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Short Communication

# Visualization of avian influenza virus infected cells using self-assembling fragments of green fluorescent protein

Katsushi Kanehira [a,*], Yuko Uchida [a], Takehiko Saito [a,b]

[a] Influenza and Prion Disease Research Center, National Institute of Animal Health National Agriculture and Food Research Organization (NARO), 3-1-5 Kannondai, Tsukuba, Ibaraki 305-0856, Japan
[b] United Gradual School of Veterinary Sciences, Gifu University, 1-1 Yanagido, Gifu City 501-1193, Japan

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ABSTRACT

*Background:* Avian influenza viruses (AIVs) are influenza A viruses which are isolated from domestic and wild birds. AIVs that include highly pathogenic avian influenza viruses (HPAIVs) are a major concern to the poultry industry because they cause outbreaks in poultry with extraordinarily high lethality. In addition, AIVs threaten human health by occasional zoonotic infection of humans from birds. Tools to visualize AIV-infected cells would facilitate the development of diagnostic tests and preventative methods to reduce the spread of AIVs. In this study, a self-assembling split-green fluorescent protein (split-GFP) system, combined with influenza virus reverse genetics was used to construct a visualization method for influenza virus-infected cells.
*Results:* The viral nucleoprotein (NP) segment of AIV was genetically modified to co-express $GFP_{11}$ of self-assembling split-GFP, and the recombinant AIV with the modified NP segment was generated by plasmid-based reverse genetics. Infection with the recombinant AIV in cultured chicken cells was visualized by transient transfection with a $GFP_{1-10}$ expression vector and fluorescence was observed in the cells at 96 hours post-inoculation. Virus titer of the recombinant AIV in embryonated eggs was comparable to wild type AIV titers at 48 h post inoculation. The inserted sequence encoding $GFP_{11}$ was stable for up to ten passages in embryonated eggs.
*Conclusions:* A visualization system for AIV-infected cells using split-GFP was developed. This method could be used to understand AIV infection dynamics in cells.



## 1. Introduction

Highly pathogenic avian influenza virus (HPAIV) affects the poultry industry by causing large-scale disease outbreaks in many parts of the world [1]. In addition, HPAIVs and low pathogenic avian influenza viruses (LPAIVs) have occasionally impacted human health as zoonotic infectious agents [2,3,4]. Establishing tools to dissect the dynamics of avian influenza virus (AIV) in infected host cells is essential to understand the host-pathogen interaction and to develop preventative measures for both livestock and public health.

Fluorescent-protein-based technology is broadly used to visualize the localization and dynamics of molecules, organs and cells, and is useful for understanding and describing biological events such as intracellular signaling, cell cycles and infection with pathogens [5,6,7,8,9,10]. Intracellular localization, viral protein functions and virus-infected cells have been visualized using methods that combine an influenza virus reverse genetics system [11] and green fluorescent gene (GFP) based technologies. Perez et al. [12]. described the design and construction of an artificial viral RNA (vRNA) which encoded a GFP reporter fused to non-structural protein 1 (NS1-GFP) and a nuclear export protein and rescued recombinant NS1-GFP influenza virus [12]. The recombinant NS1-GFP influenza virus had the genetic backbone of mouse-adapted A/Puerto Rico/8/34 (H1N1) for analysis of infection dynamics in mice [8]. Fukuyama et al. [9] have improved the fluorescent influenza virus system to stably express four different fluorescent proteins by serial passage in mice [9]. They used a fluorescent HPAIV based on A/Vietnam/1203/2004(H5N1) combined with a PR8 derived NS gene to express mouse-adapted NS1-Venus to construct a bright version of modified GFP [9]. Although the pathogenicity of the fluorescent HPAIV constructed with mouse-adapted NS1-Venus in B6 mice was comparable to that of the original A/Vietnam/1203/2004(H5N1) [9], a fluorescent AIV for avian cells is not yet reported. In this study, we constructed a fluorescent visualization system based on a LPAIV, A/chicken/Yokohama/aq55/2001(H9N2; CY55), which used a self-assembling split-green fluorescent protein (split-GFP) mechanism [13] for visualizing infected chicken cells. The artificial NS genetic segment ($GFP_{11}$-2A-NP) vRNA was designed as follows. To shorten the inserted sequence, a 16 amino acid (AA) $GFP_{11}$

