoblotting as previously mentioned [8]. Rabbit anti-human-IRF3 (Abcam; Catalogue number: ab68481; RRID: AB_11155653) and mouse anti-GAPDH (EMD Millipore, USA; Catalogue number: AB2302; RRID: AB_10615768) primary antibodies were used at a dilution of 1/1000. Goat anti-rabbit Cy5 (GE Healthcare; Catalogue number: PA45012; RRID: AB_772204) and goat anti-mouse Alexa 488 (Molecular probes; Catalogue number: A-11001; RRID: AB_2534069) secondary antibodies were used at a dilution of 1/10,000. Rabbit anti-human IRF3 antibody detected IRF3 in both human (MRC-5) and bat (Efk-3) cells. Although we observed other bands in the blot (Figure 2B), we used a cocktail of two sets of small interfering RNA (siRNA; Table 1) designed against human and bat IRF3 to specifically knockdown IRF3 protein (~50 kDa) in these cells. Dicer-ready siRNA (DsiRNA) specific to big brown bat and human IRF3 were designed and obtained through Integrated DNA Technologies (IDT). A 100 nM final concentration of a 1:1 mixture of two DsiRNAs per cell line (Table 1) targeting separate regions on the big brown bat and human IRF3 transcript was transfected into Efk3 and MRC5 cells using Lipofectamine 2000 (Invitrogen). Scrambled non-specific DsiRNA (NC DsiRNA; IDT) was used as a negative control. Cells were harvested for immunoblots after 48 h. siRNA treatment in both cell types knocked-down the specific protein band that was observed at the 50 kDa mark in the immunoblot (Figure 2B).

We quantified the amount of total nuclear IRF3 fluorescence intensity using Image J (Version 1.49) for different fixation strategies. 10% NBF fixed bat and human cells displayed significantly more fluorescence in the nucleus than both methanol-only and methanol + 10% NBF-fixed cells (Figure 2C). Methanol-only-fixed bat cells displayed the lowest nuclear staining for IRF3 amongst other fixed bat cells, whereas nuclear IRF3 fluorescent intensity in methanol-only-fixed human cells did not vary significantly from methanol + 10% NBF-fixed human cells (Figure 2C). Thus, different cell types may exhibit varying levels of IRF3 nuclear localization in response to the choice of fixative. Since it was imperative to use either 10% NBF or 4% PFA to inactivate MERS-CoV before taking the samples out of bio-containment level 3, we were unable to use either of the two fixation strategies.

Studying the cellular location of transcription factors often allows us to monitor how a cell responds to a stimulus. In immunology, it allows us to predict the cellular response that the external stimulus may produce. However, we must exercise caution while fixing the cells. Our data clearly demonstrate that different fixatives may have varying degrees of influence over the apparent cellular location of transcription factors, such as IRF3. This phenomenon

