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Relative infectivity of the SARS-CoV-2 Omicron variant in human alveolar cells

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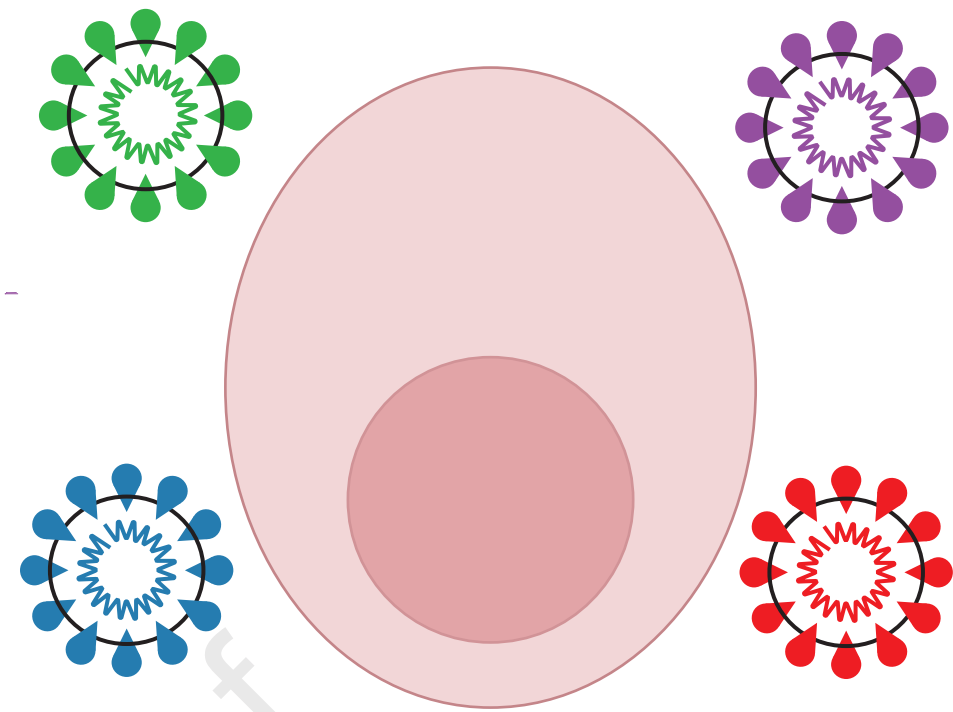
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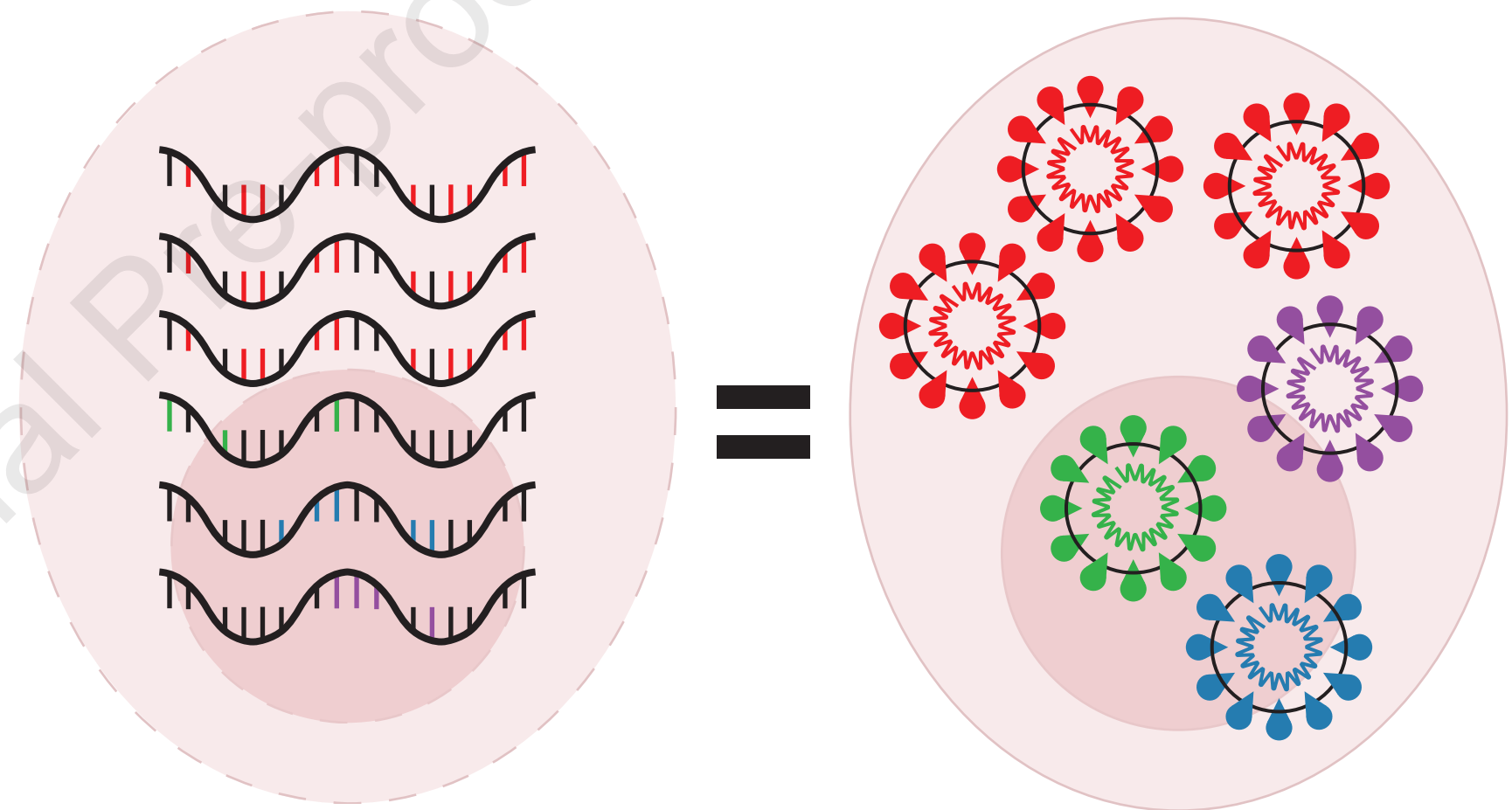
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A rapid system for evaluating the relative infectivity of viral variants : human alveolar organoid + single-cell full-length transcriptome

