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Cell-based analysis of Chikungunya virus membrane fusion using baculovirus-expression vectors

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Abstract

Chikungunya virus infection has emerged in many countries over the past decade. There are no effective drugs for controlling the disease. To develop cell-based system for screening anti-virus drugs, a bi-cistronic baculovirus expression system was utilized to co-express viral structural proteins C (capsid), E2 and E1 and the enhanced green fluorescence protein (EGFP) in *Spodoptera frugiperda* insect cells (Sf21). The EGFP-positive Sf21 cells fused with each other and with uninfected cells to form a syncytium, allowing characterization of cholesterol and low pH requirements for syncytium formation. Western blot analysis showed three structural proteins were expressed in baculovirus infected cells. The structural proteins of Chikungunya virus that is required for cell fusion was determined with various recombinant baculoviruses bearing different lengths of the viral structural protein genes. Protein E1 was required for cell fusion and indicating that Chikungunya viral membrane fusion was a class II membrane fusion. It was also demonstrated that the heterologous expression of alphavirus monomeric E1 can induce insect cell fusions. Furthermore, this cell-based system provides a model for studying class II viral membrane fusion.

Key words: baculovirus; Chikungunya virus; internal ribosome entry site; syncytium; viral membrane fusion

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1. Introduction

Chikungunya virus (CHIKV) belongs to the *Alphavirus* of *Togaviridae* family. It is transmitted by *Aedes* mosquitoes and causes the disease known as Chikungunya fever. Chikungunya typically involves an acute phase with fever and skin rashes, followed by painful arthralgia that can last for months. CHIKV epidemics were reported in the Democratic Republic of Congo in 2000 (Pastorino et al., 2004), Indonesia during 2001–2003 (Laras et al., 2005) and India during 2005–2006 (Yergolkar et al., 2006). CHIKV has also emerged as an important infection in South-East Asia and in the Pacific regions (Mackenzie et al., 2001; Thaikruea et al., 1997). Because there is no effective antiviral treatment for Chikungunya fever, understanding the pathogenesis of virus infection is important.

CHIKV is an enveloped virus with a positive-stranded RNA genome of 11,826 nucleotides (CHIKV S27 strain, AF369024). Its genome contains two open reading frames (ORFs) embedded between the non-translated regions, 5'UTR and 3'UTR. Based on studies of other alphaviruses and sequence similarity (Solignat et al., 2009), the first ORF located at the 5'-end encodes a polyprotein precursor of nonstructural proteins (nsP1, nsP2, nsP3 and nsP4), which function in viral replication. The second ORF encodes the polyprotein of the structural proteins (C, E2 and E1), which form the viral nucleocapsid and envelope. The C–pE2–6k–E1 polyprotein is encoded by the 26S

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subgenomic RNA. Co-translational and post-translational cleavage results in mature structural proteins of Semliki forest virus (SFV) during viral replication (Liljestrom and Garoff, 1991). Autoproteolytic serine proteinase releases SFV capsid from the N-terminus of the nascent polyprotein (Melancon and Garoff, 1987). The envelope polyprotein that inserts into the endoplasmic reticulum (ER) bilayer through an N-terminal signal sequence is processed into the pE2 and E1 proteins, which are associated with each other and translocated to the plasma membrane (Strauss and Strauss, 1994). The SFV 6K short peptide sequence serves as a signal sequence for the E1 protein to insert into the ER membrane (Liljestrom and Garoff, 1991). During transport, and probably just before its arrival at the cell surface, pE2 is cleaved by host furin or proteinases into E2 and E3 (Ozden et al., 2008; Zhang et al., 2003).

Previous studies of SFV and Sindbis virus (SINV), two well-characterized alphaviruses, revealed that alphavirus infection is established through clathrin-dependent endocytosis. Delivery of SFV RNA genomes into the cytoplasm is via a membrane fusion event which is triggered by endosomal low pH (Kielian et al., 1990). Viral membrane fusion mediated by viral fusion protein is a critical step of enveloped virus infection for releasing viral genome into host cell (Harrison, 2008). On the alphavirus infection, this membrane fusion event is mediated by the E1 glycoprotein, a class II fusion protein (Klimjack et al., 1994; Omar and Koblet, 1988;

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Sanz et al., 2003). During fusion, a low-pH environment in the endosome induces a conformational change of the SFV envelope proteins, dissociation of the E2–E1 heterodimers , and E1 trimerization (Sanchez-San Martin et al., 2009; Wahlberg et al., 1992; Wahlberg and Garoff, 1992). The crystal structures of SINV and CHIKV envelope proteins have been revealed (Li et al., 2010; Voss et al., 2010). The extended intermediate of the E1 protein inserts into the target membrane via its hydrophobic fusion peptide, becomes trimerized, and refolds to form a hairpin-like structure (Bressanelli et al., 2004; Gibbons et al., 2004). Low-pH dependant cell-cell fusion has been observed with both alphavirus- (Boggs et al., 1989; Kempf et al., 1987; Sanz et al., 2003; Wahlberg and Garoff, 1992) and flavivirus-infected cells (Randolph and Stollar, 1990). In addition to the low-pH dependence for viral membrane fusion, cholesterol is required for both fusion and budding during alphavirus infection (Chatterjee et al., 2000; Kielian and Helenius, 1984; Smit et al., 1999; White and Helenius, 1980). However, CHIKV infection is established through Eps15-dependent endocytosis (Bernard et al., 2010) and whether the requirements for CHIKV membrane fusion are similar to other alphaviruses is not known.

In the present study, a cell-based assay system has been developed to understand the mechanism of CHIKV-mediated membrane fusion. A bi-cistronic baculovirus expression system (Chen et al., 2005) that co-expressed CHIKV structural proteins and

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enhanced green fluorescence protein (EGFP) was used to obtain EGFP-positive Sf21 cells expressing E1 and E2 on the cell surface. These EGFP-positive cells were then used to characterize the requirements for cell fusion. It is shown here that CHIKV E1 protein alone is able to induce cell fusion which is dependent on low pH and the presence of cholesterol. Similar findings have been reported with SFV and SINV (Boggs et al., 1989; Chanel-Vos and Kielian, 2004; Kempf et al., 1987; Lanzrein et al., 1993). Those results suggest that the insect cell fusion induced by baculovirus–based expression of the monomeric E1 protein can be used to screen for anti-CHIKV inhibitors.

2. Materials and methods

2.1. Cells, viruses, and transfection

The *Spodoptera frugiperda* IPBL-Sf9 (Sf9) and IPBL-Sf21 (Sf21) cell lines (Grace, 1962) were cultured in either TNM-FH or Sf-900 II insect medium that contained 8% heat-inactivated fetal calf serum (FCS) at 27°C (Wu et al., 2000). Preparation of viral stocks and determination of viral titers followed previously described standard protocols (O'Reilly et al., 1992). Cellfectin (Invitrogen, Carlsbad, CA, USA) was used for transfection according to the manufacturer's protocol.

2.2. Construction of transfer vectors

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DNA preparations and manipulations were performed using standard methods (Sambrook et al., 1989) or protocols provided by the manufacturers of the reagents. An established bi-cistronic baculovirus transfer vector, pBac-Rhir-E (Fig. 1A), containing the RhPV 5'UTR IRES (Chen et al., 2005) was used as a backbone for construction. The cDNA for the 26S subgenome of the Chikungunya virus (CHIKV 26S RNA) was cloned into the multiple cloning site (MCS) between the polyhedrin promoter and the RhPV-IRES-EGFP sequence. Briefly, the cDNA fragment containing the full-length structural gene (7559-11293) of Chikungunya virus strain S27-African prototype (AF369024) was first cloned into the *Bam*HI and *Eco*RV sites of the pcDNA4/HisMax A vector (Invitrogen, Carlsbad, CA, USA) to create the plasmid, pcDNA4/HisMax A-CHIKV-26S. Subsequently, the 3.7-kb *Bam*HI-*Xba*I fragment from pcDNA4/HisMax A-CHIKV-26S was subcloned into the *Bgl*II and *Xba*I sites of pBac-Rhir-E, and the resulting plasmid was named pBac-CHIKV-26S-Rhir-E (Figure 1B). Two truncated forms of the CHIKV 26S cDNA were also cloned into the pBac-Rhir-E. The plasmid pBac-CHIKV-6K-E1-Rhir-E (Fig. 1C), which includes the *6K-E1* gene of 26S cDNA, was constructed by replacing a 3.7-kb *Nhe*I-*Not*I fragment of pBacCHIKV-26S-Rhir-E with a 1.8-kb *Nhe*I-*Not*I fragment that was amplified by PCR using pBacCHIKV-26S-Rhir-E as a template and a pair of primers (Forward: 5'-AGGGCTAGCATGGCAGTGGGGATGTGCATG-3'; Reverse:

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5'-GTGTGCGAAGGGATTATAGG-3'). The plasmid pBac-CHIKV-26S-ΔE1-Rhir-E (Fig. 1D), which contained an *E1* deletion, was derived by replacing a 2245-bp *Sac*II-*Xba*I fragment of pBac-CHIKV-26S-Rhir-E with a 769-bp *Sac*II-*Xba*I fragment that was amplified by PCR using pBac-CHIKV-26S-Rhir-E as a template and a pair of primers (Forward: 5'-AAAAAGGATCCGACAACTTCAATGTCTATAAAGCCA-3'; Reverse: 5'-AGGTCTAGAACGCGCTCACAGTGTGG-3'). All construct sequences were confirmed by DNA sequencing.

2.3. Recombinant virus production, titer determination and serial passage

Using Cellfectin (1 µl), Sf21 cells were seeded at 2 x 10^5 cells/well in a 24-well plate and co-transfected with 0.25 µg of the linearized viral DNA Bac-N-Blue (Invitrogen, Carlsbad, CA, USA) and 0.8 µg of the plasmids, pBac-CHIKV-26S-Rhir-E, pBac-CHIKV-S26-ΔE1-Rhir-E or pBac-CHIKV-6K-E1-Rhir-E, respectively. Recombinant viruses were collected from Sf21 cell cultures emitting green fluorescence under a fluorescence microscope (Nikon, Tokyo, Japan). These recombinant viruses were purified by a series of three end-point dilutions, and the resulting viruses were named vAc-CHIKV-26S-Rhir-E, vAc-CHIKV-6K-E1-Rhir-E and vAc-CHIKV-S26-ΔE1-Rhir-E, respectively. The viral titer was determined by end-point dilution and fluorescence detection in a 96-well plate and was calculated according to the 50% tissue culture infectious dose (TCID50)

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method (Reed, 1938).

