# SPECIFIC DETECTION AND REPLICATION KINETICS OF SARS-CoV-2 USING smRNA-FISH

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**Rajiv Pathak**[1], Carolina Eliscovich[1], Updesh Dixit[1], Nacho Mena[2], Adolfo Garcia-Sastre[2], James M. Pullman[3], Robert H. Singer[1], Ganjam V. Kalpana[1]

[1]Albert Einstein College of Medicine, Bronx, NY, United States, [2]Icahn School of Medicine at Mt Sinai, New York, NY, United States, [3]Montefiore Medical Center, Bronx, NY, United States

## BACKGROUND

SARS-CoV-2 is a positive-sense single-stranded RNA virus and its replication begins after the synthesis of virally encoded polymerase complex that is required for replication and transcription of genomic RNA (gRNA) within the infected cells. Despite the global interest in the study of SARS-CoV-2, the kinetics of SARS-CoV-2 RNA replication and transcription during the early phase of viral infection is poorly understood. Here, we used the single-molecule RNA fluorescence in situ hybridization (smRNA-FISH) for sensitive detection of SARS-CoV-2 at the single molecule level and to determine the replication of genomic RNA (gRNA) and sub-genomic RNA (sgRNA) in the infected cells, at very early stages of infection.

## METHODS

We designed highly specific smRNA-FISH probes targeted to gRNA and Spike gene sgRNA of SARS-CoV2 virus, using stellaris method. We performed in silico probe sequence specificity analysis against the whole RefSeq genome or transcriptome assembly using 'bowtie2' (2.4.4) (**Fig. 1**). We optimized the method to simultaneously visualize these two RNAs at single-cell and single-molecule levels using SARS-CoV2-infected Vero E6 cells (**Fig. 2**). Because of the high sensitivity of our probes, we applied smRNA-FISH technology to detect SARS-CoV-2 positive cells from autopsy samples obtained from diseased COVID-19 patients. Furthermore, we used high resolution and high-speed scanning microscopy to detect the extent of infection in cell models of SARS-CoV-2 and in COVID-19 patient samples.

Fig-1a:

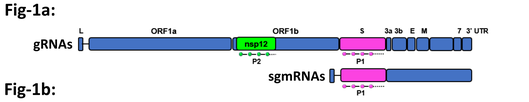



Fig-1b:

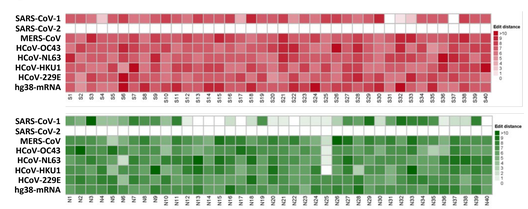



**Figure 1(a):** Top Panel: Cartoon representation of genomic and spike sub-genomic RNA. **(b) Bottom two panels**: The uniqueness of the probes represented as "edit distance".

Fig-2a:

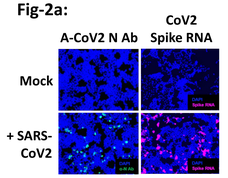



Fig-2b:

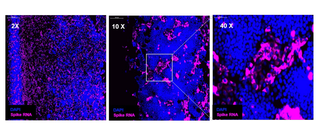



**Figure 2: (a)** Specificity of SARS_CoV2 spike RNA Probe (P1): Vero cells with or without SARS_CoV2 24 hours post-infection (MOI: 0.5 PFU/cell) probed with anti-N antibody and spike RNA probe (P1), respectively. **(b).** Detection of plaque formation at SARS-CoV2 24-hour post-infection (using spike RNA-FISH probe P1).

- We have successfully applied smRNA-FISH to detect both gRNA and spike sgRNAs of SARS-CoV2 at the single-molecule level.
- Single molecules of gRNA of SARS-CoV-2 could be detected as little as 30 min to 2 hr. post-infection.
- Distinct "Replication Centers" (RC) began to appear 1 to 2 hr. post-infection and the sgRNAs began to migrate out of these RCs.
- The method was successfully applied to the patient samples.
- The signals of gRNA and spike sgRNA along with the spike proteins co-localized within the same cells of the lung autopsy samples of SARS-CoV-2 infected patients.

## RESULTS

A time-course analysis of SARS-CoV-2 replication indicated that single molecules of gRNA could be detected as little as 30 min to 2 hr. post-infection (**Fig. 3**). Distinct "Replication Centers" (RC) began to appear one to two hours post-infection and the sgRNAs began to migrate out of these RCs (**Fig. 4**). Replication after the initial delay appeared to be rapid and gRNA and sgRNAs dispersed throughout the cell within 4-5 hours post-infection forming multiple RCs **(Fig. 5)**. We found that our RNA-FISH correctly detected the SARS-CoV2 positive samples from patient autopsy samples that were characterized by qRT-PCR or immunological detection methods (**Fig. 6**). The signals of spike gRNA and sgRNA (**Fig. 7**) along with the spike proteins (**Fig. 8**) co-localized within the same cells of the SARS-CoV-2 infected patients within the cells of lung samples. Taken together, RNA-FISH offers the ability to directly and specifically visualize viral RNA in single cells and is ideal to address questions both about the cell types infected by SARS-CoV2 and the subcellular localization of viral transcripts.

