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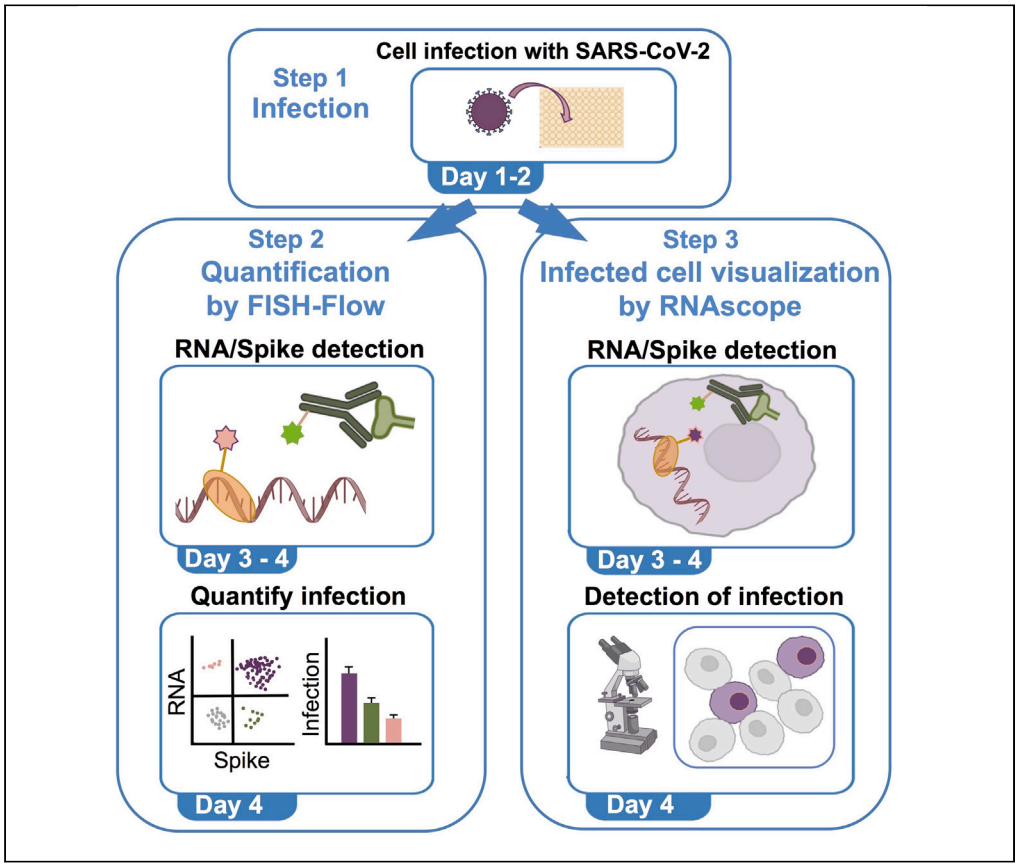
Protocol to detect infectious SARS-CoV-2 at low levels using *in situ* hybridization techniques



Low and persistent levels of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) RNA/protein/virus can be detected in clinical samples months after infection, possibly related to the emergence of SARS-CoV-2 variants or development of long coronavirus disease. Here, we present a protocol to detect low levels of viral RNA together with protein using flow cytometry and microscopy. We describe steps for cell infection with SARS-CoV-2 and quantification by fluorescence *in situ* hybridization-flow cytometry. We then detail procedures for visualization using immunolabeling and RNAscope. This approach is directly applicable to clinical samples.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Protocol to quantify low level of infectious SARS-CoV-2 at the single-cell level

Immunolabeling and *in situ* hybridization details to detect spike protein and viral RNA

Combining morphological and flow cytometry quantifications for improving robustness

Guidance to deal with background and to improve signal-to-noise ratios

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Protocol to detect infectious SARS-CoV-2 at low levels using *in situ* hybridization techniques

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SUMMARY

Low and persistent levels of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) RNA/protein/virus can be detected in clinical samples months after infection, possibly related to the emergence of SARS-CoV-2 variants or development of long coronavirus disease. Here, we present a protocol to detect low levels of viral RNA together with protein using flow cytometry and microscopy. We describe steps for cell infection with SARS-CoV-2 and quantification by fluorescence *in situ* hybridization-flow cytometry. We then detail procedures for visualization using immunolabeling and RNAscope. This approach is directly applicable to clinical samples.

For complete details on the use and execution of this protocol, please refer to Zhu et al. (2022).[1]

BEFORE YOU BEGIN

The protocol below describes the specific steps for using Vero E6 cells (referred to as Vero cells) as reporter cells for detection of low amounts of SARS-CoV-2. This protocol can be adapted for other infected cell culture supernatants or biological other fluids from clinical samples.[1]

The protocol describes two complementary techniques for detection of *in situ* hybridized SARS-CoV-2 RNA: one quantitative at the single cell level using flow cytometry, also known as FISH-flow[2,3] and the other semi-quantitative using microscopy from samples processed for *in situ* hybridization using the "RNAscope" technology.[3,4]

Design of FISH-flow probes

Timing: 1 h for design, 1–2 weeks for delivery of probes, 1 h for preparing probe set stocks

1. Design the Cy5-tagged probe sets for SARS-CoV-2 gene targets using the LGC Biosearch Technologies Stellaris Probe designer tool, a software free with registration, accessed at https://www.biosearchtech.com/support/tools/design-software/stellaris-probe-designer.
   a. Create an account at the LGC Biosearch Technologies website: https://www.biosearchtech.com/stellaris-designer
   b. Log in with username and password.
   c. Complete the required fields.

i. For organism, select "Other."
ii. Masking level defaults to "2."
iii. Set number of probes to "48," oligo length to "20," and min spacing length to "2."

d. Enter the target sequences provided below into the provided field.
e. Select "Design probes."

⚠ CRITICAL: The tool designs probes that are antisense to the input sequence. It corresponds to the present protocol established for the detection of positive-sense vRNA. Therefore, enter the 5′→3′ cDNA target sequence; the resulting probes will be anti-positive-sense vRNA.

f. After selecting "Design probes," the "Review design results" screen displays. By selecting "Order", the probes can be purchased directly through LGC Biosearch Technologies. Select Quasar 670 as the desired fluorophore and add the item to the cart. Fluorophore selection depends on the optical system that will be used for analysis and must be chosen such as the optical system used can detect signal in this wavelength.

Note: In this FISH-flow assay, we used a combination of 4 different probe sets (40–48 probes each) covering 2 regions of the *RdRp* gene, and the *S* and *N* genes (Wuhan strain, reference genome: GenBank : NC_045512) as described in the table below. In contrast, targeting only the most variable *S* gene failed to detect SARS-CoV-2 infected cells obtained from samples from infected patients (data not shown). The detection of SARS-CoV-2 variants may require the design of variant-specific probes, although the combination of probes described here detected alpha, beta, delta, and omicron variants (see below). It should be noted that using only probe set 1 that targets the *RdRp* gene did not detect SARS-CoV-2 alpha infection.

