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Colocalization of intracellular specific IgA (icIgA) with influenza virus in patients' nasopharyngeal aspirate cells.

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Abstract

Inhibition of viral replication by icIgA antibodies has only been observed with *in vitro* studies using epithelial cell lines in transwell cultures. This effect appears to involve an interaction between polymeric immunoglobulin A (pIgA) and viral particles within an intracellular compartment, since IgA is transported across polarized cells. Polyclonal guinea pig antisera against purified influenza A virus and mouse antisera prepared against Influenza A/H3N2 hemagglutinin (HA_0) cleavage loop peptides, were used in confocal fluorescence microscopy to show specific staining of wild-type influenza H1N1 and H3N2 viruses in clinical specimens. The HA_0 cleavage loop peptides used for intranasal immunization of mice were designed and synthesized from specific conserved regions of influenza A/H1N1 & A/H3N2 viruses. Anti-human secretory IgA antibodies were used to show co-localisation of influenza A virus and icIgA. The results showed specific immunofluorescent staining of influenza A/H3N2 (X31) (HA_0 uncleaved)-infected MDCK cells and the presence of icIgA in respiratory exudate cells of infected patients.

Both results confirm specific co-localisation and suggest interaction between influenza A virus and icIgA in patients' respiratory exudate cells. Importantly, antisera to the mouse anti-HA_0 cleavage site were specific for wild-type virus in clinical specimens, indicating that the conserved region of HA_0 was present in the uncleaved form. Similar staining and colocalization patterns between icIgA and virus were observed with polyclonal guinea pig antisera against influenza A virus. These are the first observations of co-localization of influenza A virus and intracellular IgA in clinical specimens.

Role of icIgA: This report shows the co-localization of influenza A virus HA_0 and icIgA antibodies in respiratory exudate cells of patients who were culture and viral RNA positive, suggesting that icIgA directed against the conserved HA_0 site may have a privileged and unique opportunity to act on immature virus and thus prevent HA_0 cleavage, maturation and subsequent cycles of viral replication. The precise mechanism by which icIgA mediates intracellular viral neutralization remains to be fully elucidated.

Significance: The above findings in clinical specimens would contribute strongly to our understanding of the mechanisms and kinetics of icIgA neutralization in relation to viral entry and early replication steps of mucosal viral infections. A rapid, objective and sensitive assay - by ex vivo enumeration of respiratory epithelial cells that have co-localized influenza virus and icIgA – would contribute to further mucosal immunity

studies and inform the design of more effective vaccines against influenza and other viral infections transmitted via the mucosal route e.g. respiratory syncytial virus, rotavirus.

Keywords: Intracellular IgA; Co-localisation; Influenza; Hemagglutinin; HA_0 Cleavage loop

Short Communication

Intracellular IgA and influenza virus

Prevention of influenza infection requires mucosal and serum neutralizing antibodies, especially those directed against hemagglutinin (HA) and neuraminidase (NA) epitopes (Belshe et al., 2007; Belshe et al., 2004; Brown et al., 1990; Wareing and Tannock, 2003). Different mechanisms are involved in virus neutralization that can minimize or prevent viral replication (Armstrong and Dimmock, 1992; Reading and Dimmock, 2007). One commonly accepted mechanism is interference of virus entry by antibody-mediated blocking of HA attachment to the host cell sialic acid receptor. Other possible mechanisms may involve viral uncoating and nuclear entry.

Studies on humoral immune responses to influenza have largely focused on the role of serum IgG and IgM antibodies as protection markers (Cox et al., 1994). The role of local secretory and intracellular IgA (icIgA) have been less well studied (Brandtzaeg, 2009; Reading and Dimmock, 2007), while that of secretory IgA (sIgA) antibodies in exclusion and prevention of virus-cell attachment is generally accepted.