2.4. Preparation of rabbit anti-sera against CHIKV E1, E2 and the whole virion

A 794-bp *Nco*I-*Sal*I fragment (AF369024, 10071-10865) of the *E1* gene and a 789-bp *Bam*HI-*Hin*dIII fragment (AF369024, 8551-9340) from pcDNA4/HisMax A-CHIKV-26S were subcloned into the same restriction enzyme sites of pET32a. Ligation mixtures were used to transform competent TOP10 *E. coli* cells (Invitrogen, Carlsbad, CA, USA) and to obtain expression clones, termed pET32a-PE1 and pET32a-PE2, respectively.

Single colonies harboring either pET32a-PE1 or pET32a-PE2 were grown in Luria Bertani (LB)/ampicillin broth and induced by the addition of 0.5 mM isopropyl-β-D-1-thiogalactopyranoside, when the culture reached an O.D.600 nm of approximately 0.5, for 3 hours at 37°C. Cells were pelleted, resuspended in a 1 M urea solution, disrupted by sonication and then centrifuged at 12,000 x g for 30 minutes. The resulting pellets, which contained inclusion bodies, were washed with 1 M urea solution twice and then resolved in an 8 M urea solution. Samples containing recombinant E1 or E2 proteins were ultra-centrifuged at 40,000 rpm in a 70Ti rotor for 1 hour and then filtered though a 0.22 μm filter. The Äkta FPLC system was used to purify recombinant proteins using a HiTrap HP column containing Ni-Sepharose resin (Amersham Biosciences, Little Chalfont, United Kingdom). Eluted fractions that

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showed no traces of contaminating proteins as measured by SDS-PAGE were collected and dialyzed in 0.5 L of dialysis buffer (10 mM Tris-HCl (pH 7.4), 0.15 M NaCl, 0.1% Triton X-100, and 10% glycerol) at 4℃, with the dialysis buffer being replaced 4 times at 6-hour intervals. The final concentrations of the recombinant proteins were determined by the Bradford method using bovine serum albumin as a standard (Bradford, 1976).

BHK-21 cells were infected with Chikungunya virus (S27-African prototype strain) at a multiplicity of infection (M.O.I.) of one and then cultured for two days in Dulbecco's Modified Eagles' Medium (DMEM) (Invitrogen-GIBCO, Carlsbad, CA, USA) without serum. CHIKV was inactivated by treatment of the harvested medium with 0.05% formaldehyde at 4°C overnight. A 400 ml inactivated culture supernatant was first centrifuged at 12,000 x g for 30 minutes to remove cell debris; this was followed by ultra-centrifugation at 20,000 rpm in a 45Ti rotor for 24 hours to obtain concentrated CHIKV. The inactivated CHIKV suspension obtained by dissolving the final pellet in 3 ml PBS (phosphate buffered saline) was used for raising antibodies for Western blot analysis.

The inactivated CHIKV suspension and recombinant proteins E1 and E2 were used to raise antibodies in New Zealand White rabbits. In brief, rabbits were primary immunized by subcutaneous injection with 0.5 ml inactivated CHIKV suspension or

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500 µg of recombinant protein in Freund's complete adjuvant (Sigma, St. Louis, MO, USA). After one month, rabbits were boosted three times at 15-day intervals using 0.25 ml inactivated CHIKV suspension or 100 µg of recombinant protein in Freund's incomplete adjuvant (Sigma, St. Louis, MO, USA). Anti-sera against whole CHIKV, E1 or E2 were obtained after completing the immunization program.

2.5. Western blot analysis

Sf21 cells were seeded at 2 x 10^5 cells/well in a 24-well plate and infected with recombinant viruses at a M.O.I. of 10. A 30 µg/well total proteins harvested at 2 dpi (day post infection) were resolved in Laemmli sample buffer and separated onto an SDS-PAGE (10%). After SDS-PAGE separation, proteins were electrotransferred onto a PVDF membrane (polyvinylidene difluoride; Millipore, Billerica, MA, USA). The resulting membrane was blocked with Tris-buffered saline (TBS; 100 mM Tris (pH 7.4), 100 mM NaCl, and 0.1% Tween 20) containing 5% (v/v) non-fat dry milk at room temperature for 1 hour with gentle shaking. Subsequently, the membrane was incubated with a 1:2,000 dilution of anti-E1, anti-E2 or anti-CHIKV antibodies in TBS with 5% (v/v) non-fat dry milk at 4°C overnight. Unbound antibodies were removed by three washes of 5 minutes each in TBS buffer at room temperature with shaking. The membrane was then incubated with a 1:2,500 dilution of horseradish peroxidase (HRP)-conjugated secondary antibodies (Chemicon, Billerica, MA, USA) for 1 hour at

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room temperature. HRP on the membrane was detected by an enhanced chemiluminescence kit (Pierce, Rockford, IL, USA) following the protocol provided by the manufacturer. The UVP AutoChemi Image System was used for capturing and processing the various images.

2.6. Cell fusion assay

1 x 10^5 Sf21 cells/well were seeded in 96-well plates and infected with recombinant baculoviruses at an M.O.I. of 10; after infection cultures were maintained in Sf-900 II (pH 6.4) containing 2% FCS. After 1 dpi, the culture medium was replaced with Sf-900 II containing 2% FCS with either a variety concentration of cholesterol at pH 5.8 or different pH levels (from 5.8 to 6.6) at 27°C. The syncytial formation was then examined under a fluorescence microscope at 2 dpi.

2.7. Cell fusion inhibition by rabbit anti-whole CHIKV serum

1 x 10^5 Sf21 cells/well were seeded in 96-well plates and infected with recombinant baculovirus of vAc-CHIKV-26S-Rhir-E at an M.O.I. of 10; after infection cultures, were maintained in Sf-900 II (pH 6.4) containing 8% FCS. After 1 dpi, cells were incubated with different dilutions (1:200, 1:400, 1:800, 1:1,600 and 1:3,200) of rabbit anti-whole CHIKV serum in above medium for 1 hour at 27□, following which the medium was replaced with Sf-900 II (pH 5.8) containing 2% FCS, 0.1 mg/ml cholesterol (Sigma, St. Louis, MO, USA) and a specific diluted serum. Two day later,

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the samples were then examined and photographed under an inverted fluorescence microscope Olympus IX71 (OLYMPUS, Tokyo, Japan).

2.7. Co-culture of infected and uninfected cells

Infected Sf21 cells (designated as expression cells) co-expressing EGFP and CHIKV structural proteins and emitting green fluorescence were cultured in Sf-900 II (pH 6.4) plus 2% FCS. Uninfected cells (designated as target cells) were labeled with the red fluorochrome probe CellTracker RED CMPTX (Invitrogen, Molecular Probes, Carlsbad, CA). In brief, the suspended target cells were stained with 100 μg/ml of CellTracker RED CMPTX in Sf-900 II for 45 minutes , washed with Sf-900 II to remove free dye, and then incubated for additional one hour at 27°C in Sf-900 II (pH 6.4) containing 2% FCS. The expression cells were mixed by pipeting with an equal number of target cells in Sf-900 II (pH 5.8) containing 0.2 mg/ml cholesterol . After an additional 2-hour incubation, cells were examined and photographed with a Leica TCS SP2 AOBS confocal microscope (Leica. Microsystems, Heidelberg GmbH, Germany) using various fluorescence excitation and emission settings to detect EGFP and RED CMPTX.

2.8. Cholesterol depletion and replenishment

1 x 10^5 Sf21 cells/well were seeded in 96-well plates and infected with recombinant baculovirus of vAc-CHIKV-26S-Rhir-E at an M.O.I. of 10; after infection,

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cultures were maintained in Sf-900 II (pH 6.4) with 2% FCS. After 1 dpi, the infected cells were treated with 4.5 mM methyl-β-cyclodextrin (MβCD) (Sigma, St. Louis, Mo, USA) in Sf-900 II for 1 hour at 27°C, and then culture medium was replaced with either Sf-900 II (pH 5.8) or Sf-900 II (pH 5.8) containing 0.15 mg/ml cholesterol for replenishment. The cell fusion activity was examined under a fluorescence microscope Olympus IX71 at 2 dpi.

2.9. Immunofluorescence microscopy

For the surface E1 or E2 proteins staining, Sf21 cells were infected with recombinant baculoviruses at a M.O.I. of 1 in Sf-900 II (pH 6.4) containing 2% FCS. Two dpi, cells were fixed with 3% formaldehyde and stained with either rabbit anti-CHIKV E1 or E2 sera for 30 minutes at room temperature. After washing twice with cold PBS, cells were incubated with the secondary antibody, Alexa Fluor 546-conjugated goat anti-rabbit IgG (Invitrogen, Molecular Probes, Carlsbad, CA), at a dilution of 1:10,000 for 30 minutes at room temperature and then washed twice with cold PBS.

In the living cell immuno-staining for localization of CHIKV envelope proteins (E1 and E2) on the syncytial cell surface, EGFP-expressing cells which were infected by recombinant baculovirus of vAc-CHIKV-26S-Rhir-E were incubated within Sf-900 II (pH 5.8) containing 0.15 mg/ml cholesterol for 1 hour, and then stained with rabbit

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anti-whole CHIKV serum at a dilution of 1:800 in Sf-900 II for 30 minutes at 27□. After washing twice with Sf-900 II, cells were incubated within 10 μM Hoechst 33342 for nuclear staining and with the secondary antibody, Alexa Fluor 546-conjugated goat anti-rabbit IgG at a dilution of 1:10,000 for 30 minutes at 27□. Cells were then washed twice with Sf-900 II. All samples were examined and photographed under an inverted fluorescence microscope Olympus IX71.