Probe set 1 First part of *RdRp* gene (12688-14185)	Probe set 2 Second part of *RdRp* gene (14186-16236)	Probe set 3 *N* gene (28274-29533)	Probe set 4 *S* Gene (21631-23303)
GCAACAGGACTAAGCTCATT	CAACATGTGACTCTGCAGTT	TGATTTTGGGGTCCATTATC	GAATTAGTGTATGCAGGGGG
AGCACAAGACATCTGTCGTA	CCCACTTAATGTAAGGCTTT	AACGTAATGCGGGGTGCATT	GGGTAATAAACACCACGTGT
CAAGCAGTTTGTGTAGTACC	TCTCTTCCGTGAAGTCATAT	AGTTGAATCTGAGGGTCCAC	ACAAGTCCTGAGTTGAATGT
AGCTAACGCATTGTCATCAG	ATTTGGGTGGTATGTCTGAT	GTTCTCCATTCTGGTTACTG	GGTCCCAGAGACATGTATAG
GTGCAAGTACAAACCTACCT	TGCAGAATGCATCTGTCATC	CGACGTTGTTTTGATCGCGC	GACAGGGTTATCAAACCTCT
GCCCATTTCAAATCCTGTAA	GTGGTCCAAAACTTGTAGGT	AGACGCAGTATTATTGGGTA	ACCAAAAATCCAGCCTCTTA
TCCATCACTCTTAGGGAATC	CTGAAGTGGTATCCAGTTGA	TGTTGAGTGAGAGCGGTGAA	ATAAGTAGGGACTGGGTCTT
GGTTCCAGTTCTGTATAGAT	CCTGATTATGTACAACACCT	GGGAATTTAAGGTCTTCCTT	ACACCCAAAAATGGATCATT
CACTTTAGGACCTTTAGGTG	TAGATTACCAGAAGCAGCGT	AATTGGAACGCCTTGTCCTC	ACTCTGAACTCACTTTCCAT
CCATACCTCTATTTAGGTTG	ACGTAGTGCGTTTATCTAGT	CATCTGGACTGCTATTGGTG	GTGCAATTATTCGCACTAGA
GTGGCAGCTAAACTACCAAG	ATTACCGGGTTTGACAGTTT	CTTCGGTAGTAGCCAATTTG	AGGCTGAGAGACATATTCAA
ATTACCAGCTTGTAGACGTA	AGAAACCCTTAGACACAGCA	ACCACGAATTCGTCTGGTAG	TTCCTTCAAGGTCCATAAGA
TACAGTTGAATTGGCAGGCA	ACCATCCTGAGCAAAGAAGA	GATCTTTCATTTTACCGTCA	CGCACTAAATTAATAGGCGT
TGTAAGCTTTAGCAGCATCT	CATAATCGCTGATAGCAGCA	TTTGTTAGCACCATAGGGAA	AAGCCGAAAAACCCTGAGGG
AATTAGTGATTGGTTGTCCC	AATACAGCCACCATCGTAAC	CAGTTGCAACCCATATGATG	GGCAAATCTACCAATGGTTC
CCAGTGTGTGTACACAACAT	TGACGATGACTTGGTTAGCA	GATTGCAGCATTGTTAGCAG	ACCTAGTGATGTTAATACCT
GTGTAACTGTTATTGCCTGA	AACCAGCTGATTTGTCTAGG	TAGAAGCCTTTTGGCAATGT	ATAATAAGCTGCAGCACCAG
TTCTTGATCCATATTGGCTT	GCATCTTGATCCTCATAACT	ACGAGAAGAGGCTTGACTGC	TCCTAGGTTGAAGATAACCC
AACACGATGCACCACCAAAG	GAGCTCTATTCTTTGCACTA	TGTTGCGACTACGTGATGAG	TCTACAGCATCTGTAATGGT
TATGTGGCAACGGCAGTACA	AGATAGAGACACCAGCTACG	GCCTGGAGTTGAATTTCTTG	TGAGAGAGGGTCAAGTGCAC
ATCCTTTAGGATTTGGATGA	GGCGGCTATTGATTTCAATA	CAGGAGAAGTTCCCCTACTG	GATTTCAACGTACACTTTGT
GGTATTTGTACATACTTACC	TTACTACAGTAGCTCCTCTA	CAGCAAAGCAAGAGCAGCAT	GATTCCTTTTTCTACAGTGA
CAGGGTCATTAGCACAAGTT	CCATAGAATTTGCTTGTTCC	AGCTGGTTCAATCTGTCAAG	AGATTCTGTTGGTTGGACTC
AGACGGTACAGACTGTGTTT	GTTTTTAACATGTTGTGCCA	TTTACCAGACATTTTGCTCT	CCAAAAGGGCACAAGTTTGT
AGCCATAACCTTTCCACATA	GTGAGGGTTTTCTACATCAC	TAGTGGCAGTACGTTTTTGC	TCTGGTGGCGTTAAAAACTT

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Continued

Probe set 1 First part of *RdRp* gene (12688-14185)	Probe set 2 Second part of *RdRp* gene (14186-16236)	Probe set 3 *N* gene (28274-29533)	Probe set 4 *S* Gene (21631-23303)
TTCGCGGAGTTGATCACAAC	GCATGGCTCTATCACATTTA	GCTTGTGTTACATTGTATGC	TCCAAGCATAAACAGATGCA
TGTGCATCAGCTGACTGAAG	CGGTGTGACAAGCTACAACA	TTTGTTCTGGACCACGTCTG	GCGGAATTATATAGGACAGA
CCGCAAACCCGTTTAAAAAC	ACTTGAGCACACTCATTAGC	TGGTCCCCAAAATTTCCTTG	GGAGACACTCCATAACACTT
TGTAAGACGGGCTGCACTTA	ACACATGACCATTTCACTCA	AGTTCCTTGTCTGATTAGTT	ATTTGTCTGACTTCATCACC
CATCAGTACTAGTGCCTGTG	TTCCACCTGGTTTAACATAT	CAATTTGCGGCCAATGTTTG	CAGCAATCTTTCCAGTTTGC
ATGTCAAAAGCCCTGTATAC	AAGTGCATTAACATTGGCCG	GAAGCGCTGGGGGCAAATTG	GATTGTTAGAATTCCAAGCT
ACCAGCTACTTTATCATTGT	CGGCAATTTTGTTACCATCA	ATGCGCGACATTCCGAAGAA	TTACCACCAACCTTAGAATC
AATTGTCATCTTCGTCCTTT	GTCTGTGTTGTAAATTGCGG	CGAAGGTGTGACTTCCATGC	GGTTTGAGATTAGACTTCCT
CATGTTTAGCAACAGCTGGA	AGTCTGTGTCAACATCTCTA	CTGTGTAGGTCAACCACGTT	GCCTGATAGATTTCAGTTGA
GTCACCGTCTATTCTAAACT	CACAACAGCATCGTCAGAGA	ATTTGGATCTTTGTCATCCA	ACCATTACAAGGTGTGCTAC
GACGTGATATATGTGGTACC	ACCTTGAGATGCATAAGTGC	TTGTATGCGTCAATATGCTT	TACTACTCTGTATGGTTGGT
TCTGCCATTGTGTATTTAGT	AAGTTCTTTATGCTAGCCAC	GGTAAGGCTTGAGTTTCATC	GGTGCATGTAGAAGTTCAAA
ATGCCTTAAAGCATAGACGA	TCAGTCCAACATTTTGCTTC	GAAGAGTCACAGTTTGCTGT	GACACATTTGTTTTTAACCA
GTGTCACAATTACCTTCATC	TGAGGTCCTTTAGTAAGGTC	ATCATCCAAATCTGCAGCAG	GCCTGTTAAACCATTGAAGT
ACAAAATCATACCAGTCCTT	AGCATTGTATGTTGAGAGCA	GGATTGTTGCAATTGTTTGG	TAGACTCAGTAAGAACACCT
GTTGGCGTATACGCGTAATA	TGGATCTGGGTAAGGAAGGT		ATCACGGACAGCATCAGTAG
AAAGCTTGGCGTACACGTTC	TCATCTACAAAACAGCCGGC		
CGCATGGCATCACAGAATTG	GTGTACCATCTGTTTTTACG		
GTACACCAACAATACCAGCA	AGACACGAACCGTTCAATCA		
CCGAAATCATACCAGTTACC	AGTGGGTAAGCATCTATAGC		
CTGGCGTGGTTTGTATGAAA	GCATACTCCTGATTAGGATG		
TCTACAACAGGAACTCCACT	CCCAATACCTTGAAGTGTTA		
GGTCAAGGTTAATATAGGCA	AAGACTGTATGCGGTGTGTA		

2. Aliquot each Quasar 670-labeled probe set separately at 12.5 μM in H_2O in 2 μL aliquots in RNA-free 1.5 mL tubes upon receipt for single use.
3. Store aliquots at −80°C. In such storage conditions, probes are stable for several years.

