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Detection of Single Influenza Viral RNA in Cells Using a Polymeric Sequence Probe

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Abstract

A polynucleotide probe, call a polymeric sequence probe (PSP), was used to detect influenza A (Influenza A/WSN/33) NA (Neuraminidase) viral RNA in Madin-Darby canine kidney (MDCK) cells. The PSP is a single-stranded DNA molecule with ~2 000 tandem repeat fluorescence binding sites and target binding sites that can bind with multiple fluorescence complementary oligos and target viral RNA using a fluorescence in situ hybridization (FISH) process. A single viral RNA labeled by PSP can be directly observed in MDCK cells. The simple FISH protocol enables the observation and quantitative analysis of the infectious process and drug effects with ultrahigh sensitivity and spatial resolution.

There has been increasing interest in studying the biological process in morphology preserved chromosomes, cells, or tissue sections at the single molecule level, for example, to understand the lifecycle of the influenza virus infection. One of the challenges is to visualize the viral RNA (vRNA) at the very early stages of infection. Reverse transcription-polymerase chain reaction (RT-PCR) cannot provide the information about the locations and

distributions of the RNA molecules. Fluorescence in situ hybridization (FISH) was designed to detect and localize specific nucleic acids. However, traditional FISH probe labeled with radioactive dye has been limited by its low stability, low spatial resolution, and high safety shield requirement. The FISH probe labeled with an enzyme probe amplifies fluorescence signals using a simple procedure,(1) whereas the location of the target molecule cannot be precisely determined because the enzyme amplification products are small molecules and diffuse away from the target molecule within a short time. The FISH probe linked with a few fluorophores provides high spatial resolution, but its sensitivity is relatively low and requires expensive detectors. Recently more advanced probes have been developed to achieve single-molecule resolution and sensitivity. Plasmid-derived RNA was expressed from an engineered plasmid to include a probe tail that binds several dozen fluorophores.(2–4) The assays have achieved high sensitivity at the single molecule level, but they are limited to mRNA detection in certain types of cells that can be efficiently transfected and are not applicable to nucleic acids, such as vRNA, that cannot be engineered. The padlock probe followed by a target-primed RCA (rolling circle amplification) can be used to label native nucleic acids with high specificity,(5) but the assay requires several additional steps, such as replication, ligation, amplification, etc.

In this letter we report a probe, called a polymeric sequence probe (PSP), which can detect nucleic acid biomarkers with single-molecule sensitivity and spatial resolution in a simple FISH process. A PSP is a 100 k mer single-stranded DNA containing tandem repeat sequences of a target binding site and a labeling site. The reporting site is hybridized with a complementary oligo conjugated with a fluorophore. A single PSP can hybridize with thousands of fluorophores. Under a conventional fluorescence microscope, a PSP can be observed as a superbright spot that can be easily distinguished from the background in a cell (Scheme 1). The multiple binding sites in a PSP lead to high binding affinity between the probe and its target. With this high signal intensity and high binding affinity, the PSP-based assay needs no amplification to achieve detection sensitivity at the single-molecule level using a simple FISH protocol.

The PSPs were prepared by rolling circle amplification (RCA).(6) RCA is an isothermal process for the enzymatic synthesis of long ssDNA with repeating sequences.(7, 8) A primer is elongated along its 3' end using a rolling circle as the template to form the long probe. The probe will fold into a "ball" shape when it is dissolved in solution. After it is tagged with multiple fluorescence complementary oligos, it can be observed as a bright spot under a fluorescence microscope.(6) Scheme 1 shows the mechanism of the PSP-based assay. Initially, the infected Madin-Darby canine kidney (MDCK) cells are fixed and permeabilized. During the fixation process, nucleotides are cross-linked with proteins, and the targeted vRNA molecules are immobilized inside the cells.(9, 10) After the PSPs diffuse into the cells, they bind with the targeted vRNA, and also multiple fluorescence complementary oligos. In this experiment, we chose neuraminidase (NA) vRNA of influenza A virus as the target. Unlike mRNA which is generated after the transcription, vRNA exists at the very beginning of the viral infection and can be tracked throughout the whole viral life cycle. The observation of the vRNA can provide detailed information of the infectious process, such as starting point of the replication and the distribution of vRNA before and after replication. The high sensitivity and spatial resolution of the assay enables

the direct observation of the single vRNA molecules and their locations starting at the early stage of infection and quantitatively determine vRNA densities and distributions under the influence of antiviral drugs.