3. Results

3.1. Characterization of CHIKV structural proteins expressed in baculovirus infected Sf21 cells

The full-length cDNA of the 26S subgenomic RNA from the Chikungunya viral strain S27-African prototype (AF369024), corresponding to nucleotide residues 7559-11293, was cloned into a bi-cistronic baculovirus transfer vector, pBacRhir-E (Fig. 1A), and the resulting transfer vector was named pBac-CHIKV-26S-Rhir-E (Fig. 1B). The expression of three CHIKV structural proteins, the capsid (C), E2 and E1 proteins, in vAc-CHIKV-26S-Rhir-E-infected Sf21 cells was verified by Western blot analysis. The mobilities of the C, E2 and E1 proteins from vAc-CHIKV-26S-Rhir-E infected S21 cells were similar to those of the authentic proteins from purified CHIKV (Fig. 2A), demonstrating that a complete cleavage of the precursor of CHIKV structural proteins occurs in recombinant baculovirus infected Sf21 cells. The surface

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localization of the E1 and E2 proteins was examined by staining the intact EGFP-positive Sf21 cells with either anti-E1 or anti-E2 antibodies and then observed under a fluorescence microscope. Both E1 and E2 were present on the cell surface (Fig. 2B). These results show CHIKV structural proteins generated by recombinant baculoviruses can be processed successfully inside the infected-Sf21 cells and displayed both envelope proteins of E1 and E2 on the cell surface.

3.2. Insect cells infected by vAc-CHIKV-26S-Rhir-E induce syncytium formation

Co-expression of EGFP with CHIKV structural proteins from the bi-cistronic baculovirus expression vector not only simplified the purification of recombinant baculoviruses, i.e, vAc-CHIKV-26S-Rhir-E, but also made detection of the infected cells easier. Unexpectedly, examination of recombinant baculovirus infected cells under a fluorescent microscope revealed that vAc-CHIKV-26S-Rhir-E-infected Sf21 cells underwent cell fusion. Polykaryons were specifically present in Sf21 cells infected with the recombinant baculovirus vAc-CHIKV-26S-Rhir-E (Fig. 2B and Fig. 3A, left panel) but not in those infected with the control baculovirus, vAc-Rhir-E (Fig. 3A, right panel). Sf21 cells infected with vAc-Rhir-E did not induce syncytium suggesting that the expression of the three CHIKV structural proteins maybe necessary for syncytium formation. To investigate whether this fusion event was induced through the expression of the CHIKV structural proteins, the vAc-CHIKV-26S-Rhir-E-infected

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Sf21 cells were pre-incubated with anti-serum against whole CHIKV particles. The result shows cell-cell fusion was blocked by anti-CHIKV serum in vAc-CHIKV-26S-Rhir-E-infected Sf21 cells (Fig. 3A, middle panel) but not by the treatment with pre-immune or unrelated sera (data not shown). The inhibition of cell fusion was dependent on the concentrations of anti-CHIKV serum (diluted into 200 to 3,200 folds) (Fig. 3B).

In the next experiment, vAc-CHIKV-26S-Rhir-E-infected Sf21 cells expressing EGFP (Fig. 3C, left panel) were co-cultured with uninfected Sf21 cells labeled with the fluorochrome probe CellTracker (Fig. 3C, middle panel), which emitted red florescence. After a 2-hour incubation, cells containing multiple nuclei and emitting both green and red florescence were observed by confocal microscopy (Fig. 3C, right panel). To determine whether the CHIKV envelope proteins were displayed on the surface of syncytial cells, a living cell immuno-staining with rabbit anti-whole CHIKV serum follow by Alexa Fluor 546-conjugated goat anti-rabbit IgG was carried out. Fluorescence microscopy showed that the syncytial cells emitting green fluorescence (Fig. 3D; upper left panel) also revealed red fluorescence on the cell surface (Fig. 3D; upper right panel) indicating that the envelope proteins of CHIKV were displayed on the plasma membrane (Fig. 3D; lower right panel). In contrast, the EGFP free cells neither emitted red fluorescence nor formed polykayrons (Fig. 3D; lower panel). The

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syncytium formation observed in vAc-CHIKV-26S-Rhir-E-infected Sf21 cells thus occurs most likely through the expression of the E1 and E2 proteins on the cell surface.

3.3. Syncytium formation is pH- and cholesterol-dependent

Previous studies indicated that the E1 proteins of SFV and SINV, members of are class II fusion proteins implying that the fusion they cause is dependent on a low-pH level and on the presence of cholesterol (Kielian and Rey, 2006, Smit et al., 1999). To determine whether the fusion of vAc-CHIKV-26S-Rhir-E-infected Sf21 cells fits the features of alphavirus-induced fusion, cells were treated with different pH levels, ranging from 5.8 to 6.6, in the presence of 2% FCS and 0.2 mg/ml cholesterol. Sf21 cells infected by vAc-CHIKV-26S-Rhir-E were able to fuse at pH levels ≤ 6.4, with the highest fusion activity at pH 5.8, but could no longer fusion at a pH level of 6.6 (Fig. 4). Thus, the pH threshold for Sf21 cell fusion induced by vAc-CHIKV-26S-Rhir-E infection was approximately pH 6.4.

In previous studies on membrane fusion event about SFV and SIN, the low-pH-dependent fusion of both viruses is strongly enhanced by the presence of cholesterol in the virus-liposome fusion assay (Kielian and Helenius, 1984, Smit et al, 1999, White and Helenius, 1980). To determine whether cholesterol is required for cell fusion, vAc-CHIKV-26S-Rhir-E-infected Sf21 cells were incubated with Sf-900 II SFM (pH 5.8) containing 2% FCS and 0.2 mg/ml to 0.002 mg/ml of cholesterol. The

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cell fusion activity was enhanced in proportion to the concentration of cholesterol added (Fig. 5A). To confirm the role of cholesterol in cell fusion, the cells were grown under cholesterol-depleted conditions by addition of MβCD as described, after which cholesterol was added to specific concentration. Although the cell fusion was observed under condition of without further treatment (Fig. 5B, left panels), cell fusion was blocked completely when the cells were depleted of cholesterol (Fig. 5B, middle panels). After the repletion of 0.15 mg/ml cholesterol, the strong fusion activities were restored (Fig. 5B, right panels). Taken together, these observations indicate that CHIKV induced cell fusion is pH- and cholesterol-dependent. In the presence of 0.2 mg/ml of cholesterol at pH 5.8, cell fusion of infected cells proceeded rapidly as demonstrated by time-lapse photography. The onset of cell fusion occurred after just 30 minutes of incubation and the fusion was complete by 180 minutes (Fig. 6).

3.4. *Expression of 6K-E1 can induce cell syncytium formation*

The E1 and E2 proteins of several alphaviruses form heterodimers on the viral envelope, with the E1 protein playing a fundamental role in viral membrane fusion. To determine whether the CHIKV E1 protein is critical for the induction of cell fusion, two recombinant baculoviruses (Figs. 1 C and D) carrying partial deletions of the CHIKV structural genes were generated. The first deletion resulted in a vector that expressed 6K sequence and E1 protein proteins (Fig. 1C). The 6K sequence is known

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to serve as a signal sequence for SFV E1 protein (Liljestrom and Garoff, 1991). The second recombinant virus, vAc-CHIKV-26S-ΔE1-Rhir-E encodes C and E2, but contains an *E1* deletion (Fig. 1D). The protein expression profiles and cell fusion abilities of Sf21 cells infected with these three baculoviruses were verified by Western blot analyses and fluorescence microscopy. Figure 7A of Western blot shows that the E1 protein was expressed correctly in both vAc-CHIKV-26S-Rhir-E (lane 1) and vAc-CHIKV-6K-E1-Rhir-E-infected Sf21 cells (lane 3) but not in vAc-CHIKV--26S-ΔE1-Rhir-E (lane 2) or vAc-Rhir-E-infected cells (lane 4). Cells infected with vAc-CHIKV-26S-ΔE1-Rhir-E did not fuse, whereas cells infected with vAc-CHIKV-6K-E1-Rhir-E which expressed E1 protein only showed a clear evidence of fusion (Fig. 7B). The other protein bands appeared in the Western blot (Fig. 7A) may represent the non-specific binding of the anti-E1 polyclonal antibodies to the cell extracts of recombinant baculoviruses infected Sf21 cells. Thus, the E1 protein of CHIKV is necessary and sufficient to induce fusion at infected Sf21 cells.

4. Discussion

In this study, a baculovirus bi-cistronic expression system was used to demonstrate the co-expression of EGFP and CHIKV structural proteins in Sf21 cells and induction of Sf21 cell fusion. Advantages of co-expression of EGFP are to facilitate the identification of recombinant baculoviruses and the determination of viral titer (Chen

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et al., 2005). More importantly, it made it possible to analyze cell fusion events and to demonstrate CHIKV membrane fusion requires low pH and cholesterol.

One might argue that the observed Sf21 cell fusion is not mediated by CHIKV proteins but by baculovirus gp64 protein since gp64 protein can induce insect cell fusion (Blissard and Wenz, 1992). Two lines of evidence support that Sf21 cell fusion is induced by CHIKV proteins but not baculovirus gp64. First, induction of cell fusion by baculovirus gp64 protein occurs only at pH of 5.5 or lower while Sf21 cells expressing CHIKV proteins fuse with other Sf21 cells, no matter expression of CHIKV proteins or not, can occur at pH of 6.4 (Fig. 4). Second, Sf21 cells infected by the control recombinant baculovirus, vAc-Rhir-E, which expressed EGFP but no CHIKV proteins did not undergo cell fusion at the same culture media condition (Fig. 3A, right panel). Furthermore, Sf21 cell fusion was blocked proportionally by adding various dilutions of anti-CHIKV sera (Fig. 3B), indicating that the cell fusion event was mediated specifically by the CHIKV proteins but not the baculovirus. However, weaker fusion ability at pH of 5.6 was observed at vAc-Rhir-E-infected Sf21 cells when comparing with vAc-CHIKV-S26-Rhir-E-infected Sf21 cells (data not shown). The successful induction of fusion between infected Sf21 cells is attributed to the proper cleavage of the CHIKV polyprotein into individual structural proteins (Fig. 2A) and the proper targeting of the E1 and E2 proteins to the cell surface (Fig. 2B).