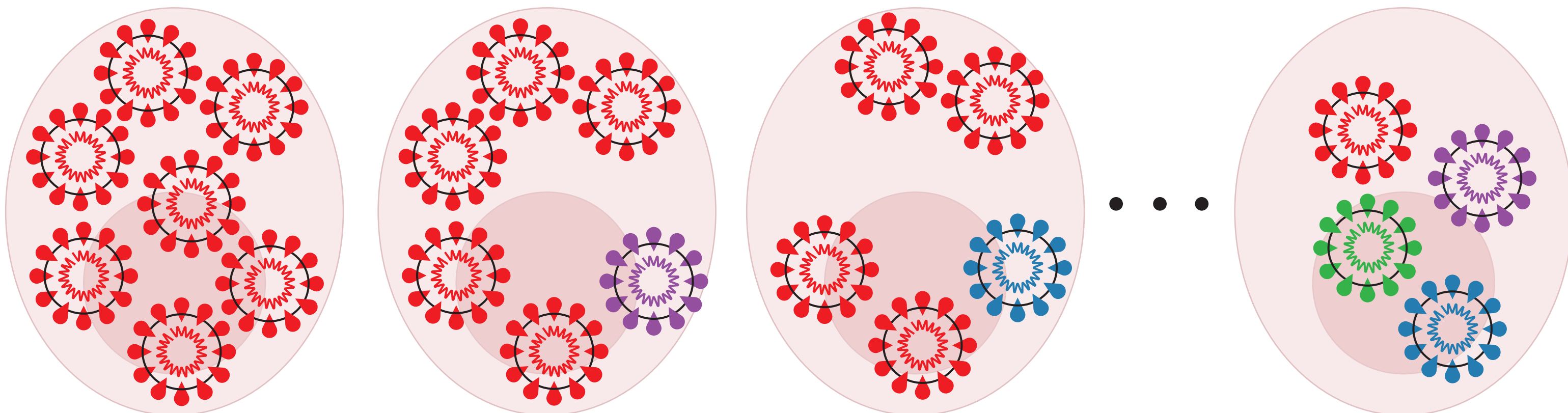
Incubate type-2 alveolar cell with
a pool of viruses



Calculate the fraction of viruses with
unique mutations



Omicron dominantly infected alveolar cells



Relative infectivity of the SARS-CoV-2 Omicron variant in human alveolar cells

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Summary

With the continuous emergence of highly transmissible SARS-CoV-2 variants, the comparison of their infectivity has become a critical issue for public health. However, a direct assessment of the viral characteristic has been challenging due to the lack of appropriate experimental models and efficient methods. Here, we integrated human alveolar organoids and single-cell transcriptome sequencing to facilitate the evaluation. In a proof-of-concept study with four highly transmissible SARS-CoV-2 variants, including GR (B.1.1.119), Alpha (B.1.1.7), Delta (B.1.617.2), and Omicron (BA.1), a rapid evaluation of the relative infectivity was possible. Our system demonstrates that the Omicron variant is 5- to 7-fold more infectious to human alveolar cells than the other SARS-CoV-2 variants at the initial stage of infection. To our knowledge, for the first time, this study measures the relative infectivity of the Omicron variant under multiple virus co-infection and provides new experimental procedures that can be applied to monitor emerging viral variants.