Experimental Section

Polymeric Sequence Probe Production

The nucleic acid polymeric sequence probe was prepared by mixing 1 μ L of 100 μ M circle DNA, 3 μ L of 100 μ M primer, 5 μ L of 10 $\times$ T4 DNA ligase reaction buffer, and 1 μ L of T4 DNA ligase (Fermentas) in 40 μ L of DI water. The solution was incubated at room temperature for 1 h, then the T4 DNA ligase was deactivated at 65 $^{\circ}$ C for 10 min. The RCA reaction was made by adding 5 μ L of the ligation product, 0.5 μ L of 10 mM dNTP, 1 μ L of 10 unit/ μ L Phi 29 Polymerase (Epicentre Biotechnologies), 5 μ L of 10 $\times$ RCA buffer, and 1 μ L of 1% BSA into 37.5 μ L of DI water. The solution was incubated at 30 $^{\circ}$ C for 1 h to form PSPs, which were stable in storage buffer at room temperature. The sequences of the oligonucleotides used in the PSP assay are listed in Table 1.

Cell Culture and Viral Infection

MDCK cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Cellgro) supplemented with 10% fetal bovine serum (FBS, Omega) and 1% P/S (penicillin streptomycin, Gibco). Cells were cultured in tissue culture plates at 37 $^{\circ}$ C and 10% CO₂ and passaged using 0.25% Trypsin-EDTA (Gibco). Influenza A/WSN/33 viruses were generated by reverse genetics following the protocol reported previously(11) and aliquoted and stored at -80 $^{\circ}$ C. The viruses were later titrated using a plaque assay in MDCK cells. MDCK cells were seeded on glass slides in 60 cm plates overnight. During the viral infection, MDCK cells were incubated with the virus at a multiplicity of infection (MOI) of 5 at 37 $^{\circ}$ C and 10% CO₂.

Drug Treatment

MDCK cells were seeded on glass slides in 60 cm plates overnight. Influenza A/WSN/33 viruses were added to MDCK cells at MOI of 5 and incubated at 37 $^{\circ}$ C and 10% CO₂. Ribavirin (Santa Cruz Biotechnology) with a concentration of 120 μ g/mL was added during the infection.

Sample Preparation

After infection, cells were fixed on glass slides with PBS containing 4% paraformaldehyde and permeabilized by incubating in 0.5% Triton X-100 for 1 min followed by a PBS wash. After that, the cells were dehydrated through a series of 70%, 90%, and 99.5% ethanol for 3 min each.

Fluorescence in Situ Hybridization Process

The whole assay was performed at room temperature. The cells fixed on glass slides were prehybridized for 5 min in prehybridization buffer (2 $\times$ SSC, 1 $\times$ Denhardt's solution, 1 mM EDTA). Then the probe hybridization solution (PSP in 1 $\times$ SSC buffer) was added to the slides. To ensure the detection of single vRNA, the density of the PSPs was significantly

higher than the possible density of vRNA inside the cells. After incubation, the slides were thoroughly washed with 2× SSC and then 1× SSC for 3 min each. A solution of 400 nM fluorescence complementary oligos in 4× SSC with 10% formamide and actin stain (Alexa Fluor 488 phalloidin from Invitrogen) was added to the slides and incubated for 30 min. The slides were washed with PBS twice for 3 min each. Before imaging, the slides were mounted with ProLong Gold (Invitrogen) mounting medium with DAPI. The fluorescence images were taken by a Nikon E400 fluorescence microscope with Chroma filters for Cy3.5, FITC and DAPI. The PSP bounded with the vRNA inside each cell were recognized and counted from the fluorescence images by Nikon NIS Element software.

Results and Discussion

NA (Neuraminidase) vRNA in MDCK cells infected by the influenza (A/WSN/33) viruses with a MOI of 5 was detected by PSP. The fluorescence images of the infected MDCK cells at a series of post infection (P.I.) time points ranging between 20 min and 6 h are shown in Figure 1. The cell nuclei were stained with DAPI, and actin were stained with Alexa Fluor 488 phalloidin. The single vRNA molecule can be observed clearly inside the cells, and each red bright dot in the images represents a single vRNA molecule bound with a PSP. No PSP dot was observed in a large amount of mock cells without viral infection, and no PSP dot was observed beyond the cells, which indicate that the PSP has an extremely low nonspecific binding rate. The numbers of the vRNA molecules in the cells can be quantitatively measured by counting the PSP dots, and the average number of the vRNA molecules per cell is shown versus the P.I. time in Figure 2. The vRNA was detected in the cytoplasm as early as 20 min after infection. The average number of vRNA per cell increases linearly versus the P.I. time between 20 and 40 min, indicating that the vRNAs gradually entered the cell from the environment at this stage. The replication of vRNA started after 1 h of infection. The average number of vRNA per cell increases exponentially versus the P.I. time between 1 and 6 h (Figure 2). The average number of vRNA per cell kept increasing versus P.I. time after 6 h P.I. time until it reached a saturation point. However, at the late stage of infection, the density of the PSP dots was too high to identify the single vRNA molecules in fluorescence images. The error bars in Figure 2 show the distribution of the virus to each cell (not the system error). Each data point was derived from 50 independent cells. It can be seen from Figure 2 that the vRNA molecule per cell varies from cell to cell.