Preparation of Vero cell cultures

Timing: 2 days

4. Get Vero cells ready for infection.
   a. Culture Vero cells DMEM supplemented with Penicillin/streptomycin (100 U/mL) and 10% FCS (D10) in standard conditions (5% CO_2, 37°C).
   b. Trypsinize Vero cells using Trypsin 0.05% v/v for 5 min at 37°C.
   c. Neutralize trypsin with an equal volume of D10.
   d. Centrifuge cells.
   e. Resuspend cells in D10.
   f. Seed 50,000 cells per well of a 96-flat well plate.
   g. Place the plate at 37°C until the next day (> 15 h).

Preparation of buffers

Timing: 2 h

5. Prepare buffers (as described in the Materials and Equipment section) and a fresh paraformaldehyde 4% solution in PBS from the 16% stock using Nuclease-free reagents.

⚠ CRITICAL: Paraformaldehyde is highly toxic. It is a strong irritant and has caustic effects on skin, eyes and mucous membranes. There is limited evidence of carcinogenic effects. Handle it in a well-ventilated workspace and handle the 16% stock in a fume hood to avoid breathing vapor.

⚠ CRITICAL: Growing the cells until confluence ensures optimal detection of infectious particles. In contrast, over-confluence is detrimental to the infection of the cells.

⚠ CRITICAL: Plan to have at least three infected cell replicates per virus dilution and per sample to ensure robustness of detection and analyzes.

Setting incubators

Timing: 2 h

6. Set two different non-CO_2 incubators to 37°C and 40°C.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-SARS-CoV/SARS-CoV-2 (COVID-19) spike antibody (1A9), mouse IgG1 monoclonal (concentration: 2 µg/mL)	GeneTex	GTX632604
Alexa Fluor 488 AffiniPure goat anti-mouse IgG1 (dilution 1:8000)	Jackson ImmunoResearch	115-545-205
SARS-CoV-2 spike S1 subunit Alexa Fluor 488-conjugated antibody, monoclonal mouse IgG_{2A} clone# 1035226 (dilution 1:250)	R&D Systems	FAB105805G-100UG
Biological samples		
Horse serum	Vector Laboratories	FV-93951-74
Human serum	Sigma-Aldrich	H4522
SARS-CoV-2 variants of concern (VOCs)	Desmarets et al.[9]	Desmarets et al.[9]
Chemicals, peptides, and recombinant proteins		
Paraformaldehyde 16%	Electron Microscopy Sciences	15710
Trypsin 0.05%	Gibco	25300-024
TPCK-treated trypsin	Sigma	T1426
Phosphate-buffered saline without calcium and magnesium	Gibco	14190-114
10× PBS nuclease-free	Thermo Fisher Scientific	AM9939
RNase-free BSA	Thermo Fisher Scientific	AM2616
Nuclease-free H_2O	Thermo Fisher Scientific	10977-035
Tween 20	Sigma	P1379
Saponin	Sigma	S-7900
20× saline-sodium citrate (SSC) buffer	Sigma	SRE0068
Formamide	MP Biomedicals	FORMD002
Dextran sulfate	Calbiochem	265152
Yeast tRNA	Thermo Fisher Scientific	15401029
10× TRR, RNAscope target retrieval reagents	Advanced Cell Diagnostics (ACD)	322000
Protease Plus, provided in RNAscope H_2O_2 and Protease Plus reagents	ACD	322330
AMP1-3, HRP-C1, HRP blocker, TSA buffer are provided in RNAscope multiplex fluorescent v2	ACD	323110

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
50× wash buffer, RNAscope wash buffer reagents	ACD	310091
Opal 570	Akoya Biosciences	FP1488001KT
ibidi mounting medium	ibidi	50001
DAPI	Sigma-Aldrich	D8417-10MG
Gelatin from cold water fish skin (45%)	Sigma-Aldrich	G7765-1L
EDTA (0.5 M)	Sigma-Aldrich	03609
BSA (fraction V)	Sigma-Aldrich	10735086001
Viobility 405/452 fixable dye	Miltenyi	130-130-420
Experimental models: Cell lines		
Vero E6 cells	ATCC	CRL-1587
DMEM	Gibco	41966-029
Fetal calf serum (FCS)	Eurobio	CVFSVF06-01
Penicillin/streptomycin	Gibco	15140-022
Oligonucleotides		
Probe set 1 First part of *RdRp* gene (12688-14185)	LGC Biosearch Technologies	This study
Probe set 2 Second part of *RdRp* gene (14186-16236)	LGC Biosearch Technologies	This study
Probe set 3 *N* gene (28274-29533)	LGC Biosearch Technologies	This study
Probe set 4 *S* gene (21631-23303)	LGC Biosearch Technologies	This study
RNAscope SARS-CoV-2 spike probe: RNAscope Probe - V-nCoV2019-S	ACD	848561
Software and algorithms		
Stellaris probe designer tool	LGC Biosearch Technologies	https://www.biosearchtech.com/support/tools/design-software/stellaris-probe-designer
FlowJo	FlowJo (BD)	Version 10.7.2
GuavaSoft	Millipore	Version 3.4
Other		
Poly-L-lysine-coated slides: poly-prep slides	Sigma-Aldrich	P0425-72EA
Hydrophobic pen: ReadyProbes Hydrophobic Barrier Pap Pen	Thermo Fisher Scientific	R3777
Flow cytometer	Merck	Guava easyCyte 12HT System
Confocal microscope	Olympus	IXplore, spin microscope

MATERIALS AND EQUIPMENT

PBS-FISH

Reagent	Final concentration	Amount
10× PBS Nuclease-free	1×	5 mL
RNase-free BSA	0.2 mg/mL	0.2 mL
Nuclease-free H_2O	N/A	44.8 mL
Total	**N/A**	**50 mL**

⚠ CRITICAL: Pass PBS-FISH through a 0.22 μm filter to sterilize and keep at 4°C for 2 months maximum for storage.

Stock solution of permeabilization buffer (10% PERM-FISH-Tween Stock)

Reagent	Final concentration	Amount
Tween 20	10% v/v	5 mL
10× PBS Nuclease-free	1×	5 mL

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Continued		
Reagent	Final concentration	Amount
Nuclease-free H_2O	N/A	40 mL
Total	**N/A**	**50 mL**

⚠ CRITICAL: Pass 10% PERM-FISH-Tween Stock through a 0.22 μm filter to sterilize and keep at 20°C–23°C for a maximum of 12 months. This later is a stock solution for long-term storage.

⚠ CRITICAL: Tween 20 is viscous and needs to be pipetted slowly, rinsing the pipette several times into the solution for proper mixing. Avoid formation of bubbles. Pipettes with big orifices are also useful for pipetting viscous solution like Tween 20.

Permeabilization buffer (PERM-FISH-Tween)		
Reagent	Final concentration	Amount
10% PERM-FISH-Tween stock	2% v/v (corresponding to 0.2% v/v final for Tween 20)	1 mL
10× PBS Nuclease-free	1×	5 mL
Nuclease-free H_2O	N/A	44 mL
Total	**N/A**	**50 mL**

⚠ CRITICAL: Pass PERM-FISH-Tween through a 0.22 μm filter to sterilize and keep at 20°C–23°C. This is the solution to be used in the reaction and to be discarded after use

Alternatively prepare Permeabilization buffer Saponin (PERM-FISH-Sapo)		
Reagent	Final concentration	Amount
Saponin 10% in nuclease-free H_2O	0.1% (dilute 100×)	500 μL
FCS	2%	1 mL
PBS 1×		48.5 mL
Total	**N/A**	**50 mL**

⚠ CRITICAL: Pass PERM-FISH-Sapo through a 0.22 μm filter to sterilize and keep at 20°C–23°C. This is the solution to be used in the reaction and to be discarded after use.