Introduction

During the global spread of the coronavirus disease 2019 (COVID-19), many novel SARS-CoV-2 variants of concern (VOC) have emerged, posing an increased risk to global public health and of quarantine ¹⁻³. International communities, such as GISAID ⁴, PANGO ⁵, and Nextstrain ⁶, have been monitoring and assessing the evolution of SARS-CoV-2 using periodic genomic sequencing of viral samples. The sequencing results have identified a few major SARS-CoV-2 variants, including GR (B.1.1.119) with the D614G variant ⁷, Alpha (B.1.1.7) (first detected in UK), and Delta (B.1.617.2) (first detected in India) (WHO). In Nov 2021, the Omicron variant (BA.1), characterized with 32 mutations in the spike protein, emerged from South Africa and is currently the dominant variant in many countries ⁸.

To understand the functional impacts and pathological characteristics of each VOC, various approaches have been conducted including epidemiological studies ⁹, spike binding affinity assay ¹⁰⁻¹², experimental model studies ¹²⁻¹⁴, and genetically engineered virus comparison studies. The epidemiological studies illustrate characteristics of viral transmission and clinical severity, but their underlying cellular and molecular mechanisms cannot be investigated. The spike binding assay measures the affinity between the virus spike protein and human receptor, but its biological impact is cryptic. For the experimental model studies, including animal models ¹⁵ and cell lines, the issue of viral tropism is inherent. Often, genetically engineered viruses with a specific mutation of interest, rather than natural viral variants (e.g., D614G (GR, Alpha, Delta, and Omicron) ¹⁶, N501Y (Alpha and Omicron) ¹⁷ or P681R (Delta) ^{18,19}), are used in infection studies, but these engineered viruses may not reflect the full characteristics of natural VOCs.

Despite all these efforts, the direct measurement of the relative infectivity of multiple VOCs, particularly the impact of the natural virus on physiological human tissues, has not been investigated. Here, we developed a rapid, fully-controlled virus competition system by integrating normal human type-2 alveolar cell (hAT2 cell) organoid and single-cell full-length transcriptome sequencing. We successfully trace the viruses responsible for an infected alveolar cell. Furthermore, we compared the relative infectivity of viral variants under multiple virus co-infection at the single-cell level.

Results

A rapid, fully-controlled virus competition system

First, hAT2 cells in alveolar organoids are single-cell dissociated and then exposed to a mixture of SARS-CoV-2 variants (**Figure 1A**). After minutes- to days-long culture, the full-length transcriptomes of the infected cells are sequenced at the single-cell resolution (adopting the SMART-seq3 technique)²⁰ to capture the viral genomic mutations. These mutations are used to identify which VOCs are responsible for an individual cell's infection.

As a proof-of-concept study, we selected four SARS-CoV-2 variants, the GR clade virus (B.1.1.119), Alpha (B.1.1.7), Delta (B.1.617.2), and Omicron (BA.1), which are known to have been highly transmissible viruses during the pandemic. For our infection experiments, we used viral stocks which were collected from Korean patients and maintained by the Korea Disease Control and Prevention Agency (KDCA). RNA sequencing of these viral variants identified 118 clonal genomic alterations for tracing viral variants (**Figure 1B; Table S1**), 94% of which (n=125) are known to be present in the standard genome sequences of GR, Alpha, Delta, and Omicron variants (**Figure S1A**).

In our optimized infection experiments, viral incubation of alveolar cells was conducted at a multiplicity of infection (MOI) of 10 collectively, with each of the SARS-CoV-2 variants equally allocated for the incubation (**STAR Method**). On average, an alveolar cell interacted with 10 viral plaque-forming units (PFU), and each viral variant had an equal chance of cellular infection. We checked the number of infective (viable) viruses used for viral variants mixture with plaque assay (**Figure S1B**). The relative proportion of infective viruses was substantially balanced within 1.5-fold among the four viral variants (**Figure S1C**).

Furthermore, the viral incubation time was mostly five minutes (86%; the others were incubated for 60 minutes), which was sufficient for infection of hAT2 cells. Then, single alveolar cells were isolated in a microchip after checking the number of cells under the bright-field and fluorescence microscopy (**Figure 1A; Figure S1D**).