The drug effect on virus infection was also studied using the PSP assay. Ribavirin, a known inhibitor of influenza virus RNA synthesis,(12–14) was added at the beginning of the infection. Figure 3A showed the fluorescence images of the infected cells with or without ribavirin treatment at 2, 4, and 6 h P.I. time points. At all the three time points, the ribavirin treated cells showed a significant reduction in vRNA counts. Figure 3B shows the tracking effect of ribavirin at 2, 4, and 6 h P.I. It is observed that vRNA count in the ribavirin treated cells was approximately reduced by a factor of 2.3 in comparison with the untreated cells at 2 h P.I. time point, by a factor of 5.2 at 4 h P.I. time point, and by a factor of 11.9 at 6 h, P.I. time point. The average number of vRNA per cell remained unchanged from 2 to 4 h P.I. with ribavirin, which may be due to the vRNA molecules degradation after 2 h P.I.(15) The average number of vRNA per cell increased slightly after 4 h P.I., indicating the decrease of

the drug effect after 4 h P.I.(13) No vRNA was observed in mock (no infection) or drug only control cells (6 h incubation with ribavirin but without virus infection), which confirms that the PSP probe has an extremely low nonspecific binding rate.

Conclusions

In this letter we report the detection of single influenza A (influenza A/WSN/33) NA (neuraminidase) vRNA in MDCK cells by a PSP. The PSP is a single-stranded DNA molecule with ~2 000 tandem repeat fluorescence binding sites and target binding sites that bind with multiple fluorescence complementary oligos and the target vRNA in a FISH process. Single vRNA molecules hybridized with the PSP can be visualized under a conventional fluorescence microscope with ultrahigh sensitivity and spatial resolution. The vRNA can be detected in the cells as early as 20 min after the infection. The average number of vRNA per cell increases linearly versus the postinfection time between 20 and 40 min, indicating that the vRNA gradually enters the cell from the environment at this stage. The replication of vRNA started about 1 h after infection, and then the average number of vRNA per cell increased exponentially versus the post infection time between 1 and 6 h. A significant reduction of vRNA counts was observed in the infected cells treated with ribavirin. The PSP-based assay enables the quantitative analysis of the viral infection process and drug effect with ultrahigh sensitivity and spatial resolution.

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Figure 1.

Fluorescence images of MDCK cells infected by influenza viruses at different postinfection time points. A red dot represents a vRNA labeled by a PSP, and cell nuclei are stained with DAPI (blue). Scale bar: 10 μm .

Figure 2.

Average number of vRNA molecules per cell versus the postinfection time. The insert shows the data of the postinfection time within 1 h. The error bars indicate 1 standard division of the counted vRNA molecules numbers in different individual cells. A dashed line indicates the exponential fitting to the data with the postinfection time between 1 and 6 h. In the insert, a dashed line indicates the linear fitting to the data of the postinfection time within 1 h.

Figure 3.

(A) Fluorescence images of MDCK cells infected by influenza viruses with ribavirin (top row) and without ribavirin (bottom row) at different postinfection time points. A red dot represents a vRNA labeled with a PSP. Cell nuclei are stained with DAPI. (B) Tracking effect of ribavirin in influenza A-infected MDCK cells. Bar graph shows the average number of vRNA molecules per cell with and without ribavirin treatment. No vRNA was observed in mock (no infection) or drug only control cells (6 h incubation with ribavirin but without virus infection). Scale bar: 10 μ m.

Scheme 1.

Fluorescence in Situ Hybridization (FISH) Process to Detect Influenza Viral RNA (vRNA) Inside a Cell Using a Polymeric Sequence Probe (PSP), a Single-Stranded DNA Molecule with ~2 000 Repeat Target Binding Sites, and Fluorescence Oligos

^aAfter the cell is permeabilized, the PSP can diffuse into the cell, bind with targeted vRNA and multiple fluorescence complementary oligos. A single vRNA molecule labeled by the PSP is shown as a red dot in an infected MDCK cell with its nuclei stained by DAPI and actin stained by Alexa Fluor 488 phalloidin, respectively, in an inserted fluorescence microscope image.

Table 1

Sequences of the Oligonucleotides Used in the PSP Assay

circle DNA	5' phosphate-AAA TAC AAC GGC ATA ATA ACT GAA ACC ATA AGA GCA GAC AAC GAG GAC ACG CAT AA-3'
primer	5'-GTT GTA TTT TTA TGC GTG TC-3'
flcomp	5' Alexa 546-GA GCA GAC AAC GAG GAC ACG -3'