Inhibition of infectivity of several viruses by antibodies at the intracellular level has been observed from *in vitro* studies using epithelial cell lines in transwell culture systems viz.

influenza (Mazanec, Coudret, and Fletcher, 1995a; Mazanec et al., 1992; Mazanec et al., 1995b), parainfluenza (Fujioka et al., 1998), measles (Zhou et al., 2011) and rotavirus (Corthesy et al., 2006). This appears to involve intracellular binding of virus and polymeric immunoglobulin A (pIgA) that is transported across the polarized cells (Mazanec et al., 1995b; Reading and Dimmock, 2007). The precise mechanism by which IgA mediates intracellular viral neutralization remains to be fully elucidated with *in vitro* or *in vivo* studies.

This short communication reports co-localization of influenza A virus and icIgA in respiratory exudate cells from patients who were culture and viral RNA positive for influenza A virus. We believe that these are the first observations of co-localized influenza A virus and icIgA in clinical specimens. This report provides *in vivo* evidence (from patients' nasopharyngeal aspirates - NPA) that support the earlier *in vitro* studies by Mazanec and colleagues (Mazanec et al., 1995a; Mazanec et al., 1992; Mazanec et al., 1995b).

Confocal immunofluorescence staining in nasopharyngeal aspirate (NPA) cells from influenza A infected paediatric patients

Residual NPA samples in which complete viral diagnostic tests had been completed were used for this study. The sample volumes (< 1 ml) were limited – having been used for routine viral tests - and had been stored at 4°C for more than two days, awaiting disposal according to physical containment level 2 biosafety practice.

NPA cells were obtained by centrifugation at 1000g/5 min/RT and washed twice with PBS. The cells were resuspended in PBS with twice the pellet volume. Cell suspensions (*ca.* 20 μl) were then spotted onto Teflon-coated microscopy glass slides (Thermo Fischer Scientific, Adelaide, Australia) and allowed to dry at room temperature. The cells were then fixed by immersing in cold methanol at 4°C for 10 min, dried briefly and reacted with primary antibody (diluted 1:80 in 1% skim milk in PBS) for 30 min at 37°C in a moist chamber. They were then washed using three changes of fresh PBS with constant stirring for 15 min.

The primary virus specific antibodies used were *viz.* (i) HA_0 cleavage loop antisera and (ii) polyclonal influenza A specific antisera (guinea pig). The HA_0 antisera were produced in mice that were immunized intranasally with influenza A/H3N2 (X31) HA_0 cleavage loop overlapping peptides (designed against conserved region of HA_0) and α-

galactosylceramide as adjuvant (Miller et al., 2011). Influenza A/H3N2 (X-31) strain is a reassortant of Influenza A/PR/8/34 (PR8, H1N1) with Influenza A/Aichi/2/68 (H3N2) and carries the H3N2 surface glycoproteins. The dilution of HA_0 peptide-specific antisera was close to that used to stain influenza A/H3N2 (X31) virus infected mouse lung tissues (Miller et al. 2011).

The polyclonal influenza A specific antisera (guinea pig) were produced with purified virus, characterized and validated against >8000 respiratory NPA specimens. The optimal dilution of the guinea pig antiserum used was determined by prior titration (Kok, Mickan, and Burrell, 1994; Schepetiuk and Kok, 1993). .

The secondary antibody (fluorophore-conjugated viz. FITC or Alexa Fluor 647 red) was specific for the bound primary antibodies. The secondary antibodies were diluted in accordance with the manufacturer's recommendations. In staining for co-localization, the virus specific and IgA antibodies were added together, incubated and washed as described above. After washing to remove unbound secondary antibody, one drop of ProLong Antifade reagent (Thermo Fisher Scientific) was added to the cells. A coverslip was then placed on top of the antifade reagent, avoiding air bubbles, and sealed with a

thin layer of nail varnish at the edges of the coverslip. Immunofluorescence was observed by confocal microscopy using the Bio-Rad Radiance 2100™ microscope (Bio-Rad Laboratories, NSW, Australia) equipped with 488 nm Argon-ion, HeNe 543 nm green and 637 nm red diode lasers attached to an Olympus IX70 inverted microscope. Image acquisition was performed at room temperature using LaserSharp 2000™ software (version 5.2; Bio-Rad Laboratories).