Fig-3:

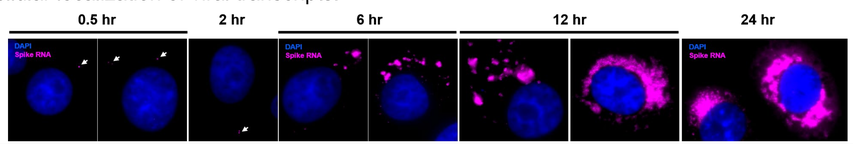



**Figure 3:** A time course analysis of early stages of SARS-CoV2 replication: The Vero E6 cells were infected for various length of period, stained with DAPI and visualized to detect single molecules. The panels represents single cells at indicated time post-infection. Viral RNA is shown in magenta.

Fig-4a:

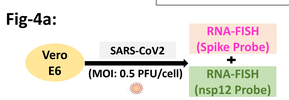



Fig-4b:




**Figure 4: Detection of genomic and sub-genomic RNAs: (a)** SARS-CoV2 infected cells were hybridized with probes against either spike RNA (P1) or nsp12 RNA (P2) or both probes together at 6 hpi (20X magnifications) **(b)** Detection of gRNA and spike sgRNA simultaneously at various times post infection (76.3X magnifications).

Fig-5:

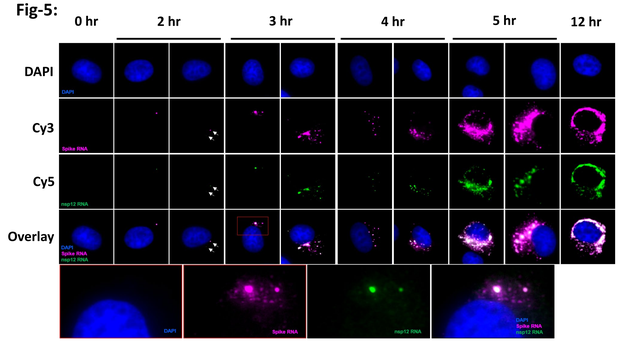



**Figure 5: Single-molecule analysis** to determine the kinetics of SARS CoV-2 replication of both gRNA and sgRNA at 0, 2, 3, 4, 5 and 12 hpi. The bottom row of panels shows zoom of a cell at 3h time point, focusing on two RCs.

Fig-6:

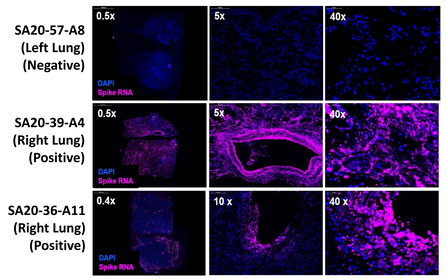



**Figure 6:** Highly sensitive and specific detection of SARS-CoV2 in autopsy samples. RNA-FISH analysis of right lung from patient SA20-39-A4; right lung from patient SA20-36-A11 and left lung from qRT-PCR negative patient SA20-57-A8.

Fig-7:

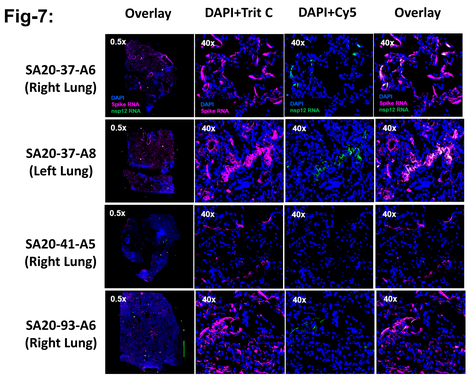



**Figure 7:** Detection of both gRNA and sgRNA from three different SARS-CoV2 infected patients lung autopsy samples, SA-20-37, SA-20-41 and SA-20-93.

Fig-8:

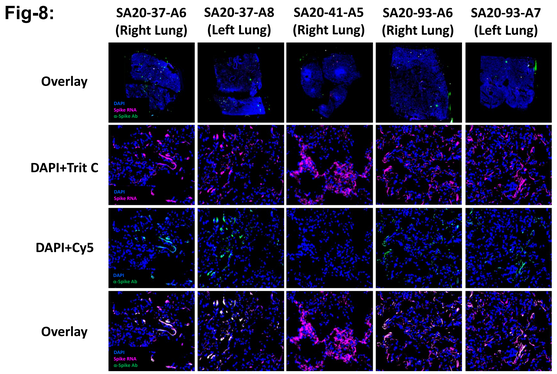



**Figure 8:** Multiplex analysis of SARS-CoV2 replication in autopsy samples using RNA-FISH and Immunofluorescence (IF). Detection of both spike gRNA and sgRNA using probe P1 along with spike protein using anti-Spike Ab from five different SARS-CoV-2 infected patient's lung autopsy samples.

## CONCLUSIONS

We propose that the specific probes and the methodology that we have developed will be highly applicable to the study of SARS-CoV-2 replication in-depth and to characterize SARS-CoV-2 infection in COVID-19 patient samples. This study may open a novel direction towards COVID-19 pathophysiology, drug screening, and diagnostics.

## ACKNOWLEDGEMENTS

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