Hybridization wash buffer (HWB)		
Reagent	Final concentration	Amount
20× SSC	2 x	5 mL
Formamide	10% v/v	5 mL
RNase-free BSA	0.2 mg/mL	0.2 mL
Nuclease-free H_2O	N/A	39.8 mL
Total	**N/A**	**50 mL**

⚠ CRITICAL: Warm Formamide stored at 4°C to 20°C–23°C before use.

⚠ CRITICAL: Formamide is a carcinogen. Work in a fume hood to avoid breathing vapor. Avoid mixing techniques that generate aerosols (i.e., pipette instead of vortex). Avoid direct skin contact. Collect all waste containing formamide and dispose according to institutional environmental health and safety guidelines.

⚠ CRITICAL: Pass HWB through a 0.22 μm filter to sterilize and keep at 4°C for 2 months maximum for storage.

Hybridization buffer 10% dextran sulfate (HB10%dx)		
Reagent	Final concentration	Amount
Dextran sulfate	10% w/v	5 g
Yeast tRNA	0.1% w/v	50 mg
20× SSC	2×	5 mL
Formamide	10% v/v	5 mL
RNase-free BSA	0.2 mg/mL	0.2 mL
Nuclease-free H_2O	N/A	Top to 50 mL
Total	**N/A**	**50 mL**

Note: Dissolve first the Dextran Sulfate and the Yeast tRNA in 30 mL of Nuclease-free water. Then add the SSC buffer, the formamide and the RNase-free BSA and top to 50 mL of total volume with Nuclease-free water.

⚠ CRITICAL: Warm Formamide stored at 4°C to 20°C–23°C before use.

⚠ CRITICAL: Formamide is a carcinogen. Work in a fume hood to avoid breathing vapor. Avoid mixing techniques that generate aerosols (i.e., pipette instead of vortex). Avoid direct skin contact. Collect all waste containing formamide and dispose according to institutional environmental health and safety guidelines.

⚠ CRITICAL: This solution is viscous: avoid formation of bubbles before aliquoting and freezing.

⚠ CRITICAL: Pass HB10%dx through a 0.22 μm filter to sterilize aliquot in RNase-free 1.5 mL tubes (1 mL/aliquot) and keep at −20°C for 12 months maximum for storage.

RNAscope Target Retrieval Reagent (TRR)		
Reagent	Final concentration	Amount
Target Retrieval Reagent 10×	1×	35 mL
dH_2O	N/A	315 mL
Total	**N/A**	**350 mL**

⚠ CRITICAL: Do not store TRR, discard after use.

RNAscope wash buffer (RWB)		
Reagent	Final concentration	Amount
RNAscope wash buffer 50×	1×	60 mL
dH_2O	N/A	2.94 L
Total	**N/A**	**3 L**

⚠ CRITICAL: Keep TRR at 37°C throughout procedure. Do not store, discard after use.

Opal 570 fluorescent dye solution (Opal 570)		
Reagent	Final concentration	Amount
Opal 570 dye	1:1,500 v/v in TSA	1 μL
TSA buffer	1×	1.499 mL
Total	**N/A**	**1.5 mL/10 samples**

⚠ CRITICAL: Keep at −20°C for 12 months maximum

RNAscope immunolabeling blocking buffer (blocking buffer)		
Reagent	Final concentration	Amount
EDTA 0.5 M	50 mM	1 mL
Gelatin from cold water fish skin	0.5% v/v	100 µL
BSA (fraction V)	1% w/v	0.1 g
Horse serum	1% v/v	100 µL
Human serum	5% v/v	500 µL
dH_2O	N/A	Top to 10 mL
Total	**N/A**	**10 mL**

⚠ CRITICAL: Pass blocking buffer through a 0.22 µm filter to sterilize, aliquot in 1.5 mL tubes (1 mL/aliquot) and keep at −20°C for 12 months maximum.

STEP-BY-STEP METHOD DETAILS

For both assays, cells need to be infected following steps 1–14. For quantifying infection by the FISH-flow assay, follow steps 15–23, and for evaluating the infection with the morphological RNAscope assay, follow steps 24–57.

Infection of Vero cells

Timing: 24 h

In this step, Vero cells are incubated with various amounts of infectious virus with indicated multiplicity of infection (MOI) ranging from 0.01 to 0.00001, thus including a condition with low infectious virus concentration (< 10 infectious virus/mL), not detectable in techniques such as plaque forming assays or RT-qPCR, this later one being sensitive but unable to discriminate infectious from non-infectious virus.

This protocol applies to the detection of low levels of infectious virus produced by different cells in *ex vitro* or *in vitro* culture systems.

1. Seed cells in D10 in a 96-flat well plate so that a layer of confluent cells can be infected with the virus on the day of infection (usually 50,000 cells/well the day before). Anticipate having at least 3 replicates per dilution of virus tested and per sample in order to ensure robustness of the detection.
2. Perform 10-fold serial dilutions of viral stocks or biological samples in DMEM supplemented with Penicillin/streptomycin (100 U/mL) and 2% FCS (D2).

⚠ CRITICAL: Addition of 1% final TPCK-treated trypsin might increase detection of infectious particles from biological samples in Vero E6 cells that lack expression of the protease TMPRSS2.[5]

3. Infect cells with 50 µL viral dilution for 1–3 h at 37°C.
4. Replace inoculum with 100 µL fresh pre-warmed D2.
5. Incubate Vero cells for 24 h at 37°C, 5% CO_2.
6. Wash two times Vero cells with PBS-FISH.
7. Detach Vero cells by incubating them in Trypsin 0.05% v/v solution for 5 min at 37°C, 5% CO_2.
   a. Add 50 µL trypsin/well.

b. After the 5 min incubation, add 100 μL D2 to neutralize trypsin, and then transfer cells to a round bottom plate.
c. Wash the wells of the culture plate with 100 μL PBS and transfer cells to the corresponding wells of a round bottom plate.

8. Centrifuge cells at 300 × *g* for 10 min and remove media.
9. Before staining with RNA probes for FISH-flow, perform a viability staining with 50 μL Viobility 405/452 diluted in PBS (final dilution 1:100), 5 min on ice.
10. Add 200 μL PBS and centrifuge cells at 300 x g for 5 min to remove the excess of Viobility staining solution.
11. Resuspend cell pellets with 4% v/v paraformaldehyde and incubate for 30 min for cell fixation.
12. Remove fixative by washing cells three times with PBS-FISH using the same centrifugation settings as in step 8.
13. Reserve cells in suspension for FISH-flow.
14. Make dry spots of the fixed Vero cells on poly-L-Lysine-coated slides for RNAscope.
    a. Resuspend 10^5 cells in 20 μL of PBS without calcium and magnesium.
    b. Drop the 20 μL of cell suspension on the poly-L-Lysine-coated slide. Deposit a maximum of two to three drops per slide.
    c. Allow drying at 20°C–23°C for 2 h.
    d. Store slides at 4°C until the RNAscope assay is run.

Quantification of infection by FISH-flow

Note: In the FISH-flow assay, *in situ* hybridization step is performed **after** immunolabeling.

Immunolabeling for FISH-flow

Timing: 1 h

In this step, cells will be permeabilized for intracellular immunolabeling of the SARS-CoV-2 spike protein. This step is performed **before** *in situ* hybridization.

15. Resuspend cells in solution of primary antibodies diluted in PERM-FISH-Tween or PERM-FISH-Sapo buffer.
    a. Dilute Alexa Fluor 488-conjugated anti-S1 antibody at 1:250 in any perm buffer.
    b. Add 50 μL of diluted antibody solution per well.
    c. Mix the cells.
16. Incubate for 30 min at 20°C–23°C.
17. Add 200 μL PERM-FISH-Tween or PERM-FISH-Sapo to the wells.
18. Wash out PERM-FISH-Tween buffer containing antibody by pelleting down the cells at 300 x g, 5 min, 20°C–23°C and resuspend cells in 200 μL HWB.

Pause point: Immunolabeled cells can be stored up to 16–18 h in HWB at 4°C.