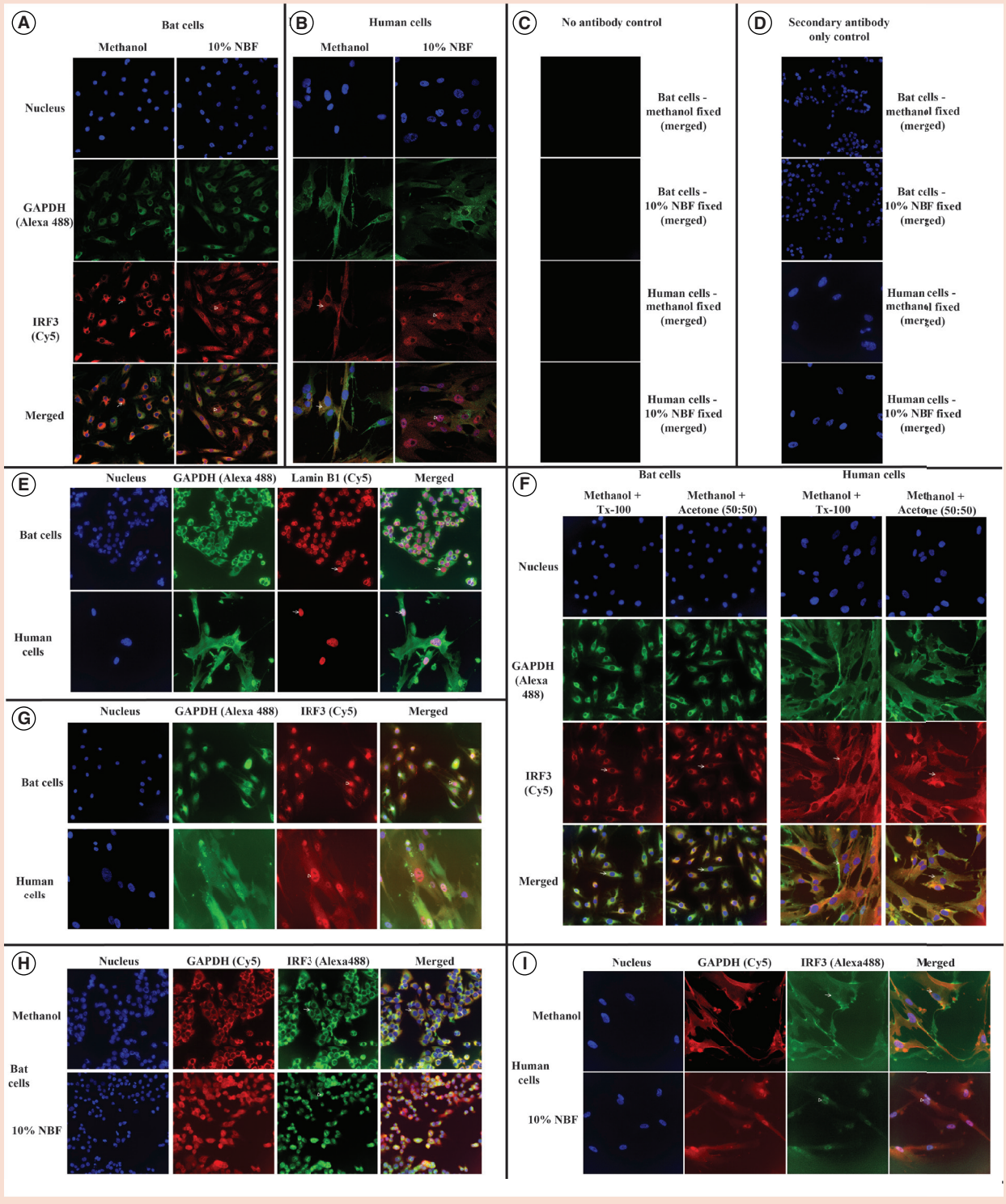

Figure 1. Fixation with 10% neutral buffered formalin causes nuclear translocation of IRF3 in cells. IRF3, a key transcription factor involved in antiviral cell signaling, remains predominantly in the cytoplasm of unstimulated cells. Bat kidney epithelial and human lung fibroblast cells were seeded in chamber slides and were stained for IRF3 the following day. **(A)** IRF3 was present in the cytoplasm of methanol-fixed bat cells (arrow), whereas it was present in both the cytoplasm and the nucleus in 10% NBF-fixed bat cells (arrow head). **(B)** IRF3 was present predominantly in the cytoplasm of methanol-fixed human cells (arrow), whereas, it was concentrated in the nucleus in 10% NBF-fixed human cells (arrow head). **(C)** No background fluorescence was observed in the no antibody control. **(D)** Secondary antibody control with Hoechst 33258 stained the nucleus of bat and human cells. No visible background staining was observed. **(E)** To detect if methanol fixation permeabilizes bat and human cells, we fixed both cell types in methanol and stained these cells for Lamin B1, an inner nuclear membrane protein. Lamin B1 (red) was detected in the nucleus of both bat and human cells that were permeabilized with methanol only. **(F)** To further rule out the possibility of partial permeabilization of cells with methanol, bat and human cells were fixed with methanol and additionally permeabilized with Triton X-100 (Tx-100). In a separate experiment, both cell types were also fixed and permeabilized using a 50:50 methanol:acetone fixative. IRF3 was detected predominantly in the cytoplasm of human and bat cells that were fixed and permeabilized using either strategy. **(G)** To rule out the possible effects of extended periods of fixation with 10% NBF on the nuclear detection of IRF3, we fixed bat and human cells in 10% NBF for 30 min and stained them for IRF3. IRF3 could still be detected in the nucleus of both cell types. **(H)** To determine if the choice of secondary antibody influenced the visualization of IRF3, we used different secondary antibodies. Using alternate secondary antibody (anti-mouse Cy5 for GAPDH and anti-rabbit Alexa 488 for IRF3) did not inhibit the detection of IRF3 in the nucleus of 10% NBF-fixed bat cells (arrowhead) as compared to methanol-fixed cells (arrow). **(I)** Similarly, using alternate secondary antibody (anti-mouse Cy5 for GAPDH and anti-rabbit Alexa 488 for IRF3) did not inhibit the detection of IRF3 in the nucleus of 10% NBF-fixed human cells (arrowhead) as compared to methanol-fixed cells (arrow). NBF: Neutral buffered formalin.