Co-localization of influenza virus and intracellular IgA in NPA cells.

NPA cells from patients with either influenza A/H3N2 or A/H1N1 infection were co-stained to detect specific virus and human icIgA (Gardner and McQuillin, 1974; Kok, Li, and Gardner, 2000). These patients were culture and PCR positive for seasonal influenza A virus (H1N1 & H3N2), as confirmed by tests reported previously (Schepetiuk and Kok, 1993; Stone et al., 2004). Intracellular IgA (icIgA) was stained (green) using FITC-rabbit-anti-human IgA (dilution 1:80) (Dako, NSW, Australia) (Fig. 1a). In Fig.1b, it is noted that the three centre cells showed specific Influenza A virus staining (red) with antisera produced against Influenza A H3N2 (X-31) HA_0 cleavage site overlapping peptide sequences (Miller et al., 2011). The labelled antibody was goat anti-mouse IgG (H+L) (1 μg/ml, Alexa Fluor 647 (red), Abcam). Fig. 1c is a phase-contrast image. Fig. 1d is a

merged image of Figs.1a and b, which shows co-localized (yellow) intracellular influenza A virus and human IgA antibodies. It is noted that in Fig. 1d, the two cells (right side and bottom) show influenza A virus staining (red) but colocalization was absent or minimal, unlike the left side cell which clearly shows colocalization of icIgA and virus (yellow). Fig. 2 shows similarly stained NPA cells from a different patient, with colocalized icIgA and influenza virus detected with mouse antisera against HA_0 cleavage site.

Similar specific immunofluorescence staining was observed with polyclonal guinea pig anti-influenza A serum that has been extensively used in our laboratory to detect different influenza A subtypes (H1N1 & H3N2) in patients' specimens (Kok et al., 2000; Kok et al., 1994). Fig. 3 shows composite confocal micrographs of stained respiratory epithelial cells from a different patient who was influenza A culture (Schepetiuk and Kok 1993) and specific viral RNA positive (Stone et al 2004). Fig. 3a shows icIgA staining (green) and influenza virus positive (red, guinea pig antisera against influenza A). Fig. 3c is a phase contrast image. Fig. 3d shows colocalized icIgA and influenza virus (yellow staining).

Figure 4 shows confocal micrographs of a respiratory columnar epithelial ciliated cell with icIgA staining (green, Fig. 4a) and influenza A virus (Fig. 4b, guinea pig antisera against influenza A, red), but there was no colocalization of virus and icIgA staining (Fig. 4d). In Figs. 4c and 4d, cilia may be observed on the left side of the respiratory columnar epithelial cell. The absence of colocalization suggests that the icIgA is not specific for influenza virus, although this was from a patient who was influenza A virus culture and specific viral RNA positive. It is noted that the cell in Fig. 4c shows specific staining (red) with polyclonal guinea pig antisera against purified influenza A virus. Figure 5 is a composite of confocal micrographs of a respiratory columnar epithelial cell (from a separate patient) that showed specific staining for icIgA but negative for influenza virus. As expected, there was no colocalization of intracellular IgA and influenza virus.

In a previous study, synthesized overlapping peptides to the HA_0 cleavage site were used for intranasal immunization of BALB/c mice. The antisera from the immunized mice interacted with the uncleaved form of HA_0 within influenza A virus-infected MDCK cells (Miller et al., 2011). Intracellular proteases are not known to cleave the HA_0 of influenza A/H3N2 (X31) or H1N1 virus. In the latter study, egg-grown Influenza A/H3N2 (X31)

virus was used to infect MDCK cells which were not treated with exogenous trypsin. The viral HA_0 is cleaved by extracellular proteases in the embryonated eggs (Klenk and Garten, 1994). Complete protection was observed in mice immunized with HA_0 peptides and subsequently challenged with Influenza A/(H3N2) (X31) virus via the intranasal route. Similar protection results were obtained when judged by estimation of viral RNA levels by RT PCR (Miller et al., 2011).