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Syncytium formation in Sf21 cells infected with the recombinant baculovirus expressing only the 6K-E1 protein further indicated that the 6K-E1 sequence alone is sufficient for cell fusion (Fig. 7). The analysis of viral membrane fusion has been done by virus-liposome interaction assay (Kielian et al., 1996), in which purified and labeled viruses are incubated with liposomes followed by either ultracentrifugation or immunoprecipitation and protein quantitation. These methods require virion purification and are more time-consuming and labor-intensive than the cell-based analysis developed in this study. Because the Sf21 cell is a cholesterol auxotroph and grows in a mildly acidic medium covering the pH threshold of CHIKV membrane fusion, the method described here provides a way to manipulate and study the activity of class II viral fusion without using viral particles; furthermore it can be performed under facilities of low-level bio-safety. In addition, it may be valuable for screening agents for their ability to block viral membrane fusion by CHIKV infection. Others have been shown or discussed that inhibition of viral membrane fusion using peptides, small molecules or neutralizing antibodies are effective prevent viral infections (Gollins and Porterfield, 1986; Kilby and Eron, 2003; Nybakken et al., 2005; Skehel and Wiley, 2000; Zwick, 2005; Sanchez-San Martin et al., 2009).

In summary, the results presented here show that the expression of CHIKV structural proteins by recombinant baculoviruses can induce fusion of insect cell.

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Furthermore, it is shows that the CHIKV E1 protein is necessary and sufficient for syncytium formation. This fusion event is low pH- and cholesterol-dependent that has been observed with other alphaviruses. In addition, this insect cell-based system may serve as a tool for studying class II viral membrane fusion, for insights into the process of CHIKV infection.

Acknowledgements

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Figure legends

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Fig. 1. Schematic presentation of the recombinant baculovirus transfer vectors. (A) The bi-cistronic baculovirus transfer vector pBac-Rhir-E, in which the RhPV 5'-UTR IRES is located between the six MCS cloning sites (*Nhe*I, *Bgl*II, *Pst*I, *Kpn*I, *Xba*I, and *Eco*RI) and the EGFP genes. (B) pBac-CHIKV-26S-Rhir-E, in which the CHIKV 26S subgenomic cDNA is cloned into the *Bgl*II and *Xba*I sites of pBac-Rhir-E. (C) pBac-CHIKV-6K-E1-Rhir-E, in which a 1.8-kb *Nhe*I-*Not*I fragment containing the *6K-E1* gene is cloned into the pBac-CHIKV-26SRhir-E to replace the 26S subgenomic cDNA. (D) pBac-CHIKV-26S-ΔE1-Rhir-E, in which the *E1* gene is deleted. Abbreviations: PPH, polyhedrin promoter; EGFP, enhanced green fluorescent protein gene; Rhir, RhPV 5'-UTR IRES; STOP, translational stop codon.

Fig. 2. Analyses of CHIKV structural protein expression in Sf21 cells. (A) Western blot analysis. CHIKV structural proteins were detected by rabbit anti-CHIKV E1 (anti-E1), anti-CHIKV E2 (anti-E2) and anti-whole CHIKV (anti-capsid) antibodies, respectively. Lane 1, purified CHIKV from ultra-centrifugation as a positive control; lane 2, Sf21 cells infected by vAc-Rhir-E as a negative control; lane 3, Sf21 cells infected by vAc-CHIKV-26S-Rhir-E. The E1, P62, E2 and capsid proteins of CHIKV are indicated by arrows to the right of the gels. The respective molecular weights of proteins are indicated. (B) Immunofluorescence observation of E1 and E2 on the cell surface of recombinant baculovirus infected Sf21 cells. Sf21 cells infected with either

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vAc-CHIKV-26S-Rhir-E (left panels) or vAc-Rhir-E (right panels) were directly observed under a fluorescence microscope with a FITC filter for detection of EGFP. The infected cells were fixed and stained with either anti-CHIKV E1 (E1) or anti-CHIKV E2 (E2) antibodies and examined with a rhodamine filter for detection of E1 or E2. Bar represents 25 μm.

Fig. 3. Sf21 cells infected by vAc-CHIKV-26S-Rhir-E induce syncytium formation. (A) Sf21 cells infected by vAc-CHIKV-26S-Rhir-E without further treatment (left panels), or with treatment of anti-CHIKV serum (1:200) (middle panels), and infected by vAc-Rhir-E (right panels) were examined under a fluorescence microscope with a FITC channel (upper panels) or a bright field (lower panels). The polykaryotic cells were indicated by arrows. Pictures from upper and lower panels were taken in the same field. Bar represents 25 μm. (B) The specific inhibition of cell fusion by antiserum in a dose-dependant manner. Sf21 cells were infected with recombinant baculovirus of vAc-CHIKV-26S-Rhir-E, at multiplicity of infection M.O.I. of 10 in Sf-900 II containing 8% FCS. After 1 dpi, cells were incubated with different dilutions (1:200, 1:400, 1:800,1:1600 and 1:3,200) of rabbit anti-whole CHIKV serum in growth medium for 1 hour at 27□, and then replace medium with Sf-900 II (pH 5.8) containing 2% FCS , 0.1mg /ml cholesterol and a corresponding diluted serum. The samples were then examined and photographed under an inverted fluorescence

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microscope (IX71; Olympus) at 2 dpi. The “wo” indicates the infected cells without further treatment. Bar represents 25 μ m. (C) Cell fusion between vAc-CHIKV-26S-Rhir-E-infected and uninfected Sf21 cells. Uninfected cells (target cells) were pre-stained with CellTracker RED CMPTX and then co-cultured with infected Sf21 cells (expression cells) in SF-900 II (pH 5.8) containing 0.2 mg/ml cholesterol. After 2-hour incubation at 27°C, the cell-cell fusion was observed under a confocal microscope. Expression cells emitted green florescence, target cells emitted red florescence and overlay images of both cells appeared in yellow. All pictures were taken in the same field. (D) Localizations of EGFP-expressing cells, CHIKV envelope expressing cells and polynuclear fused cells. Sf21 cells were infected with recombinant baculovirus of vAc-CHIKV-26S-Rhir-E. After 1 dpi, cells were treated with Sf-900 II (pH 5.8) containing 2% FCS and 0.1mg /ml cholesterol for 2 hours, and then stained with rabbit anti-whole CHIKV serum at a dilution of 1:800 in Sf-900 and following incubation within 10 μM Hoechst 33342 for nucleus staining and the secondary antibody, Alexa Fluor 546-labeled goat anti-rabbit IgG. The sample was then examined and photographed under an inverted fluorescence microscope (IX71; Olympus). Infected cells emitted green florescence (upper left panel), CHIKV envelope protein-expressing cells emitted red florescence (upper right panel), nucleus emitted blue florescence (lower left panel) and overlay image of above three images

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(lower right panel). And the yellow circle indicated the non-infected cells that did not express the E1 or E2 proteins as well as the green fluorescence protein. Bar represents 25 μm.

Fig. 4. The effect of pH on cell fusion. Sf21 cells were infected with vAc-CHIKV26S-Rhir-E in Sf-900 II containing 2% FCS. After 1 dpi, the culture medium was replaced with Sf-900 II containing 2% FCS and 0.2 mg/ml cholesterol at pH 5.8, 6.0, 6.2, 6.4 and 6.6, respectively, as indicated. The syncytial formation was examined under a fluorescence microscope with a FITC channel at 2 dpi. Bar represents 25 μm.

Fig. 5. The effect of cholesterol concentration on cell fusion. (A) Sf21 cells were infected with vAc-CHIKV-26S-Rhir-E in Sf-900 II containing 2% FCS. After 1 dpi, the culture medium was replaced with Sf-900 II (pH 5.8) containing 2% FCS and 0.002 mg/ml to 0.2 mg/ml cholesterol as indicated. (B) The effect of a cholesterol depleting agent on cell fusion. Sf21 cells were infected with vAc-CHIKV26S-Rhir-E in Sf-900 II containing 2% FCS. After 1 dpi, the infected cells were treated with 4.5 mM methyl-β-cyclodextrin (MβCD) for cholesterol depletion, and then culture medium was replaced with either Sf-900 II (pH 5.8) (middle panel) or Sf-900 II (pH 5.8) containing 0.15 mg/ml cholesterol (right panel) for replenishment.. The syncytial

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formation at 2 dpi was examined under a fluorescence microscope with a FITC channel. Bar represents 25 μm.

Fig. 6 Time course of cell-cell fusion induced by vAc-CHIKV-26S-Rhir-E infection. Sf21 cells were infected with vAc-CHIKV26S-Rhir-E in Sf-900 II containing 8% FCS. After 1 dpi, the culture medium was replaced with Sf-900 II (pH 5.8) containing 2% FCS and 0.2 mg/ml cholesterol. The cell-cell fusion was observed under a fluorescence microscope with a bright field at the indicated times. The early fusion events were indicated by arrows after a 30-minute incubation. Almost all Sf21 cells were fused together after 180-minute incubation. All pictures were taken in the same field.

Fig. 7. Induction of cell fusion by expression of CHIKV 6K-E1. (A) Western blot analysis of E1 protein in cell lysates from vAc-CHIKV-26S-Rhir-E (lane 1), vAc-CHIKV-26S-ΔE1-Rhir-E (lane 2), vAc-CHIKV-6K-E1-Rhir-E (lane 3) and vAc-Rir-E (lane 4) infected Sf21 cells. E1 protein was detected by rabbit anti-E1 serum staining. The molecular weights of standards (kDa) are indicated on the left, and the E1 protein is indicated by an arrow. (B) Sf21 cells were infected with indicated baculoviruses in Sf-900 II containing 8% FCS. After 1 dpi, medium was replaced by Sf-900 II (pH 5.8) containing 2% FCS and 0.1 mg/ml cholesterol. Cell fusions were examined and photographed under an inverted fluorescence microscope at 2 dpi. Bar represents 25 μm.

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Highlights

> A cell-based assay system for Chikungunya virus induced membrane fusion was established in baculovirus expression system. > Protein E1 of Chikungunya virus was required for cell fusion. > Cholesterol and low pH requirements for membrane fusion.> This cell-based system provides a model for studying class II viral membrane fusion.