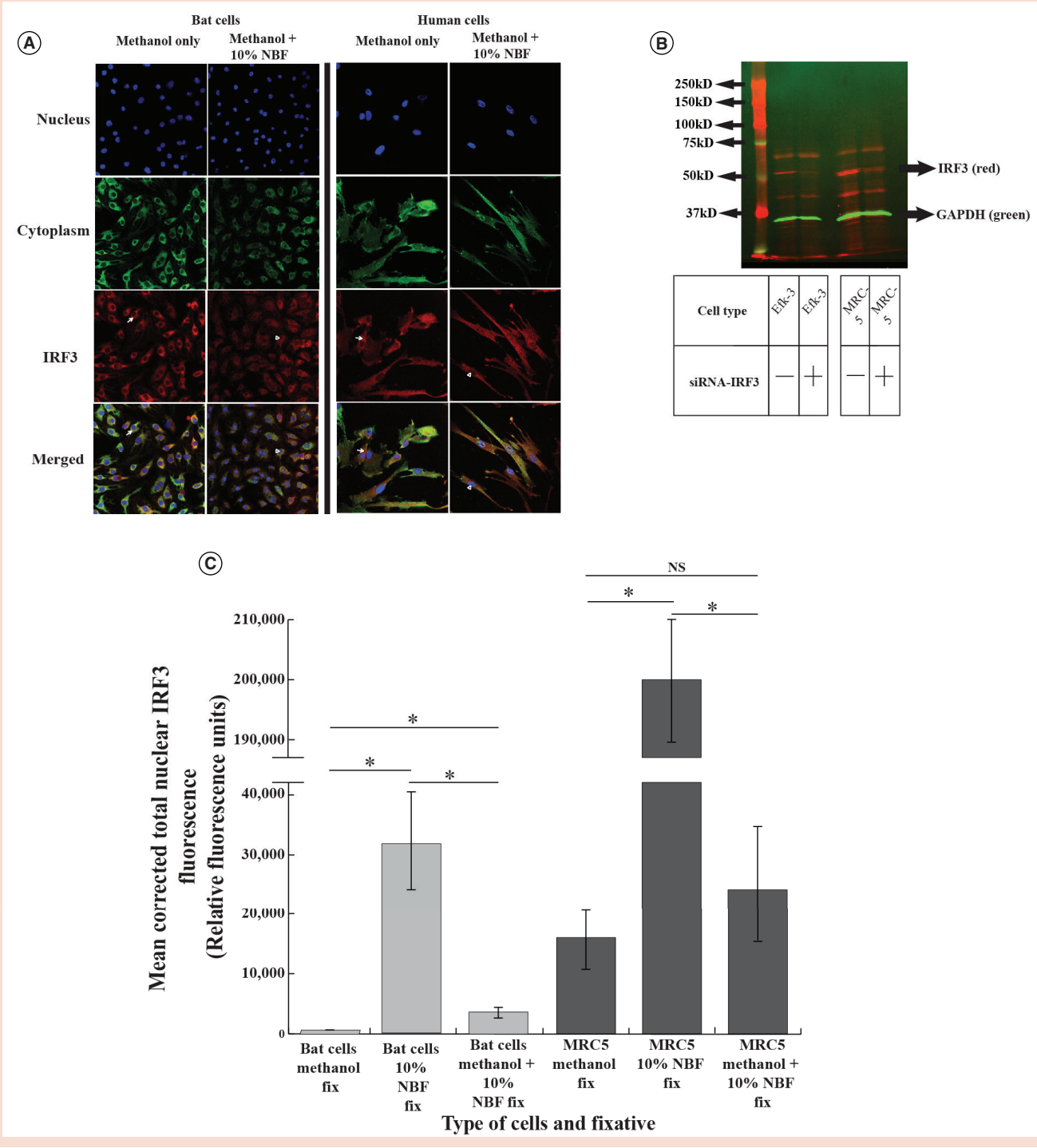

Figure 2. Simultaneous fixation with methanol and 10% neutral buffered formalin reduces (but does not eliminate) nuclear IRF3. Human (MRC-5) and bat (Efk-3) cells were seeded in six-well plates for immunoblot analysis and in chamber slides for immunofluorescence microscopy. **(A)** To determine if prior fixation with methanol reduced 10% NBF-mediated nuclear localization of IRF3, bat and human cells were fixed with methanol or a simultaneous fixation process with methanol, followed by 10% NBF. IRF3 was present in the cytoplasm of methanol-only-fixed human and bat cells (arrow). Cells fixed simultaneously in methanol, followed by 10% NBF displayed a reduced amount of nuclear IRF3 than 10% NBF-only fixed cells as seen in Figure 1A & B. However, this treatment did not eliminate nuclear staining for IRF3. **(B)** To determine the specificity of anti-IRF3 antibody, we knocked-down IRF3 in both human and bat cells using siRNA. Cells in six-well plates were transfected with siRNA or mock-siRNA. Cells were harvested after 15 h in 2x sample buffer and analysed on a reducing gel. Proteins were detected by immune-blotting. A major product was observed at the expected size of 50 kD. siRNA designed against human and big brown bat IRF3 specifically knocked-down the 50 kD protein in both human and bat cells. **(C)** To determine if the choice of fixative significantly increased the amount of nuclear IRF3 in bat and human cells, we quantified nuclear IRF3 in both cell types after fixing the cells in methanol, 10% NBF and methanol + 10% NBF. Fixing both bat and human cells with 10% NBF significantly increased the amount of nuclear IRF3 in both bat and human cells as compared