* Corresponding author.
*E-mail address:* kanehira@affrc.go.jp (K. Kanehira).
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(the $GFP_{11}$ M3 in previous study [13] is abbreviated to $GFP_{11}$ in this study) was employed. Co-expression of the $GFP_{11}$ marker peptide and native nucleoprotein (NP) was achieved by placement of the 2A 'cleavage' (to be more precise, 'ribosome skipping') peptide, a 20 AA sequence (QLLNFDLLKLAGDVESNPGP) derived from foot-and-mouth disease virus [14] between the sequences. Complementation of 3′ packaging signal, 3′ non-coding region and 60 bp derived from the 3′ end of the coding region with three mutations in the NP segment of CY55 were as previously described [10].

## 2. Materials and methods

### 2.1. Construction of recombinant virus and $GFP_{1-10}$ expression vector

The $GFP_{11}$-2A-NP vRNA/cDNA supplying vector was constructed as indicated in Fig. 1a with a tandemly arrayed coding sequence of the 3′ artificial packaging signal (Fig. 1b), Kozak sequence, GFP11, 2A peptide, full NP and 5′ untranslated region of CY55 (negative sense orientation). Since 2A peptide was used for expression of multiple poly-peptides by 'ribosome skipping' from a cDNA [14], design of $GFP_{11}$-2A-NP vRNA was intended to express two polypeptides; shorter one contains $GFP_{11}$, and another contains NP protein. $GFP_{11}$-2A-NP cDNA was artificially synthesized and cloned into pHW2000 [11] by Takara Bio Inc. (Shiga, Japan). Mixed cultures of 293 T and MDCK cells were co-transfected with $GFP_{11}$-2A-NP and PB2, PB1, PA, HA, NA, M and NS segments of CY55 cloned into pHW2000 as described previously [15]. Transfected cell supernatants were harvested after 72 h and inoculated into the allantoic cavity of 10-d old embryonated chicken eggs twice and incubated at 37°C for 48 h for amplification of the recombinant virus. Allantoic fluid was harvested and stock cultures of the recombinant AIV (rCY55/$GFP_{11}$-2A-NP) were prepared containing 1024 HAU/50 μL. The codon usage for $GFP_{1-10}$ cDNA, which was described previously [13], was optimized for chicken cells and artificially synthesized (GenScript Corp., NJ. USA). It was cloned into pCAG-Bsd expression vector (Wako Inc., Japan) to make pCAG-$GFP_{1-10}$.

### 2.2. Proliferation in embryonated chicken eggs

Virus titers were determined using 10-d old embryonated chicken eggs as described previously [16]. Briefly, serially diluted virus samples were inoculated into the allantoic cavity of the embryonated eggs and incubated at 37°C for 48 h. Fifty microliters of allantoic fluids of the eggs were collected and the hemagglutination activity against 0.55% vol/vol chicken red blood cell was examined. Fifty percent chicken embryo infectious doses per milliliter ($EID_{50}$/mL) was calculated using the positive ratio of the hemagglutination activity by the method of Reed and Muench [17].

Growth efficiency of rCY55/$GFP_{11}$-2A-NP and a reverse-genetically-constructed wild type CY55 virus (rCY55/wNP) was compared by inoculating $1.0 \times 10^2$ $EID_{50}$ of virus into allantoic cavities of twelve 10-d old embryonated eggs and incubating at 37°C for either 24 or 48 h. The allantoic fluids of the eggs were collected and $EID_{50}$/mL of the fluids was determined as mentioned above. Statistical significance of the mean titers was determined using unpaired t tests at 95% confidence level using Prism (GraphPad Software, CA).