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Fig. 1

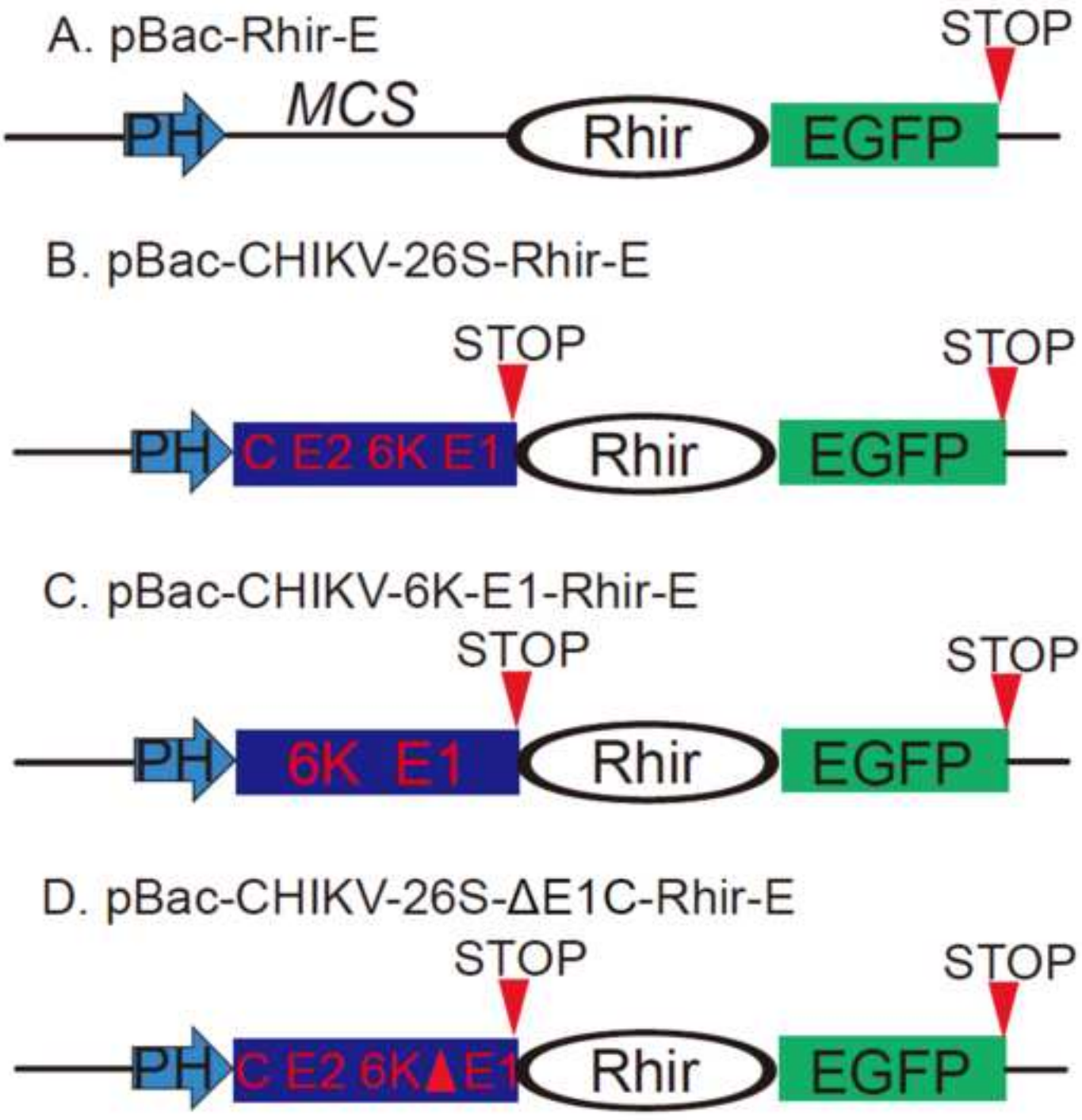

Figure 2A

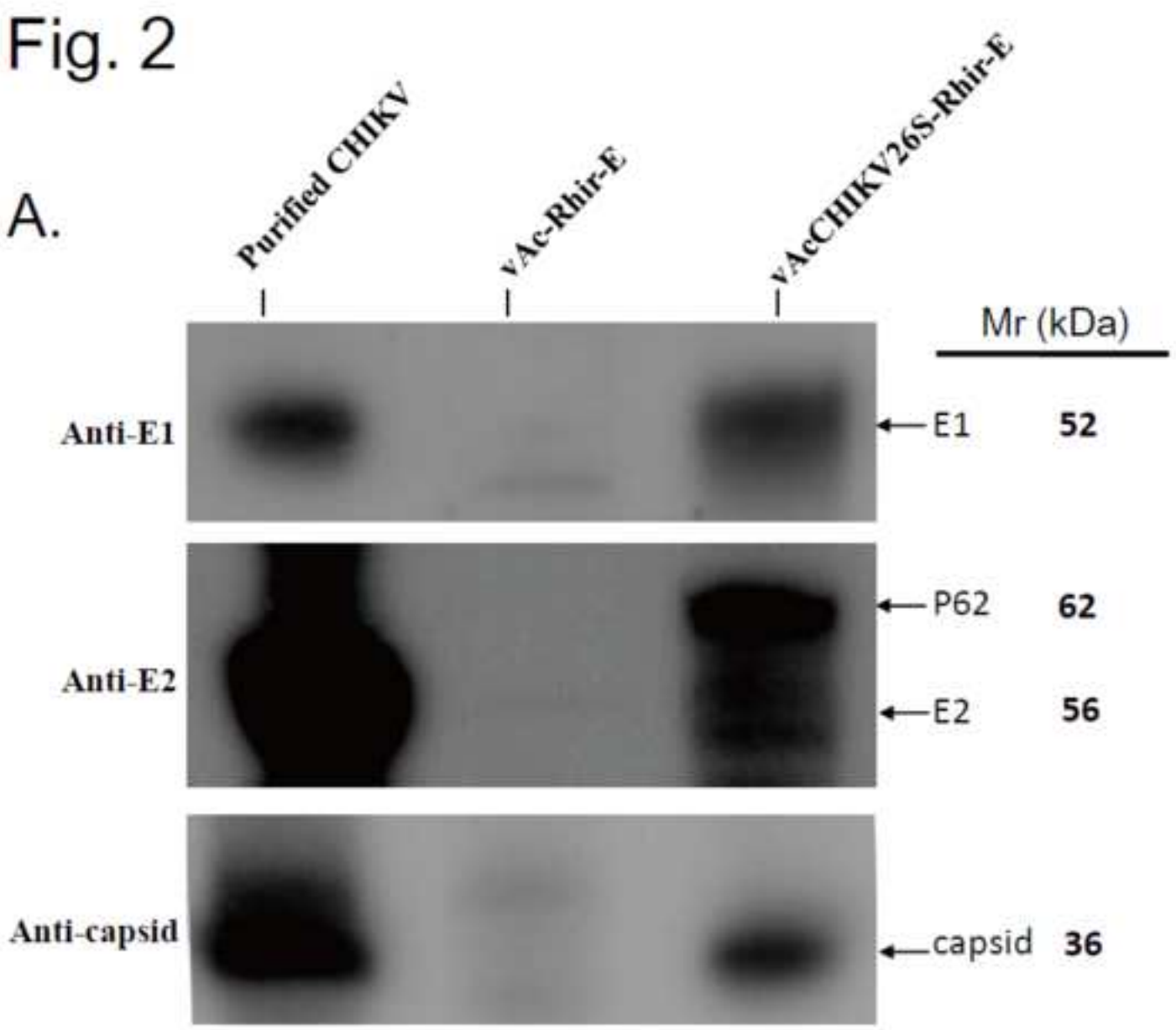
Fig. 2
A.
Purified CHIKV
vAc-Rhir-E
vAcCHIKV26S-Rhir-E
Mr (kDa)
Anti-E1
E1
52
Anti-E2
P62
62
E2
56
Anti-capsid
capsid
36