to methanol-only and methanol + 10% NBF-fixed cells (Mean ± SD; n = 5). Methanol-only-fixed bat cells had significantly lower amounts of nuclear IRF3 compared with both 10% NBF-only and methanol + 10% NBF-fixed bat cells (Mean ± SD; n = 5). The amount of nuclear IRF3 in human cells did not vary significantly between methanol-only- and methanol + 10% NBF-fixed cells (Mean ± SD; n = 5). Statistical significance was calculated using two-tailed Mann Whitney *U* test for two independent samples. *$p < 0.05$.
n: Number of cells; NBF: Neutral buffered formalin; NS: Not significant; SD: Standard deviation.

Table 1. siRNA sequences used to knock-down IRF3 in human and bat cells.

Name	Human sequence	*E. fuscus* sequence
siIRF3–1 (Duplex)	5′ rGrUrGrGrArGrGrCrArGrUrArCrUrUrCrUrGrArUrArCrCCA 3′	5′ rCrArArGrArArGrCrUrArGrUrGrArUrGrGrUrCrArArGrGTT 3′
	5′ rUrGrGrGrUrArUrCrArGrArArGrUrArCrUrGrCrCrUrCrCrArCrCrA 3′	5′ rArArCrCrUrUrGrArCrCrArUrCrArCrUrArGrCrUrUrCrUrUrGrGrU 3′
siIRF3–2 (Duplex)	5′ rArCrUrGrUrGrGrArCrCrUrGrCrArCrArUrUrUrCrCrArACA 3′	5′ rCrUrGrCrCrArArCrCrUrGrGrArArGrArGrGrArArUrUrUCA 3′
	5′ rUrGrUrUrGrGrArArArUrGrUrGrCrArGrGrUrCrCrArCrArGrUrArU 3′	5′ rUrGrArArArUrUrCrCrUrCrUrUrCrCrArGrGrUrUrGrGrCrArGrGrU 3′

r: Ribose sugar.

has previously been demonstrated in yeast, *Saccharomyces cerevisiae*, where GATA transcription factor localization was influenced by formalin. Tate and Cooper suggest that varying degrees of osmotic stress and transcription factor movement in response to formalin can occur after the beginning of fixation but before proteins become immobilized [13]. Other studies have also reported that cellular localization of proteins can be influenced by the choice of fixative [14,15].