### 2.3. Fluorescent visualization of cultured chicken cells infected with rCY55/$GFP_{11}$-2A-NP

Approximately $25 \times 10^6$ primary cultured chicken kidney (CK) cells and LMH, a chicken hepatocellular carcinoma epithelial cell line, suspended in 500 μL of OPTI-MEM (Life Technologies Inc., MD, USA) were transiently transfected with 40 μg of pCAG-$GFP_{1-10}$ by electroporation in a 0.4 cm electroporation cuvette at 950 μF and 270 V using a Gene Pulser Xcell II with PC and EC modules (Bio-Rad, CA). After electroporation, cells were diluted in 2 mL of MEM or Weymouth's medium supplemented with 10% FBS and cultured in 6-well plates. To investigate the fluorescence of rCY55/$GFP_{11}$-2A-NP recombinant virus in pCAG-$GFP_{1-10}$ transfected CK cells, $1.0 \times 10^4$ $EID_{50}$ of virus was inoculated into each well at 24 h after electroporation. After incubation, cells were fixed in 4% (vol/vol) formaldehyde/PBS for 5 min and nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). Images of transmitted light, infected cells (with fluorescence by reassembled split-GFP) and nuclei of cells (stained with DAPI) were captured using EVOS Cell Imaging system (Life Technologies Inc.) with ×40 objective lens. To investigate dose dependency of the split-GFP based visualization system, $1.0 \times 10^2$ or $5.0 \times 10^2$ $EID_{50}$, and $1.0 \times 10^2$, $2.0 \times 10^2$, $5.0 \times 10^2$ or $1.0 \times 10^3$ $EID_{50}$ of virus were inoculated into pCAG-$GFP_{1-10}$ transfected CK and LMH cells,

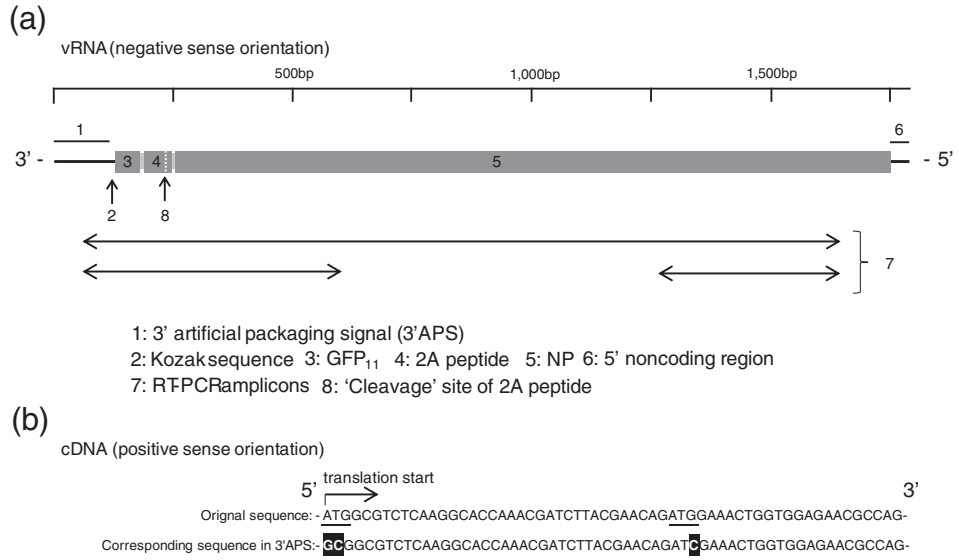


**Fig. 1.** Construction of recombinant $GFP_{11}$-2A-NP segment. (a) Schematic representation of $GFP_{11}$-2A-NP segment vRNA. The recombinant DNA was designed as a tandemly arrayed sequence as follows: 1, 3′ non-coding region and 60 bp nucleotide derived from 3′ end of the coding region with three mutations arranged as 3′ artificial packaging signal (3′APS); 2, Kozak sequence; 3, $GFP_{11}$; 4, 2A peptide; 5, NP; 6, 5′ noncoding region; and 7, RT-PCR amplicons for conformation of nucleotide mutations, 8, 'cleavage' site of 2A peptide. The RNA is shown in negative sense orientation. (b) Positive sense sequence of 3′ side of 3′APS cDNA. The 3′ side of the APS cDNA was constructed with 60 bp derived from the 5′ end of the coding region of NP cDNA with three mutations (black and white reversal).

respectively, and the numbers of fluorescent and non-fluorescent cells were calculated. The differences of ratio of fluorescent cells were statically analyzed with unpaired t tests (CK) or one-way analysis of variance and the Bonferroni's multiple comparison post-test (LMH) at 95% confidence level using Prism software.