Mouse antisera against Influenza A/H3N2 HA_0 cleavage peptides showed specific staining of wild-type influenza virus A (HA_0 uncleaved) in patient's NPA cells (Fig. 1). This observation supports the results obtained with the same mouse anti-HA_0 antisera showing intracellular specific immunofluorescence staining of uncleaved HA_0 in X31-infected MDCK cells (Miller et al., 2011). The results confirm specific co-localization and suggest interaction between influenza A virus and icIgA in respiratory exudate cells from patients. Importantly, the mouse anti-HA_0 cleavage site sera stained wild type virus in clinical specimens (Figs. 1b and 2b), indicating that the conserved region of HA_0 was present in the uncleaved form (intracellular staining).

The data indicate that binding of the mouse anti-HA_0 cleavage peptide antibodies is not restricted to a single subtype of human influenza A and that the virus was accessible to endogenous IgA antibodies within infected cells. Furthermore, the same antisera showed specific interaction with newly made virus in MDCK cells, showing the presence of common antigens with the uncleaved form of HA_0 in X31 virus (Miller et al., 2011) and wild-type virus (Figs. 1b and 2b). These findings show cross-reactions between the antibodies induced by intranasal immunization with HA_0 cleavage loop peptides and intra-cellular immature heterologous influenza A strains in mice and in patients with influenza A (H1N1 & H3N2) infections.

Taken together, these results and those of the previous report (Miller et al., 2011) provide proof of principle that: (a) the early progeny virus is accessible to intracellular IgA antibodies, (b) the antigenic specificity of the HA_0 cleavage site is indeed conserved between different strains of influenza A virus and reacts with the mouse anti-HA_0 cleavage peptide antisera and (c) potentially, intracellular IgA antibodies could inhibit viral replication from proceeding to subsequent rounds of infection, including those strains that require intracellular cleavage of HA_0 e.g. highly pathogenic avian influenza

A/H5N1 (Kido et al., 2012; Oh et al., 2009). We believe that these are the first observations of co-localized influenza A virus and icIgA in patients' NPA specimens.

Measurement of icIgA neutralizing antibodies in mucosal secretions

The traditional approach used to measure humoral immunity (e.g. virus neutralization, haemagglutination inhibition or ELISA) involves detecting specific antibodies in sera, especially with viruses that cause systemic infection. Similarly, secretory antibodies may be measured for viral infections at mucosal sites e.g. influenza, respiratory syncytial virus, poliovirus, HIV or rotavirus (Brandtzaeg, 2009; Holmgren and Czerkinsky, 2005). Measurement of local secretory antibodies (predominantly sIgA) against viruses that infect mucosal sites requires collection of secretions, but the volumes obtained are variable and the procedure requires dilution of the specimen which leads to decreased test sensitivity. Additionally, the heterogeneous properties and microbial flora in secretions can cause spurious test results. The attendant problems associated with current use of mucosal secretions prevent consistent measurement for virus specific neutralizing antibodies. We believe that measurement of epithelial cells that contain virus-specific intracellular IgA will overcome the difficulties associated with current approaches used to detect extracellular secretory antibodies.

Potential use of icIgA in vaccine evaluation

When the host encounters a new strain of influenza virus with different HA epitopes, pre-existing antibodies may not be sufficient to inhibit or prevent cell entry. Clearly, a conserved epitope that is accessible to neutralizing antibodies is the goal of a universal influenza vaccine (Krammer and Palese, 2015; Margine et al., 2013; Wilks et al., 2012). The above observation between influenza virus interaction with specific icIgA in patients' respiratory specimens will facilitate studies to identify critical viral antigenic epitopes that are recognized by the patients' mucosal immune system to produce potentially neutralizing specific icIgA. This report with clinical specimens showed that icIgA against the conserved HA_0 site has a privileged and unique opportunity to act on immature virus (human and avian influenza strains). This would prevent HA_0 cleavage, maturation and subsequent "second round" viral replication and spread to other cells. Taken together, the results in this report and Miller et al. will inform more effective vaccine design as well as studies with triggering innate and mucosal immunity (Geijtenbeek and Gringhuis, 2013; Lorin et al., 2017; Miller et al., 2011).