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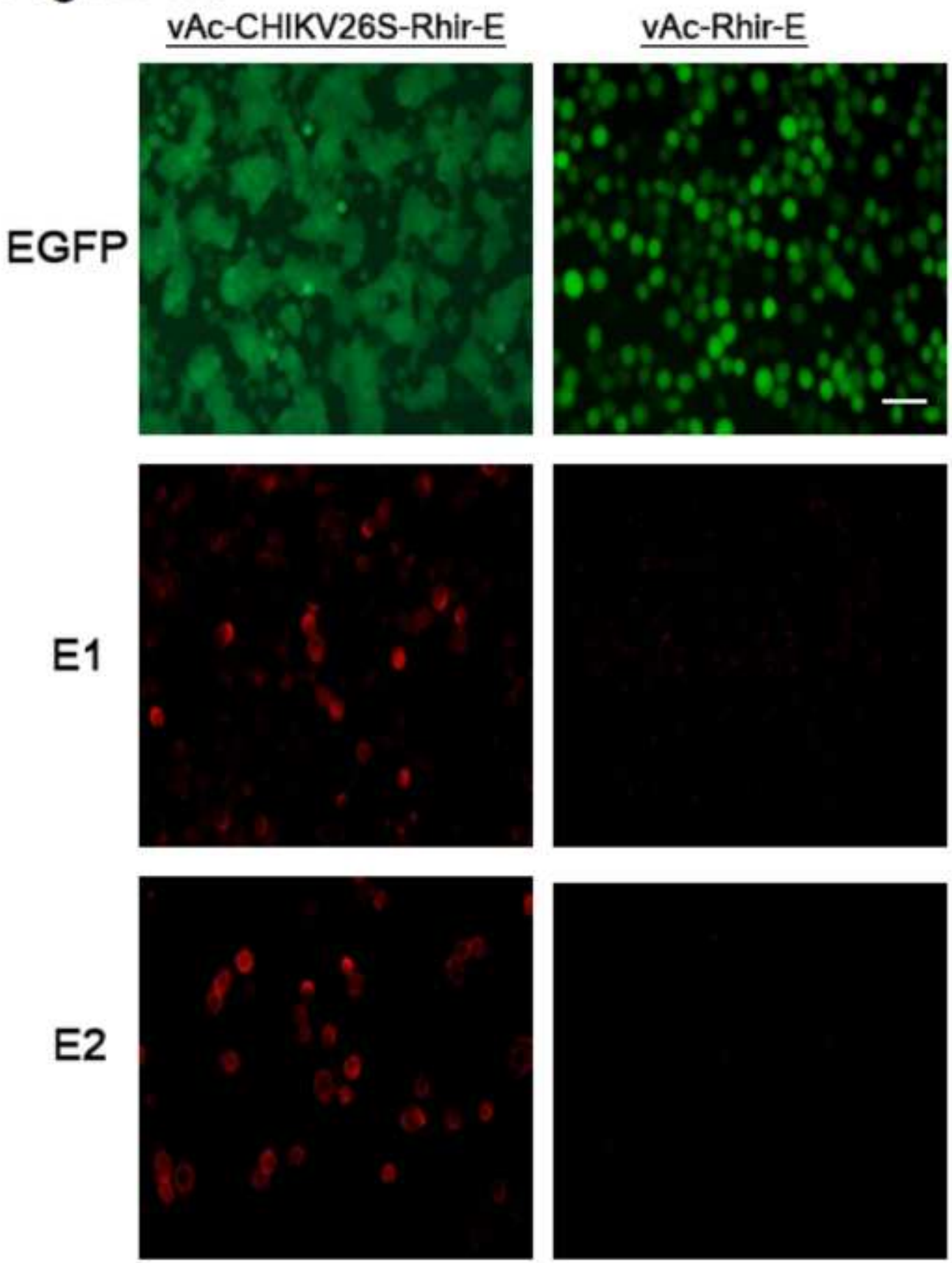
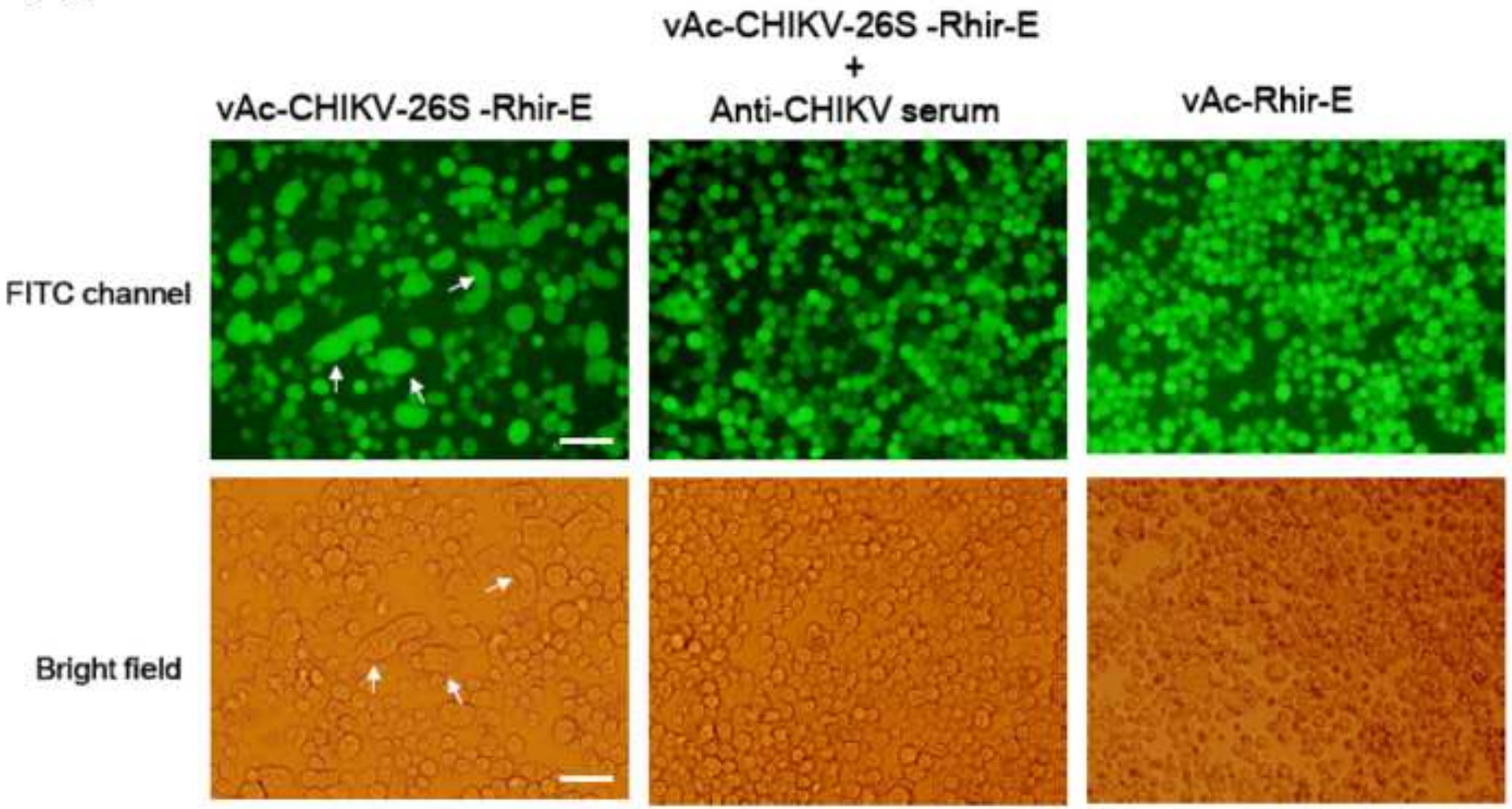
Fig. 2 B.



Figure(3A)

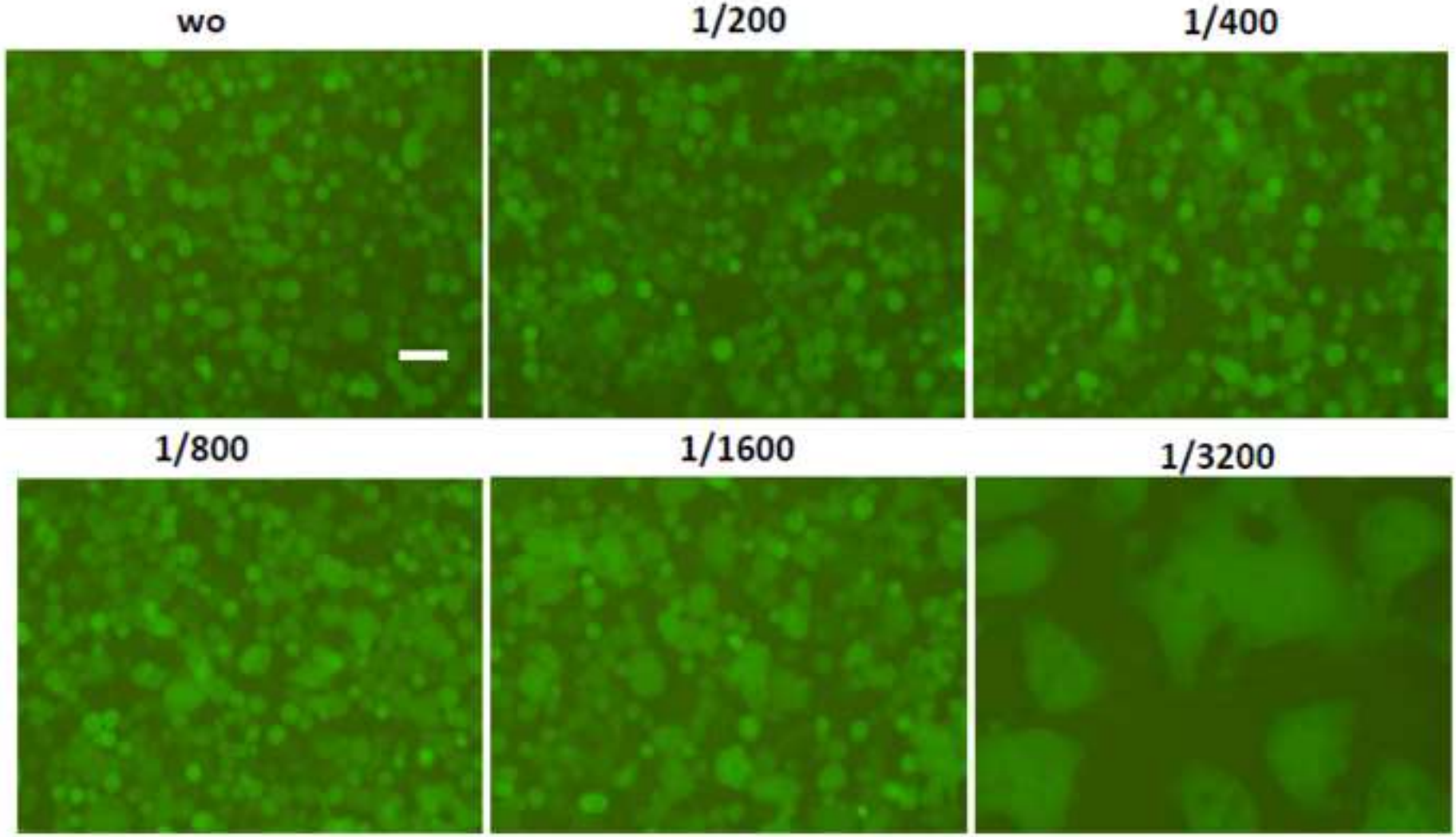
Fig. 3



Figure(3B)