## 3. Results and discussion

The stability of the inserted sequence in rCY55/$GFP_{11}$-2A-NP was evaluated by serial passage in embryonated eggs from the stock culture. Sequences of the stock culture and the $10^{th}$-passaged viruses were determined by RT-PCR (amplicons are shown in Fig. 1a), followed by direct sequencing with an ABI PRISM Genetic Analyzer (Life Technologies Inc.). Nucleotide mutations in the $GFP_{11}$-2A region of $GFP_{11}$-2A-NP genomic segment were not detected in the $10^{th}$-passaged virus (data not shown), demonstrating that the inserted artificial sequence was stably inherited by progeny viruses during propagation in embryonated chicken eggs.

Growth efficacy of rCY55/$GFP_{11}$-2A-NP and rCY55/wNP in embryonated chicken eggs was compared. The mean titer of rCY55/$GFP_{11}$-2A-NP was approximately $10^{2.11}$ $EID_{50}$/mL lower than the mean titer of rCY55/wNP at 24 h post-inoculation with statistical significance. No significant difference was observed when the incubation period was extended to 48 h (Fig. 2a). This suggests that proliferation of rCY55/$GFP_{11}$-2A-NP was slower than rCY55/wNP, although the viral titers in the plateau phases were similar in the embryonated eggs.

Replication of rCY55/$GFP_{11}$-2A-NP in $GFP_{1-10}$-transfected CK cells was visualized at 96 h post inoculation (Fig. 2b–e) and the fluorescent signal was detected in the cytoplasm and nucleus of the cells. This suggests that the $GFP_{11}$ marker translated from $GFP_{11}$-2A-NP cDNA was present in the cytoplasm separate from the NP. The $GFP_{11}$ marker and transiently transfected $GFP_{1-10}$ do not possess organelle-localization signals and can assemble in whole cell. However, the fluorescent signal in the nuclei of some cells was stronger than in the cytosol, indicating that some fractions of translated protein from $GFP_{11}$-2A-NP cDNA existed in an uncleaved form. Because the efficacy of 2A cleavage depends on the sequence and host species [18,19], modification of the 2A sequence in the artificial vRNA might have reduced the nuclear signal. In addition, it is suggested that the fused $GFP_{11}$-2A polypeptide of uncleaved form of $GFP_{11}$-2A-NP interferes the NP function in virus replication resulting the reduction of growth efficacy of rCY55/GFP11-2A-NP as mentioned above. The improvement of cleavage efficacy by modification of the 2A sequence might also improve the reduction of virus replication.

To investigate the quantitativity of the method, different doses of rCY55/$GFP_{11}$-2A-NP were inoculated and ratio of fluorescent cells was compared (Fig. 2f and Fig. 2g). Increase of fluorescent cells was shown to be in proportion to dosage of the virus inoculated to the cultured cells, although the number of fluorescent cells was found to differ between $GFP_{1-10}$ transfected CK cells and LMH cells when the same dose of rCY55/$GFP_{11}$-2A-NP virus was inoculated. Such difference appeared to reflect different susceptibility of the cells to the virus and cytopathic effect of the virus to the cells. These results suggested that the method could be used, within a range that depends on the cells used, to evaluate the number of infected cells and to follow infection by the virus.

The system described in this study will be useful for visualizing AIV-infected chicken cells. Features of our system were 1) all influenza-derived sequences of the recombinant virus genome originated from an AIV, CY55; and 2) the split-GFP system was used for visualization. Most, if not all attempts to construct recombinant influenza viruses with a GFP reporter appear to encounter difficulty with inserting relatively large fluorescent proteins within an influenza viral RNA segment. Wild type GFP isolated from *Aequorea victoria* has a molecular weight of 27 kDa and is encoded by a 714 bp cDNA [20]. Although the length of GFP reporters varies depending on biological origin and artificial modification of the reporter protein, the length of