In situ hybridization for FISH-flow

Timing: 1 day

In this step, to detect viral RNA in immunolabeled cells, RNA probe sets tagged with Quasar 670 (a fluorophore alternative of Cy5) will be hybridized to SARS-CoV-2 RNA.

19. Washout HWB and resuspend immunolabeled cells in HB10%dx buffer containing the combined probe sets.

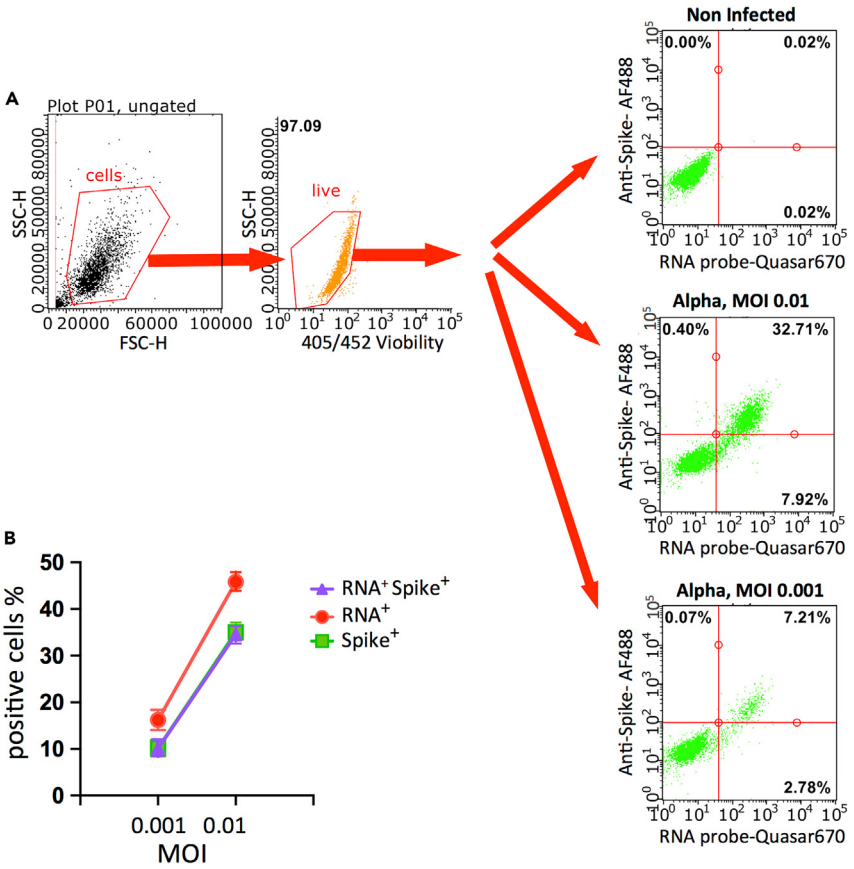

Figure 1. Quantification of Vero cell infection by FISH-flow

Cells were infected with low quantities of SARS-CoV-2 alpha variant (0.01 and 0.001 MOI) or left uninfected (non-infected) and incubated for 24 h at 37°C, labeled for viral RNA and spike protein and infection was quantified by flow cytometry.

(A) Gating strategy using non-infected cells as negative control.

(B) Quantification of cells expressing viral RNA (RNA^+), spike protein ($Spike^+$) and both the viral RNA and spike protein (RNA^+Spike^+). Values are shown as mean ± SEM (n = 3).

a. Final probe concentration is 50 nM. For the combination of 4 different probe sets, as used in this assay, use 12.5 nM of each probe set diluted in HB10%dx buffer.
b. If necessary, pre-dilute probes from probe stock solutions using Nuclease-free water as diluent.
c. Avoid bubbles when mixing probes in the HB10%dx buffer and when mixing cell with the HB10%dx buffer containing the probes.
d. Use 50 µL of HB10%dx buffer containing the probes per reaction. Distribute samples in 96-well plates with round bottom.
e. Gently mix cells and probe-containing solution.

⚠ CRITICAL: HB10%dx is viscous. Therefore prepare 10% more volume of the HB10%dx buffer solution containing the probes than actually required for the experiment.

20. Seal the 96-well plate containing samples with an adhesive film or with parafilm.
21. Incubate 16–18 h at 37°C.

⚠ CRITICAL: 16–18 h incubation must not exceed 16 h to avoid detection of false positives.

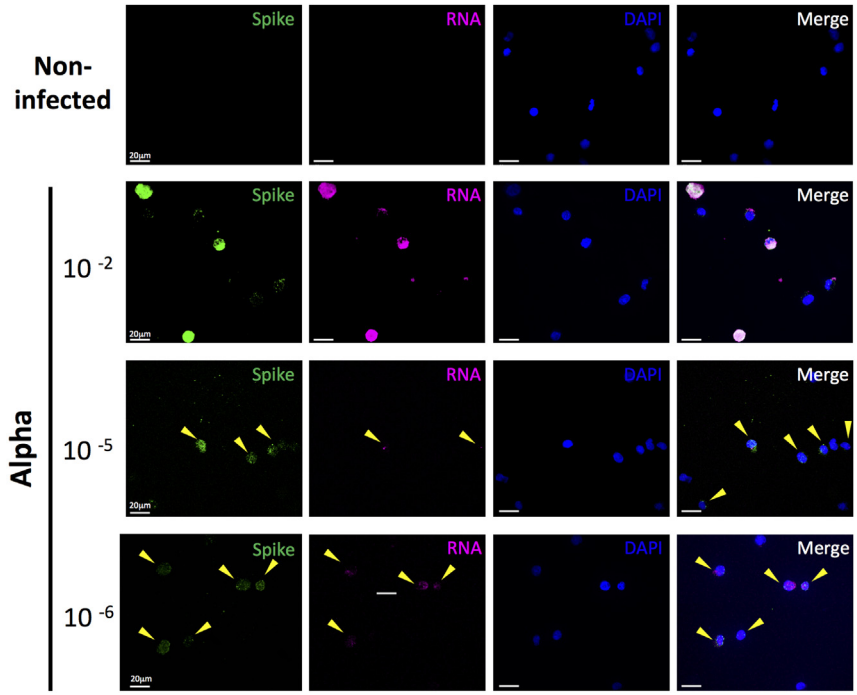

Figure 2. Detection of Vero cell infection by *in situ* hybridization using RNAscope and spike protein (Spike)-immunolabeling

Cells were infected with SARS-CoV-2 alpha variant at low MOI (10^{-2}, 10^{-5} and 10^{-6} MOI) or left uninfected (non-infected) and incubated for 24 h at 37°C. The cells were labeled for viral RNA using RNAscope (pink) and Spike (green), and counterstained for visualization of cell nuclei with DAPI (blue) and infection was analyzed by confocal microscopy using a 60× oil immersion objective. Spike, RNA and DAPI staining, and the merge of the three are individually shown. Yellow arrows point to low although specific signal observed at low MOI. Scale bar: 20 µm.

22. Add 200 µL HWB to each well to wash out the HB10%dx buffer solution containing the probes and centrifuge at 300 x g for 5 min at 20°C–23°C. Repeat the procedure once. Resuspend cells in PBS without calcium and magnesium.

Pause point: Fixed samples can be stored at 4°C for up to 7 days before flow cytometry analysis.

23. Proceed for flow cytometry acquisition, in this protocol Guava 12-HT (Millipore) was used. Further analyze the flow cytometry data with dedicated software such as GuavaSoft 3.4 (Millipore) as used in the present study, or FlowJo (BD) as in Zhou et al.[1] Outline of the gating strategy is presented in Figure 1A. As shown in Figure 1B, both viral RNA and Spike are detected in an MOI-dependent fashion. In the present context of acute infection, detection of viral RNA is usually more sensitive than that of Spike.

Morphological detection of infection by RNAscope

Note: In the RNAscope assay, *in situ* hybridization step is performed before immunolabeling.