Identification of these antigenic epitopes is necessary to enhance viral vaccine designs that will induce specific neutralizing IgA antibodies against influenza, respiratory syncytial virus, rotavirus and other viral infections transmitted via the mucosal route. These findings will provide more detailed understanding of the mechanisms and kinetics of icIgA neutralization in relation to viral entry and early replication steps with a specific focus on mucosal viral infections.

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Figure 1

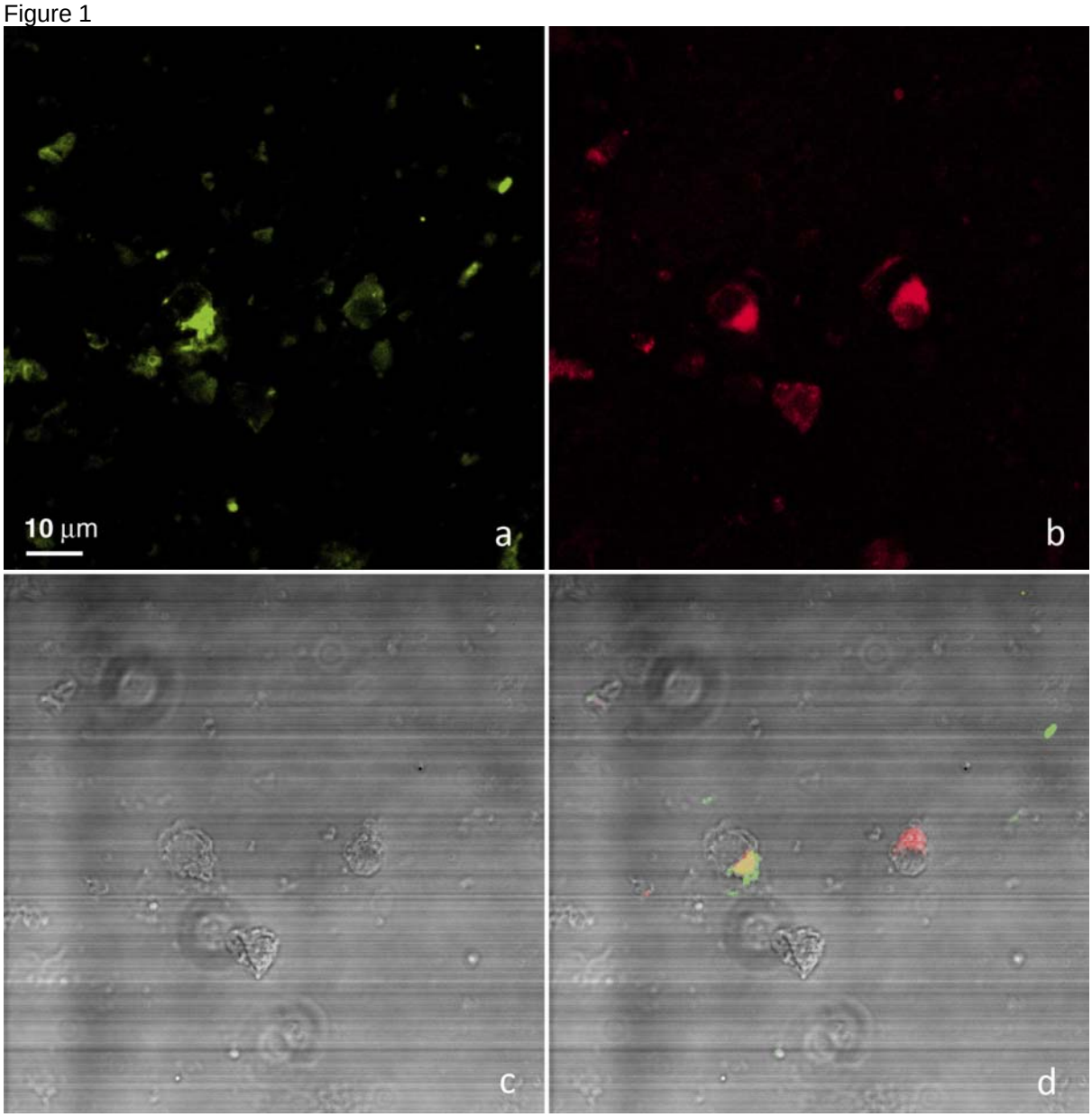

Figure 2

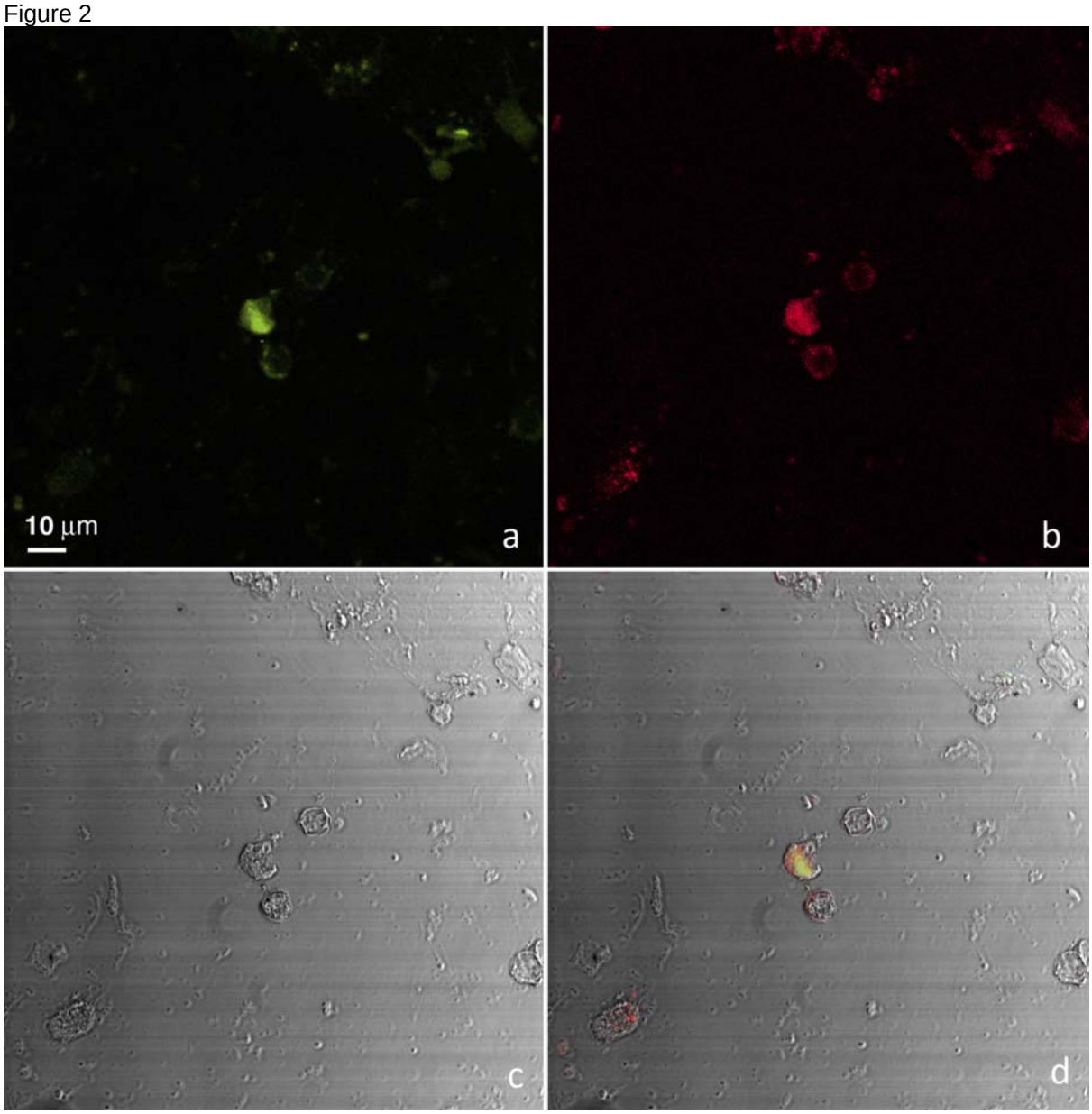

Figure 3

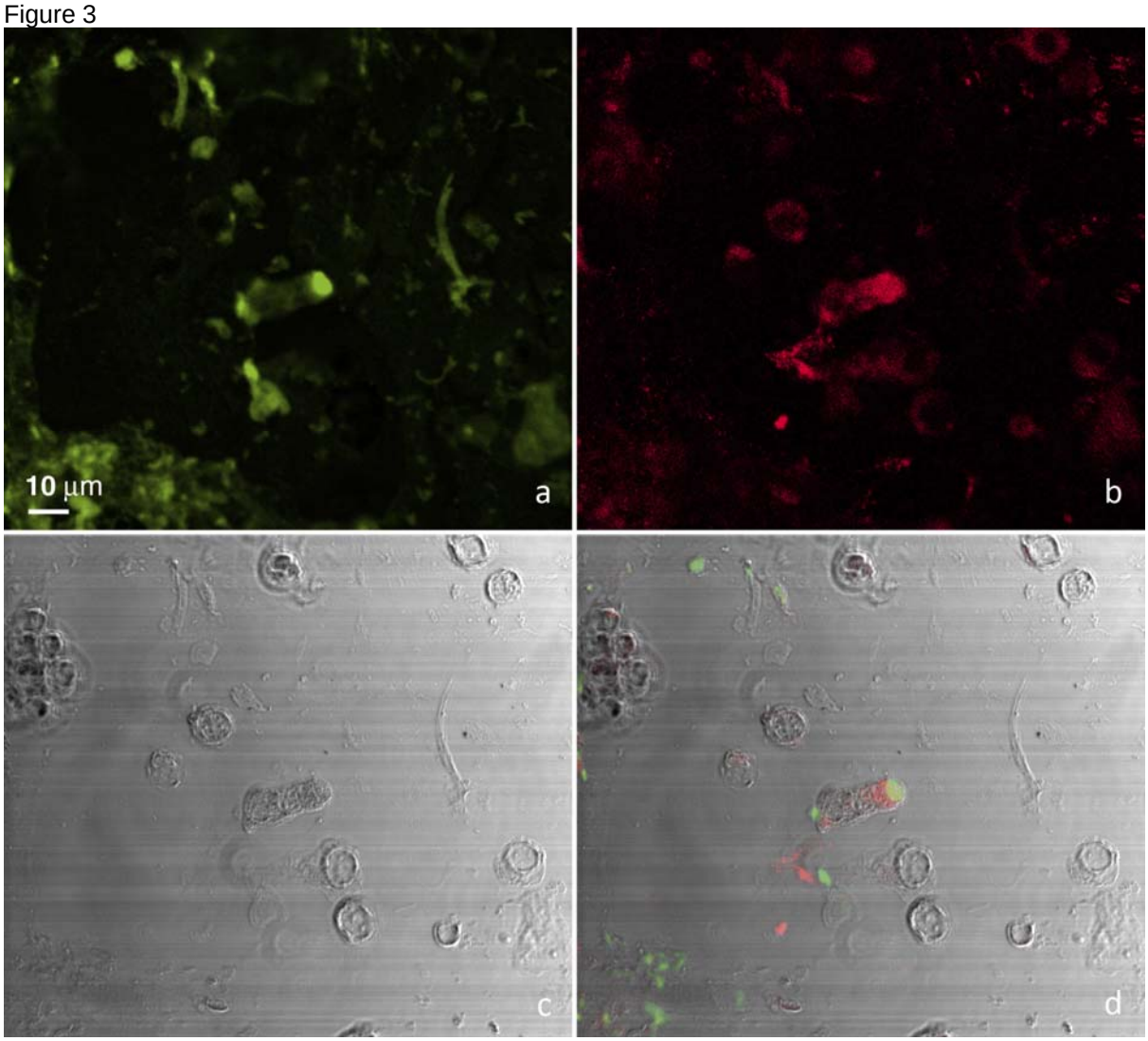

Figure 4

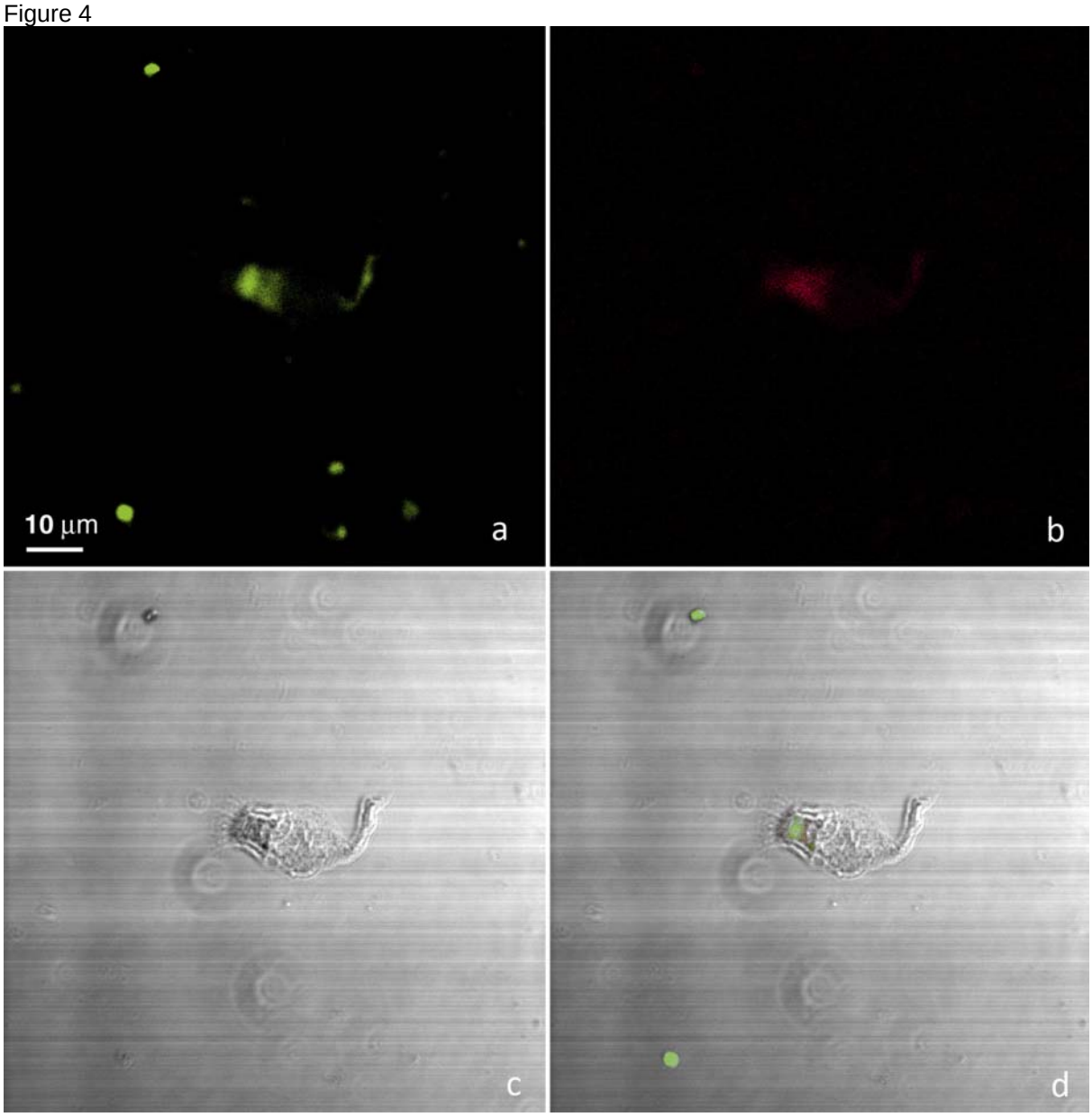

Figure 5