Fig. 3

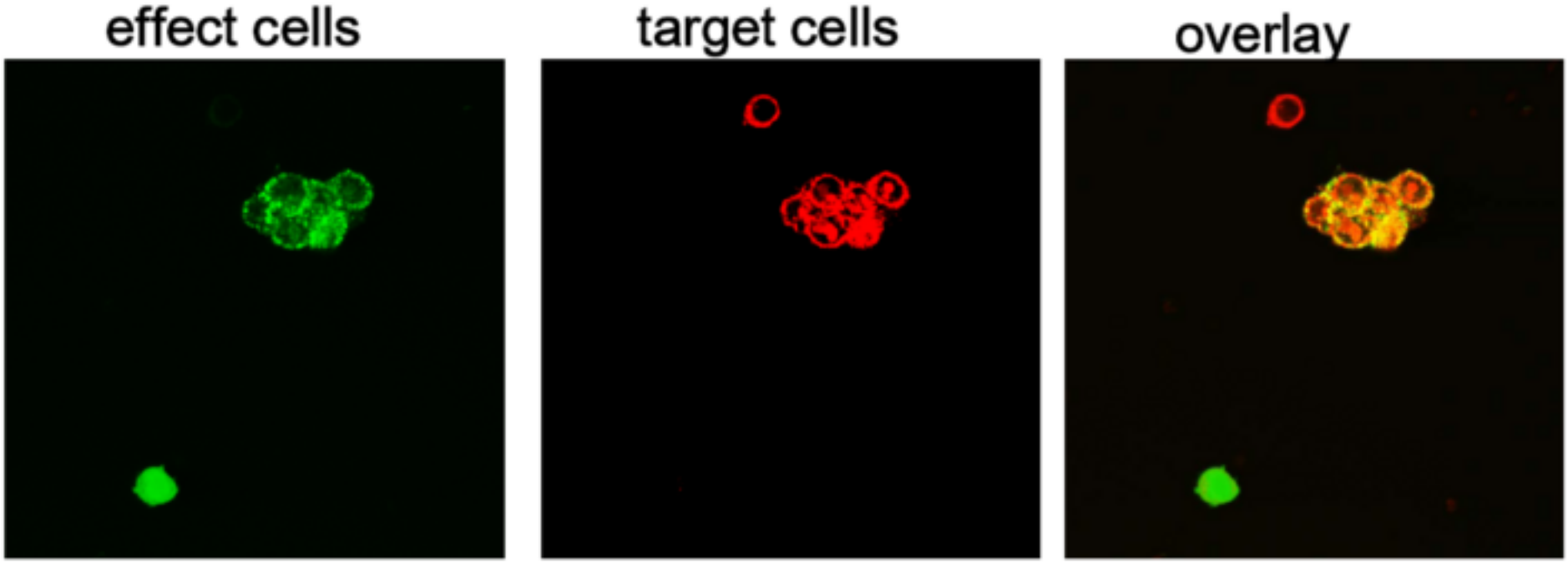
B.



Figure(3C)

Fig. 3

C.



Figure(3D)

Fig. 3

D.