In situ hybridization using RNAscope

Timing: 1 day

In this step, SARS-CoV-2 genes are hybridized with RNAscope commercial probes, using RNAscope commercial kit as follows.

24. Submerge the poly-L-lysine-coated slides containing dried spots of cell suspension in 100% v/v Ethanol solution in a Coplin jar for 1 min, for dehydration. Then, let the slide dry at 20°C–23°C.
25. Boil TRR in a heating plate until solution starts bubbling.
26. Submerge the slides in the boiling TRR, reduce the heat of the heating plate by 30% and incubate for 30 min.
27. Wash slides in dH_2O by 3–5 up-and-down movements. Repeat two times, changing dH_2O each time.
28. Wash slides in 100% v/v Ethanol by 2 up-and-down movements. Let slides dry at 20°C–23°C.
29. Create a hydrophobic barrier around dried cell spots using a hydrophobic pen. Let the hydrophobic barrier dry for 5 min at 20°C–23°C.
30. Add drops of Protease Plus solution on dried spots. Incubate slides at 40°C for 5 min.
    a. During this time, pre-warm the RNAscope SARS-CoV-2 probe at 40°C.
31. Wash slides in dH_2O by 3–5 up-and-down movements. Repeat two times by changing the dH_2O.
32. Add drops of pre-warmed RNAscope SARS-CoV-2 probe on dried spots. Incubate slides in a humidified chamber at 40°C for 2 h.
    a. During this time, prepare the RWB keeping it at 37°C (materials and equipment).
33. Wash slides by submerging them for 2 min in warmed RWB. Repeat one time by changing the RWB.
34. Add drops of AMP1 on dried cell spots. Incubate slides in a humidified chamber at 40°C for 30 min.
35. Wash slides by submerging them for 2 min in warmed RWB. Repeat one time by changing the RWB.
36. Add drops of AMP2 on dried cell spots. Incubate slides in a humidified chamber at 40°C for 30 min.
37. Wash slides by submerging them for 2 min in warmed RWB. Repeat one time by changing the RWB. Add drops of AMP3 on dried cell spots. Incubate slides in a humidified chamber at 40°C for 15 min.
38. Wash slides by submerging them for 2 min in warmed RWB. Repeat one time by changing the RWB.
39. Add drops of HRP-C1 on dried cell spots. Incubate slides in a humidified chamber at 40°C for 15 min.
40. Wash slides by submerging them for 2 min in warmed RWB. Repeat one time by changing the RWB.
    a. Prepare fluorescent dye solution Opal 570 during submersion time.
41. Add drops of Opal 570 on dried cell spots. Incubate slides in a humidified chamber at 40°C for 30 min.
42. Wash slides by submerging them for 2 min in warmed RWB. Repeat one time by changing the RWB.
43. Add drops of HRP blocker on dried cell spots. Incubate slides in a humidified chamber at 40°C for 15 min.
44. Wash slides by submerging them for 2 min in warmed RWB. Repeat one time by changing the RWB;
45. Add drops of blocking buffer on dried cell spots. Incubate slides in a humidified chamber at 20°C–23°C for 2 h.

Pause point: Alternatively, slides can be stored in blocking buffer 16–18 h at 4°C.

Immunolabeling after RNAscope

Timing: 2 h

In this step, cells will be permeabilized for intracellular immunolabeling of the SARS-CoV-2 spike protein. This step is performed after *in situ* hybridization.

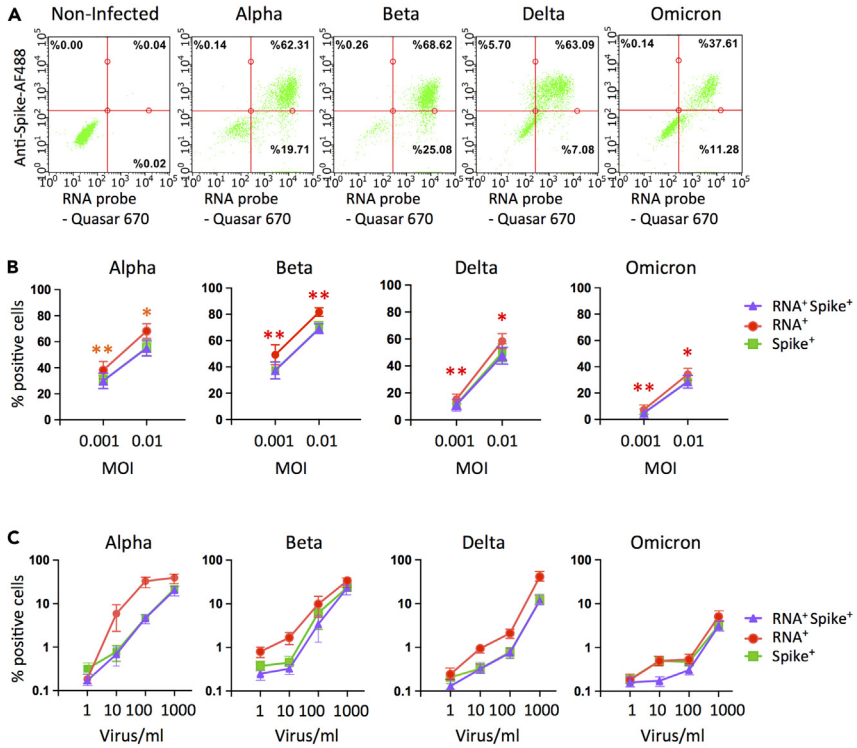

Figure 3. Quantification of infection by different variants of SARS-CoV-2 Vero cell at low MOI by FISH-flow

Cells were infected with low quantities of either SARS-CoV-2 alpha, beta, delta or omicron variants or left uninfected (non-infected) and incubated for 24 h at 37°C. The cells were labeled for viral RNA and spike protein and the infection was quantified by flow cytometry.

(A) Comparative flow cytogram of cell infection by each variant at an MOI of 0.01.

(B) Comparative quantification of cells expressing viral RNA (RNA^+), spike protein ($Spike^+$) and both the viral RNA and spike protein (RNA^+Spike^+) after infection by each variant at MOI of 0.01 and 0.001. Values are shown as mean ± SEM (n > 7/group). Statistics indicate comparison between the percentage of RNA+ cells with $RNA^+/Spike^+$ and $Spike^+$ cells by Wilcoxon test. * = : $p < 0.05$, **: $p < 0.01$.

(C) Comparative quantification of cells expressing viral RNA (RNA^+), spike protein ($Spike^+$) and both the viral RNA and spike protein (RNA^+Spike^+) after infection by each variant with very low quantities of infectious virus from 1000 up to 1 virus/mL. Values are shown as mean ± SEM (n > 6).

46. Remove blocking buffer and add drops of primary antibody diluted in blocking buffer.
    a. For antibody against SARS-CoV-2 spike protein, use 2 μg/mL concentration in 100 μL per reaction.
47. Incubate 16–18 h at 4°C in a humidified chamber.
    a. This chamber can be built using a regular plastic box containing the slides and dH_2O-soaked paper.
48. Wash slides by submerging them in PBS without calcium and magnesium for 5 min under mild tangential agitation. Repeat 2×.
49. Add drops of secondary antibody diluted in blocking buffer.
    a. For immunolabeling of anti-SARS-CoV-2 spike protein antibody, use 1:8000 (v/v) of AF488-coupled goat anti-mouse IgG1 antibody in 100 μL per reaction.
50. Incubate for 2 h at 20°C–23°C.
51. Wash slides by submersion in PBS without calcium and magnesium for 5 min under mild tangential agitation. Repeat 2×.
52. Add 20 μL drops of 5 μg/mL DAPI solution in PBS without calcium and magnesium.
53. Wash slides by submersion in PBS without calcium and magnesium for 5 min under mild tangential agitation in a shaking plate. Repeat 2×.

54. Mount slides for microscopic observation; therefore, deposit a 20 μL drop of ibidi mounting medium on the sample and cover it with a coverslip.
55. Dry at 20°C–23°C for 30 min or 16–18 h at 4°C.
56. Protect slides from light.