In our case, 10% NBF-fixed slides could have created a false impression of IRF3 translocation to the nucleus when infected with a coronavirus. This would have led us to different and potentially inaccurate conclusions. We do not know if all mammalian transcription factors would behave similarly in different fixatives, but it is worthwhile to be aware of these possibilities. Researchers should try different fixatives for individual cell lines to rule out the possibility of potential fixative-mediated artifacts in cell lines of mammalian origin. Microscopy images to determine cellular location of proteins must always be verified by cell fractionation and immunoblots where possible.

Author contributions

AB, DF and VM designed the experiments. AB performed the experiments. AB wrote the first draft and all authors reviewed the manuscript. DF and VM supervised the study.

Acknowledgments

We would like to acknowledge Eiko Kawamura for her help with microscopy.

Financial & competing interests disclosure

This study was funded by a Discovery Grant to VM from the Natural Sciences and Engineering Research Council of Canada. AB is funded by Saskatchewan Innovation and Opportunity and the Department of Veterinary Microbiology, University of Saskatchewan. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

Open access

References

1. Howat WJ, Wilson BA. Tissue fixation and the effect of molecular fixatives on downstream staining procedures. *Methods* 70(1), 12–19 (2014).
2. Janeway CA Jr, Medzhitov R. Innate immune recognition. *Annu. Rev. Immunol.* 20, 197–216 (2002).
3. Collins SE, Mossman KL. Danger, diversity and priming in innate antiviral immunity. *Cytokine Growth Factor Rev.* (2014).
4. Noyce RS, Collins SE, Mossman KL. Differential modification of interferon regulatory factor 3 following virus particle entry. *J. Virol.* 83(9), 4013–4022 (2009).
5. Lee MS, Kim YJ. Signaling pathways downstream of pattern-recognition receptors and their cross talk. *Annu. Rev. Biochem.* 76, 447–480 (2007).
6. Rodhain F. Bats and Viruses: complex relationships. *Bull Soc. Pathol. Exot.* 108(4), 272–289 (2015).
7. Hayman DT. Bats as viral reservoirs. *Annu. Rev. Virol.* 3(1), 77–99 (2016).
8. Banerjee A, Rapin N, Bollinger T, Misra V. Lack of inflammatory gene expression in bats: a unique role for a transcription repressor. *Sci. Rep.* 7(1), 2232 (2017).
9. Zhou P, Tachedjian M, Wynne JW *et al.* Contraction of the type I IFN locus and unusual constitutive expression of IFN-alpha in bats. *Proc. Natl Acad. Sci. USA* 113(10), 2696–2701 (2016).
10. Kumar M, Mazur S, Ork BL *et al.* Inactivation and safety testing of Middle East respiratory syndrome coronavirus. *J. Virol. Methods* 223, 13–18 (2015).
11. Banerjee A, Rapin N, Miller M *et al.* Generation and characterization of *Eptesicus fuscus* (big brown bat) kidney cell lines immortalized using the myotis polyomavirus large T-antigen. *J. Virol. Methods* 237, 166–173 (2016).
12. Paraformaldehyde in PBS. In: *Cold Spring Harbor Protocols.* Sever R (Ed.). (2006) pdb.rec9959.
13. Tate JJ, Cooper TG. Formalin can alter the intracellular localization of some transcription factors in Saccharomyces cerevisiae. *FEMS Yeast Res.* 8(8), 1223–1235 (2008).
14. Schimenti KJ, Jacobberger JW. Fixation of mammalian cells for flow cytometric evaluation of DNA content and nuclear immunofluorescence. *Cytometry* 13(1), 48–59 (1992).
15. Hobro AJ, Smith NI. An evaluation of fixation methods: Spatial and compositional cellular changes observed by Raman imaging. *Vibrational Spectroscopy* 91, 31–45 (2017).

First draft submitted: 26 December 2017; Accepted for publication: 29 May 2018

Address correspondence to: Vikram Misra; Department of Veterinary Microbiology, Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, S7N 5B4, Canada; E-mail: vikram.misra@usask.ca