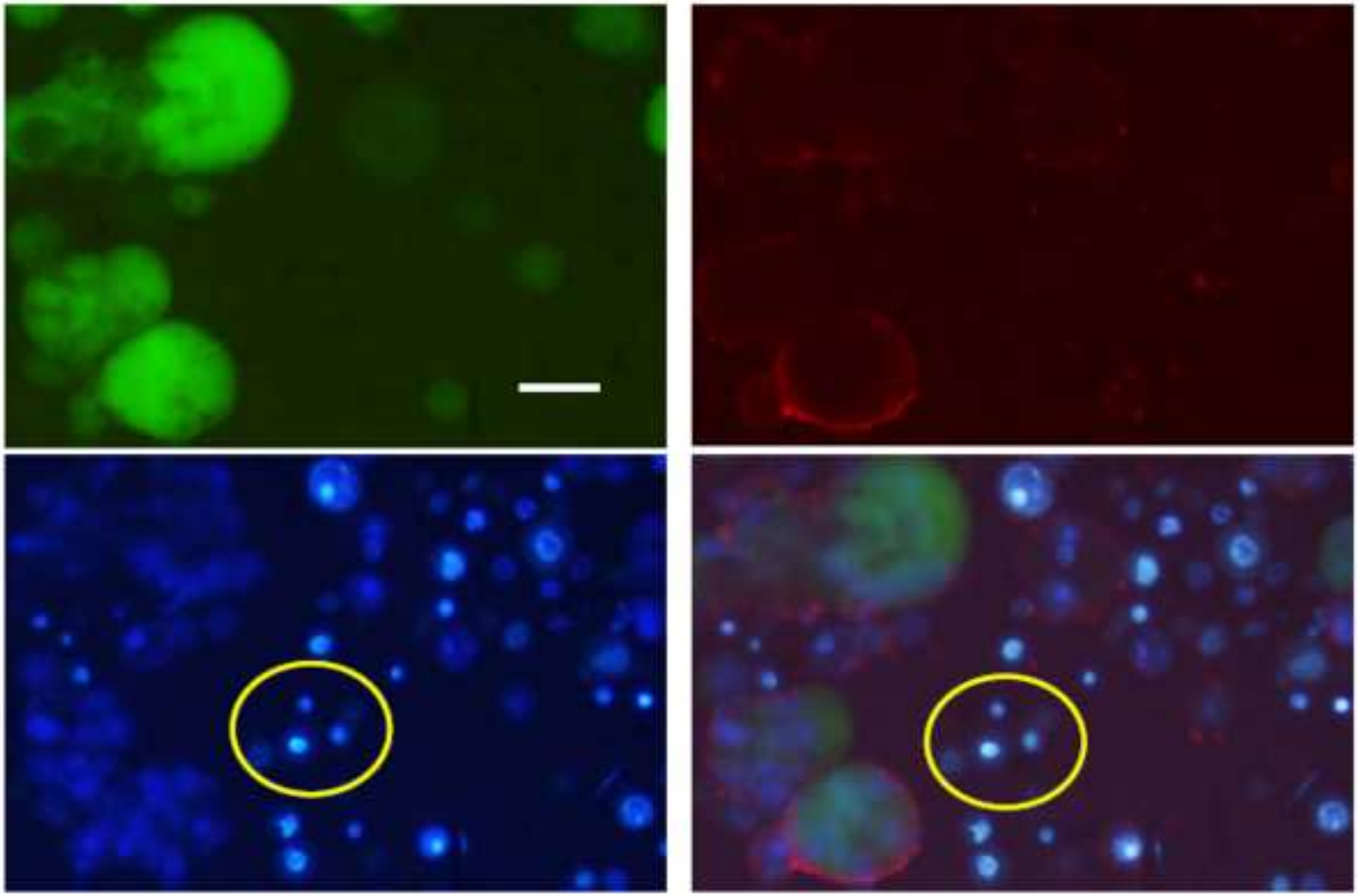


Fig. 4

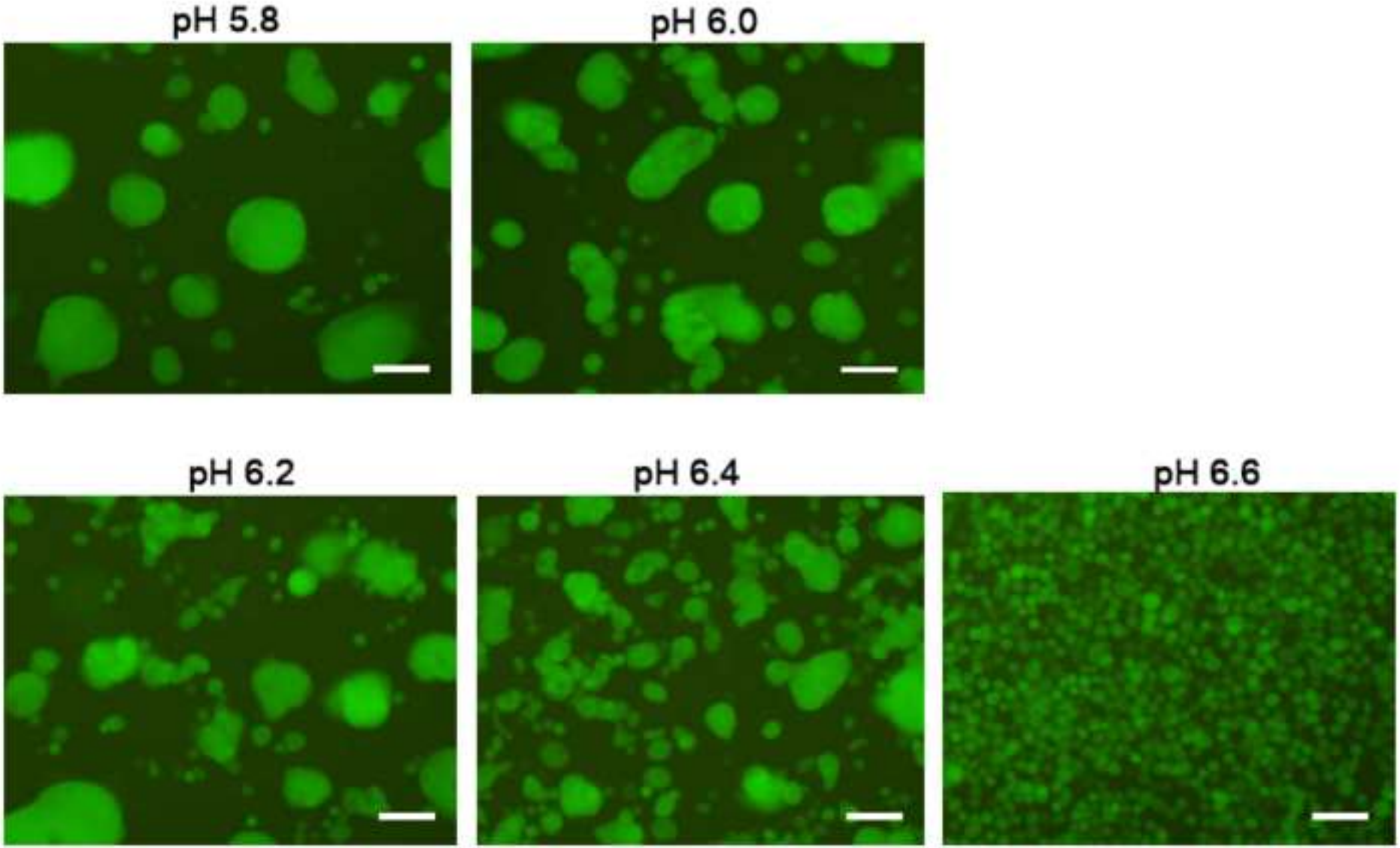


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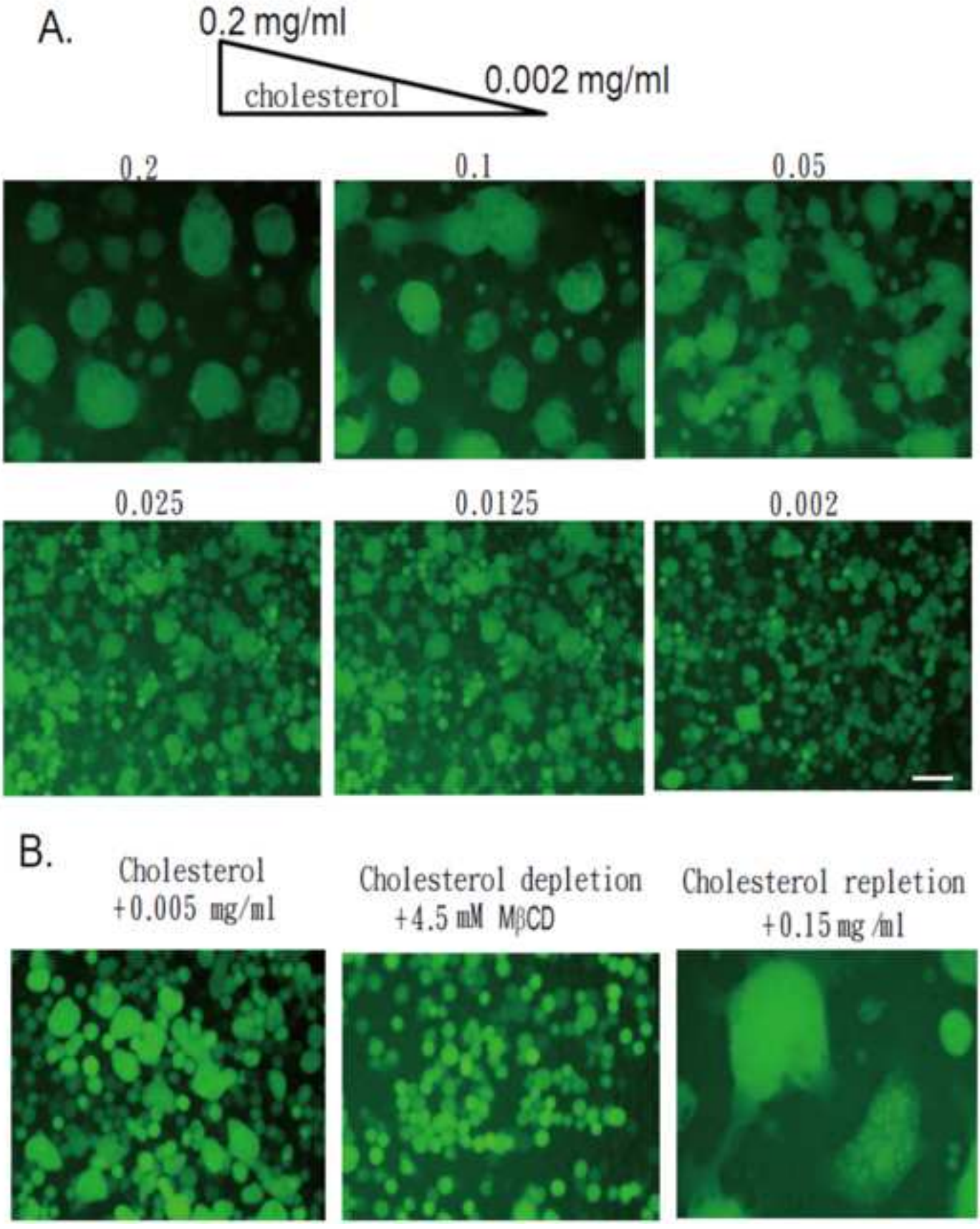
Fig. 5



Figure(6)

Fig. 6

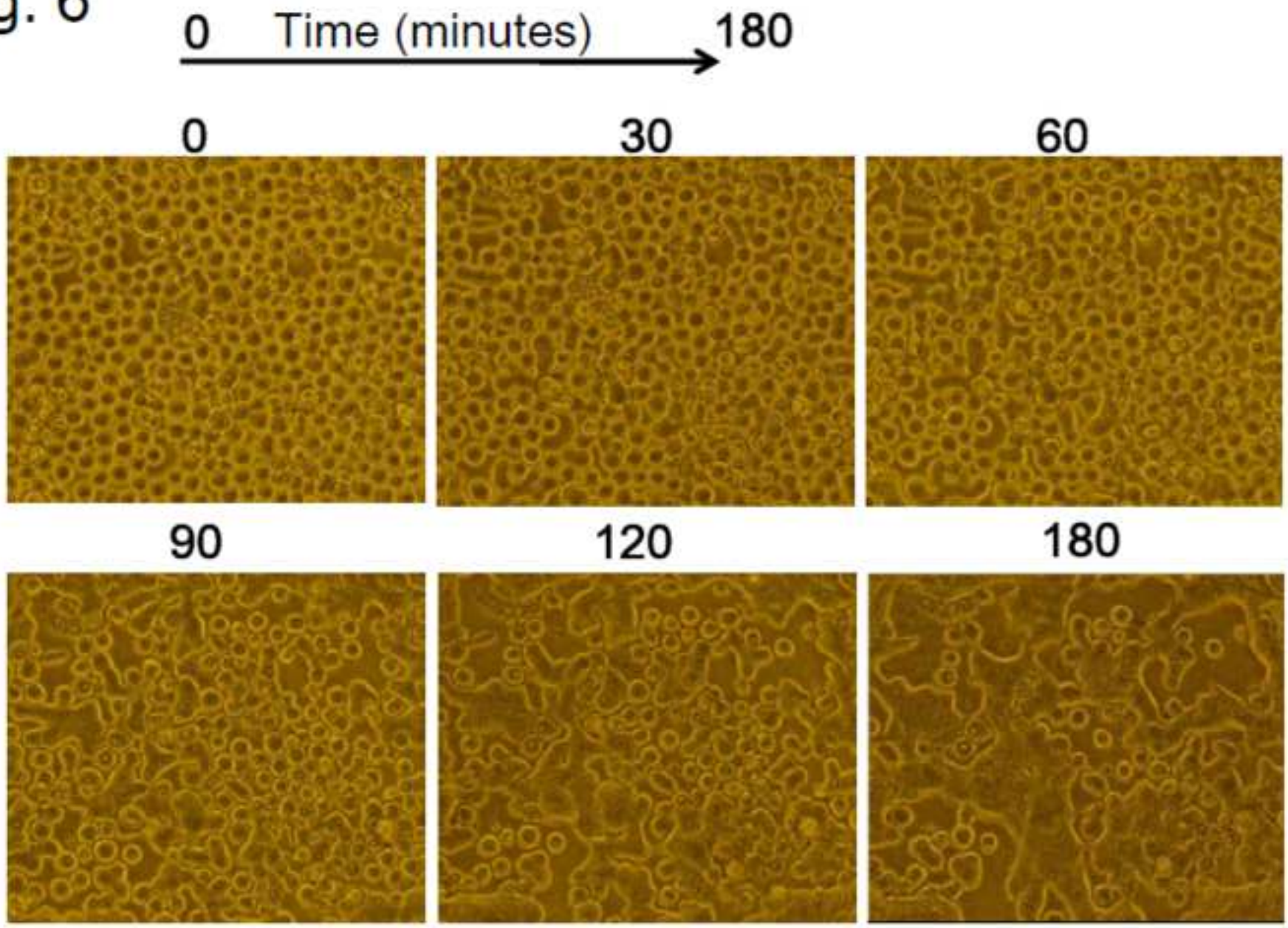
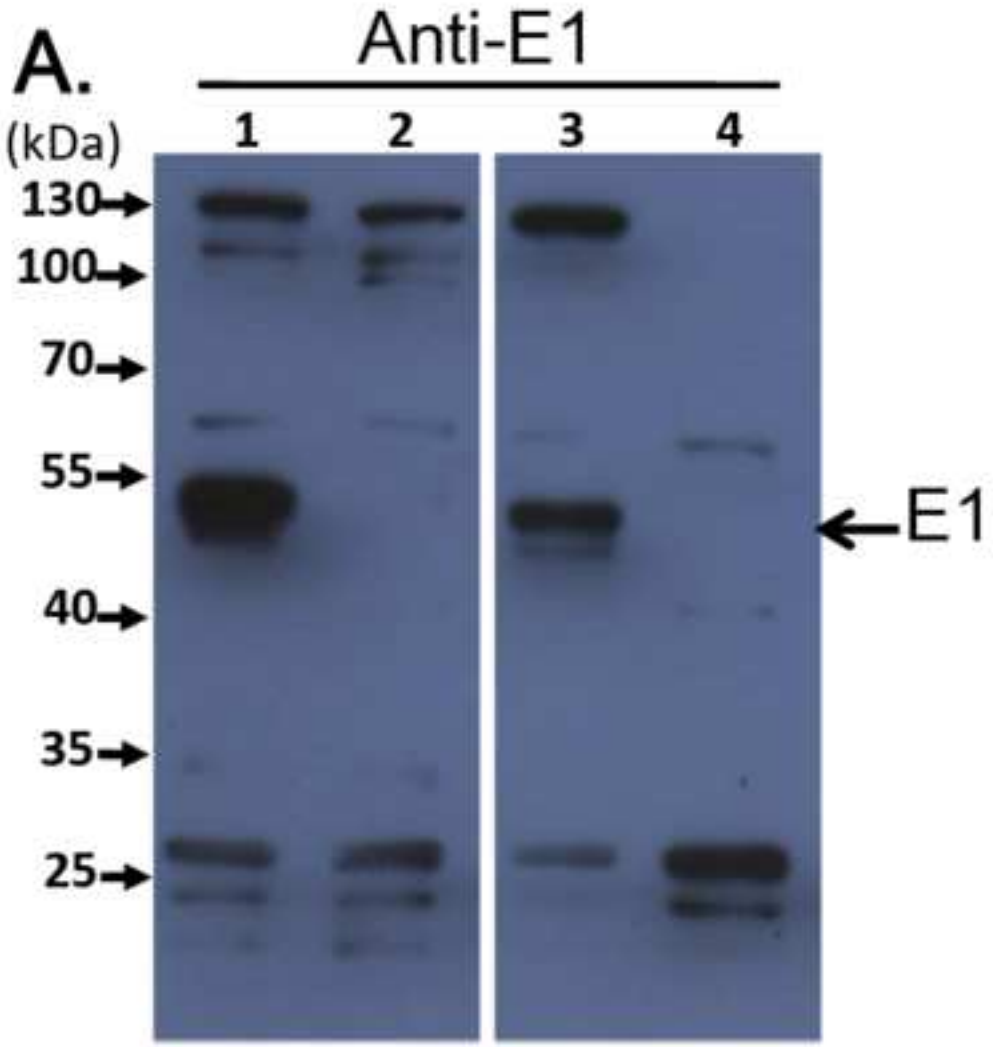


Fig. 7



Figure(7B)

Fig. 7