Pause point: Fixed samples can be stored at 4°C for up to several weeks before microscopy analysis.

57. Proceed to fluorescence microscopy, typically a confocal microscopy equipped with a x 60 oil immersion objective. As shown in Figure 2 spike protein and RNA signal are easily detected around nuclei.

EXPECTED OUTCOMES

SARS-CoV-2 is a coronavirus that spread rapidly during the global pandemic coronavirus disease (COVID-19) that began early 2020.[6] Highly sensitive, and rapid ways to measure low levels of SARS-CoV-2 (e.g., RT-qPCR) have been already developed. However, RT-qPCR fails to capture whether those low-level positive samples are truly infectious. The technique we describe here reports minimal amplification of virus on Vero cells (24 h infection time), followed by highly sensitive techniques for RNA/protein detection to demonstrate whether a sample contains infectious virus. This protocol applies for low-level detection of infectious SARS-CoV-2 in cell culture media or in biological fluids from infected individuals.[1]

We describe two assays. The first assay is a quantitative approach that combines the detection of RNA by FISH with detection of spike protein by immunolabeling at the single cell level using FISH-flow.[1–3] The second assay is a morphological approach combining RNA detection by FISH and visualization of spike protein by immunodetection both labeling analyzed by confocal microscopy.[1,3,4]

Over the years, viral variants have evolved from the ancestral Wuhan strain,[7] and numerous SARS-CoV-2 variants have been identified in humans, including the Alpha, Beta, Delta and Omicron.[8] The spike protein exposed on the surface of the virus is essential for the SARS-CoV-2 viral infection and following selection pressure, is highly susceptible to mutations that confer a selective advantage in a population. We sought to evaluate FISH-flow and RNAscope assays to detect these variants at a low rate of infection.

For the assays, Vero cells were infected with each of several variants, namely Alpha, Beta, Delta and Omicron[7,9] starting with a MOI of 0.01 for 1–3 h, after which the virus was removed and cells were further incubated 24 h. Cells were trypsinized and fixed, allowing further quantification of infection outside a confined environment (BSL3). The presence of both RNA and spike protein was quantified by FISH-flow and RNAscope and as described above.

As shown on Figures 3A and 3B, all variants tested were detected by FISH-flow using the RNA probes and spike protein-specific monoclonal antibody originally developed to target the ancestral Wuhan variant. Over 60% of cells were labeled with the SARS-CoV-2 RNA probes after infection with the Alpha, Beta and Delta variants at a MOI of 0.01, whereas the detection of RNA after infection with Omicron variant was lower, with 35% of the cells labeled. The percentage of cells positive for Spike detection was in the same range as for RNA FISH detection, although slightly lower for all variants and equal in number with cells positive for both spike protein and RNA. This suggests that after 24 h of infection most, but not all, infected cells can be detected by spike protein staining. In case of lower viral input, a longer infection time up to 48 h will likely increase the sensitivity of detection.

Detection of SARS-CoV-2 RNA and spike protein was concentration dependent, with the percentage of infected cells decreasing threefold for Alpha and Beta variants and fivefold for the Delta and Omicron variants, when infected with a MOI of 0.001 compared to 0.01. The lower detection of Omicron variant may be explained by the greater phylogenetic distance of the omicron variant from the alpha variant,[7] especially in the *Spike* gene and consequently in the protein, compared to the other tested variants.

These mutations may alter the recognition of the *S* gene and the Spike protein by the RNA- and Spike protein-specific probes that were originally designed against the ancestral Wuhan variant. The present study therefore established a protocol describing a robust method for the detection of all variants.

The infectious virus content in biological fluids can be low at later stage of the disease[10] or in case of viral persistence, as has been proposed in patients with post-COVID-19 symptoms referred to as Long COVID-19.[11] We therefore titrated the minimal amount of infectious virus detectable by FISH-flow from 1000 to 1 virus/mL using the protocol described here. As shown in Figure 3C, the technique detected as low as 1 infectious viruses/mL for all variants. The assay was setup with minimal inoculum volumes and incubation times to facilitate the experimental protocol. However, this protocol could be adapted to higher volume, different numbers of replicates and different infection times to improve the detection of infectious content from low input samples. Although the signal may be very low due to the small viral inoculum, the combination of RNA FISH and antibody probes greatly increases the robustness of detection.

The 96-well plate format used in the FISH-flow assay for the detection of SARS-CoV-2 in samples accounts for the high throughput of the assay, while the sample dilution replicates accounts for robustness. In addition, the combined detection of viral protein and RNA increases the sensitivity of the assay and downstream analysis, especially for samples with low viral content. As illustrated in the Figure 1A illustrating the gating strategy for Vero cells infected with Alpha with different MOI, the method is very specific as the anti-s spike protein antibody and the RNA FISH probes have no background signals in uninfected cells, which was consistently observed over experiments. In contrast, upon infection, cells double-labeled for spike protein and viral RNA are clearly detected. Furthermore, additional cells labeled only for RNA may represent cells at an earlier stage of infection, prior to detectable viral spike protein synthesis. As expected, cells labeled for spike protein but not RNA were not detected. Finally, although the background increases slightly in uninfected cells, it did not affect detection of infection.

The sensitivity of the method, and its applicability to multiple SARS-CoV-2 variants was evaluated. After infection with serial dilution up to 1 infectious virus/mL of all viral variant stocks tested at the same time, infection was detected by FISH-flow in all conditions assessed (Figure 3C). These data confirm the robustness of FISH-flow, therefore a suitable high throughput method. Referring experimental infection to a standard curve using viral production of known titers may allow accurate quantification of infectious content in the sample.[1]

We also assessed the ability of the RNAscope to detect infection with the alpha, beta and omicron variants at an MOI of 10^{-2} to 10^{-6}. As shown in Figure 4, viral RNA was detected after infection of Vero cells with the alpha, beta and omicron variants at an MOI of 10^{-5} and even 10^{-6} for the alpha and beta variants. RNA and spike protein signals were very strong at an MOI of 10^{-2}, always remaining cytosolic as expected, and decreased in a dose-dependent manner with decreasing MOI. At lower MOI, the RNA and spike protein staining pattern appeared punctate, as expected for RNA, which resides in compartments of the viral factory, and for spike protein, a glycoprotein that associates with the membrane of the secretory pathway. The combined detection of RNA FISH and antibody probes greatly increases the sensitivity and robustness of detection. In addition, the background was negligible. Therefore this morphological technique can reliably detect a few infected cells, even with the current probes originally designed for the Wuhan ancestral variant by the manufacturer.

Overall, our protocol can be applied to infections by different SARS-CoV-2 variants, although for each new variant, the optimal conditions for detection of RNA and spike protein may slightly differ from those reported here. Finally, additional cell markers can be detected alongside with viral RNA and spike protein, as we described previously.[1,12] This multiparametric labeling can allow accurate phenotyping of infected cells or quantification of infected cells at the single cell level in a mixed cell population (biological fluid, cell suspension prepared from tissues),[1,12–14] and therefore valuable for rare human clinical sample analyzes.