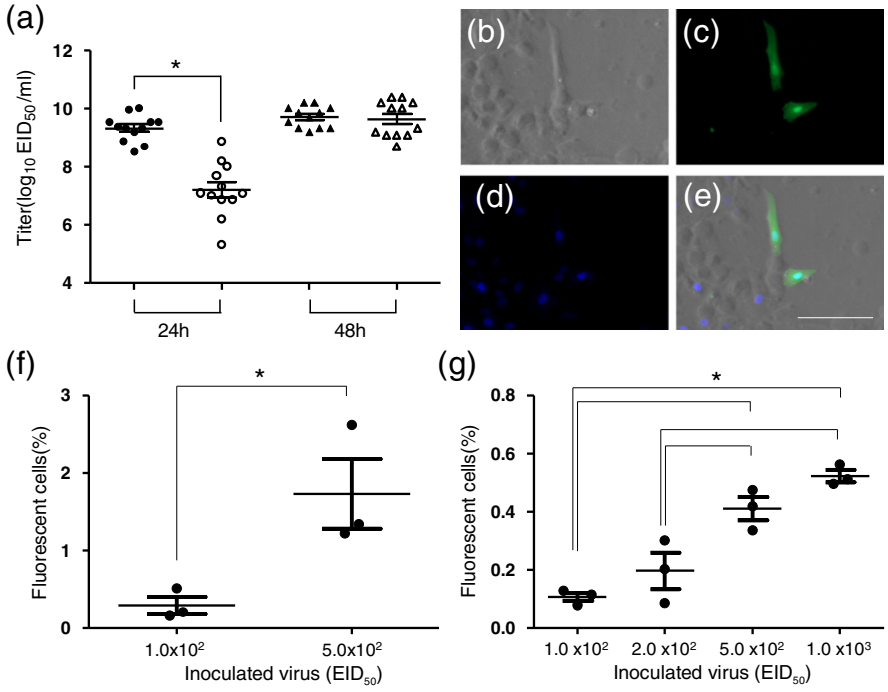


**Fig. 2.** Replication of rCY55/GFP11-2A-NP. (A) Viral replication in embryonated eggs. Allantoic cavities of embryonated chicken eggs were inoculated with rCY55/wNP (closed symbols) and rCY55/GFP11-2A-NP (open symbols). The eggs were incubated at 37°C for 24 h (circles) or 48 h (triangles) and were transferred to 4°C. Fifty percent chicken embryo infectious doses per milliliter ($EID_{50}$/mL) of collected allantoic fluid samples were determined. *: $P < 0.05$. (B–E) Visualization of infected cells by self-assembling split GFP system. Primary chicken kidney cells which have been transfected with the $GFP_{1-10}$ plasmid were infected with rCY55/$GFP_{11}$-2A-NP. Images were obtained using EVOS FL cell imaging system with light cubes for the (b) transmitted light, (c) GFP and (d) DAPI, and merged into an image (e). Scale bars = 100 μm. (f and g) Comparison of ratio of fluorescent cells between the $GFP_{1-10}$ transfected (f) CK and (g) LMH inoculated with indicated doses of rCY55/$GFP_{11}$-2A-NP. *: $P < 0.05$.

eight-influenza virus RNAs ranged from approximately 890 to 2340 nt. The split-GFP system is an ingenious strategy to reduce the size of the insertion into an influenza viral segment. Previously, Avilov et al. [7] used the system to produce a recombinant A/WSN/33 virus which allowed expression of individually fluorescent PB2 polymerase subunits in infected cells [7]. In their study, $GFP_{11}$ was fused to the PB2 protein and translated as a fusion protein in infected cells [7]. In our study, $GFP_{11}$ was encoded as a marker rather than fused to viral proteins in rCY55/$GFP_{11}$-2A-NP. The inserted marker protein was designed so that it would not interfere with the function of NP because the expressed NP translated from the artificial $GFP_{11}$-2A-NP vRNA only possesses three additional AA (P, which is derived from the carboxyl C-terminal end of 2A, and EF, which are translated from the linker sequence between 2A and NP) on the N-terminal side. This strategy may also be useful for generation of recombinant AIVs which express external genes for further studies.

In summary, a novel method for the construction of a visualization system in AIV-infected chicken cells was reported in this study. This method provides the basis for understanding AIV infection dynamics in cells of the natural host.

## Conflict of interest

None to declare.

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