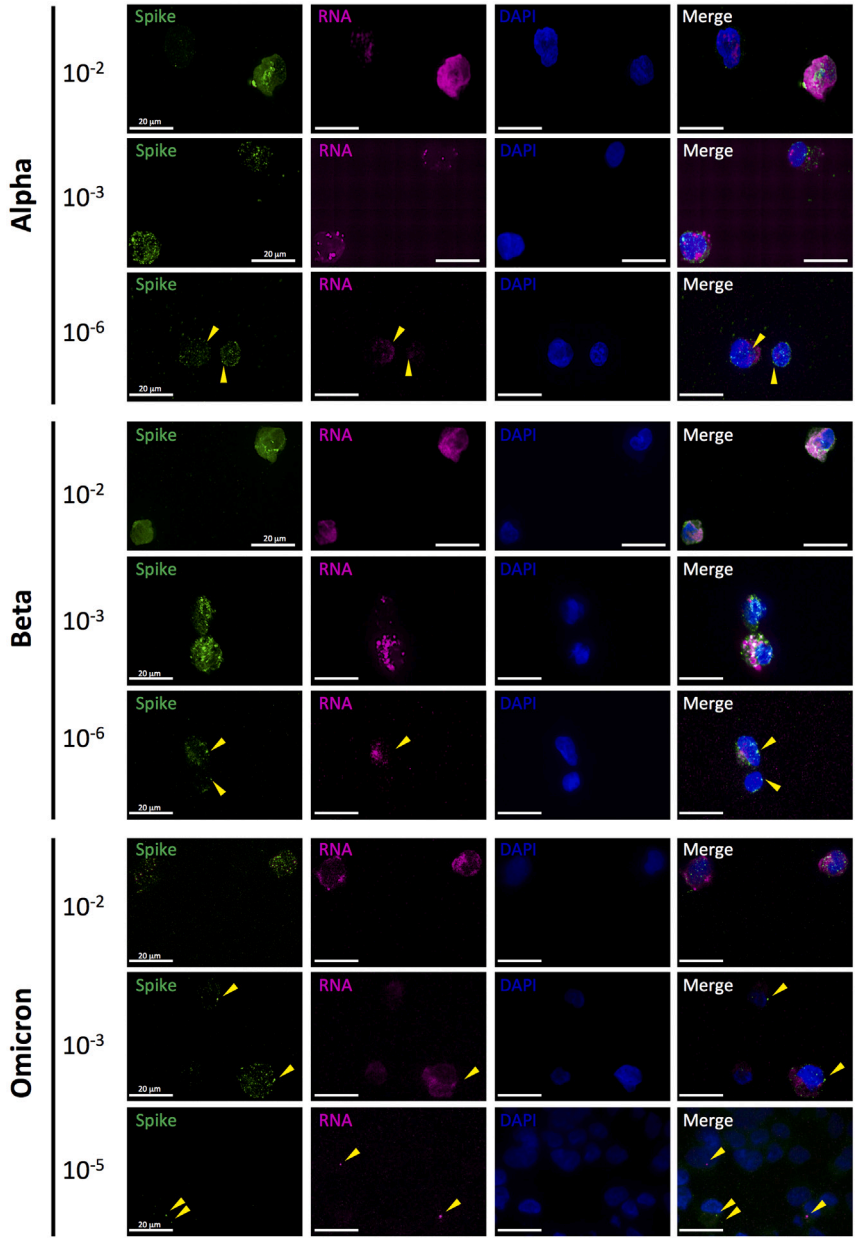

Figure 4. Detection of Vero cell infection by different variants of SARS-CoV-2 by RNAscope and spike protein (Spike)-immunolabeling

Cells were infected with SARS-CoV-2 alpha, beta or omicron variants (MOI of 10^{-2} up to 10^{-6}) or left uninfected (non-infected) and incubated for 24 h at 37°C, labeled for viral RNA using RNAscope (pink) and Spike (green), and counterstained to visualize cell nuclei with DAPI (blue) and infection was analyzed by confocal microscopy. Spike, RNA and DAPI staining, and a merge of the three are individually shown. Yellow arrows point to low although specific signal observed at low MOI. Scale bar: 20 µm.

QUANTIFICATION AND STATISTICAL ANALYSIS

The results are represented as the means with respective standard errors of the mean using GraphPad Prism 8. Statistical tests were performed using Wilcoxon test. Asterisks indicate significant differences considering p values below 0.05 and 0.01, as indicated.

LIMITATIONS

For samples with very low concentration of infectious particles (1–10 virus/mL), it might be necessary to increase the technical replicates in order to detect signal in at least one well.

As exemplified in Figure 3, all variants will replicate viral RNA and produce viral proteins during infection, but their relative abundance may vary depending on the SARS-CoV-2 variant. This will have a direct impact on the detection of infection using either FISH-flow and/or RNA-scope. With the present technique, we were able to detect infected cells with all tested variants, which demonstrates the robustness of the method.

FISH-flow does not allow spatial resolution of the labeling, which is why RNAscope has been developed. This lack of spatial resolution in FISH-flow could be resolved by quantifying FISH-Flow with an imaging cytometry system such as Image Stream (Amnis), instead of standard flow cytometry.

TROUBLESHOOTING

Problem 1

Bubbles in HB10%dx; Assay step 19.

Potential solution

Try to gently pipette carefully up and down samples with probes using a smaller volume of probes containing HB10%dx < 50 μL (such as 25 μL) and by decreasing aspiration/dispensing speed of the pipet. Centrifuge plate at low speed (50 x g for 3 min).

Problem 2

All cells appear to be stained for 405/452 Viobility dye; Assay step 23.

Potential solution

Increase dye final dilution below the recommended concentration starting from 1:200.

Decrease staining time to 3 min.

Problem 3

RNA probe signal too weak; Assay step 23.

Potential solution

Ensure that the cell layer reach confluency before infection by increasing seeding cell number to 100 000 per well.

Increase the duration of the infection time (>24 h, i.e., 36 h or 48 h) to allow sufficient amplification of viral RNA and therefore its detection.

Problem 4

No detection of infectious particles in all the infected wells; Assay step 23.

Potential solution

In the case of low input, detection of positive signals is not expected in all infected wells, which could explain discrepancies in statistical spread of the virus(es) in the wells. To improve detection of low infectious virus concentration, increase the inoculum volume up to 100 μL.

Problem 5

No signal detection of a known to be positive sample; Assay step 23.

Potential solution

Ensure that TPCK-treated trypsin is added to the inoculum at 1 μg/mL final concentration. Biological samples such as bronchoalveolar lavage also contain other compounds/microorganisms that may interfere with infection. Although counterintuitive, samples could be diluted with DMEM containing 10% FCS, 1% Gentamicin 1% Amphotericin B and incubate at 37°C for 20 min. Mix samples thoroughly and centrifuge at 500 x g for 5 min before cell infection.

Problem 6

High background in RNA probe emission channel; assay step 23.

Potential solution

Increase the number of washes to 3 times or more at step 22. Check cell autofluorescence and treat cells with 100 mM Glycine 75 mM NH_4Cl, pH 7.4 for 10 min, remove the buffer and wash one time with PBS. Decrease the probe concentration by 10 or 20%. Make sure probe staining does not exceed 16 h.

Problem 7

High background in antibody staining; Assay step 23.

Potential solution

Antibody staining might need titration down (by two-fold dilution) in your setup to ensure optimal staining.

Problem 8

High heterogeneity in markers staining; Assay step 23.

Potential solution

Make sure to mix carefully after addition of antibody and/or RNA-probe solution to the cells.

Problem 9

Abnormal FSC-H and/or SSC-H profile; Assay step 23.

Potential solution

Reduce trypsin treatment time to 3 min. Reduce centrifugation speed while cells are not fixed (do not exceed 300 x g). Handle cells more carefully before fixation, for instance avoid giving mechanical stress and producing bubbles during vortexing steps.

Problem 10

Tissue dries during treatment; Assay steps 24–52.

Potential solution

Add enough reagents to completely cover tissue/cells. Make sure that the humidified paper is still moist at the end of the incubation periods in the chamber. Decrease number of slides processed at the same time. Check incubation temperature is as indicated.

Problem 11

Tissue dries during treatment; Assay step 56.

Potential solution

Ensure that cells have been properly fixed to the slide before the beginning of treatment for RNAscope staining (Step 24). Alternatively, double the quantity of cells deposited.

Problem 12

High Background; Assay step 56.

Potential solution

Establish pre-treatment conditions specific to your samples by reducing boiling and protease treatment time and concentration, as suggested by the RNAscope manufacturer (https://acdbio.com/technical-support/solutions).

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Morgane Bomsel (morgane.bomsel@inserm.fr).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate new unique reagents.

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS

Method, design, and writing, A.C.-C., F.H., A.Z., F.R., and M.B.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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