Robust infection in alveolar cells

The full-length transcriptome for SARS-CoV-2 infected single cells by SMART-seq3 was sequenced by short-read paired-end sequencing with ~314Mb of sequencing throughput per cell, or ~2.1 M reads with 150 bp per cell, on average. The transcriptional profiles of the host genes confirmed that the infected human cells are hAT2 cells (**Figure 1C**).

In the single-cell transcriptome of 244 infected cells that passed the quality check and threshold of the infection criteria (**STAR Method**), the proportion of viral sequences over total sequences

ranges from 0.08% to 60% (**Figure 1D**). For the infected alveolar cells, the expression profile of viral RNA transcripts is consistent with the previous report ²¹ that the 3' genomic regions of the viral genome showed much higher RNA expression levels (**Figure 1E**). Of note, the full-length transcriptome method, SMART-seq3 has more uniform coverage than the 3' enriched transcriptome method, such as 10X Chromium ²². Therefore, SMART-seq3 can detect more viral genomic mutations which are missed by 10X Chromium.

Decomposition of viral variants in infected alveolar cells

Considering the 118 clonal viral genomic mutations as viral variant barcodes (**Figure 1B and 1E, Table S1**), we decomposed the fraction of each viral variant responsible for an individual cell's infection by using variant allele fraction (VAF). We used two algorithms, the average VAF method and the non-negative matrix factorization (NMF) (**STAR Method**). The results of the two algorithms, the fraction of viral variants in infected alveolar cells, were overall concordant with each other (**Figure 1F**). Only eight cells (3.3%) showed an insufficient cosine similarity (<80%). In these cells, a large fraction of viral mutation loci was stochastically uncovered in the full-length single-cell transcriptome sequencing (**Figure 1G**).

The Omicron variant dominantly infected alveolar cells

Of the 244 infected cells, 97 (39.8%), 92 (37.7%), and 52 (21.4%) cells were dominantly infected by single, double, and multiple viral variants, respectively, suggesting that multiple viral entries are possible in the experimental condition. For the remaining 3 cells (1.2%), unique viral variants could not be assigned.

Despite the equal chance of infection, each SARS-CoV-2 variant showed strikingly different frequencies in the infected cells (**Figure 2A**). For instance, of the 97 cells with a single variant infection, 63 (65.0 %) were caused by the Omicron variant, followed by Alpha (n=20; 20.6 %), GR (n=13; 13.4%), and Delta (n=1; 1.0%). The Omicron variant was 2.60-fold more frequently observed than the random expectation (95% confidence interval = [2.18, 2.97]; $p = 3.0 \times 10^{-19}$), implying an ~7.4-fold higher infectivity than the other viruses by odds ratio under the same infectivity among viruses (**STAR Method**).

A similar conclusion was robustly drawn from a parallel analysis with all 244 cells, including the ones infected by two or more variants. Here, the Omicron variant was found in 199 cells (81.6%), followed by 114 (46.7%), 75 (30.7%), and 56 (23.0%) for the Alpha, GR and Delta variants, respectively, which is also biased toward the Omicron variant ($p = 5.26 \times 10^{-43}$) (**Figure 2A**). Taking into consideration the relative viral burden of each variant in an infected cell (a weighted average), the Omicron variant involved 143.2 cells (58.7%), out-competing the other variants. This result means that the Omicron variant was observed 2.34-fold more frequently than the random

expectation (95% confidence interval = [2.09, 2.60]; $p = 1.37 \times 10^{-33}$) and showed a 5.6-times higher infectivity than the other viruses. This result was concordant with the results drawn from the cells with the single variant infection.

In the pairwise comparison with the other variants by odds ratio, the Omicron variant showed ~4.8 (against the Alpha), ~9.7 (against the GR), and ~31.6 (against the Delta), times higher infectivity in the assay. We believe that the dominance of the Omicron is robust because the trend was replicated in 9 independent batches (**Figure 2B**). Of note, in an experiment with a longer viral incubation time (60 min), the predominance of the Omicron variant was even higher (**Figure 2C**).

Our calculation of the relative infectivity is conducted under an assumption of equal infection chance among the four viral variants. However, in our plaque assays (**Figure S1C**), the fraction of infective Omicron variant in the source was ~20.5% on average, slightly lower than 25%. The higher infectivity of the Omicron variant will be further enhanced if we take its original fraction into consideration.

Viral mutations during virus stock preparation

Through the VAF of the mutation, we are able to trace the probable stage when the mutation was acquired. Since we passaged one more time to make viral stock for the virus competition experiment after receiving the passage 3 viral stock which originated from the patient sample, we can distinguish whether mutations were acquired before passage 3 or between passage 3 and passage 4. Using a total of 48 viral stock (passage 4) RNA sequencing with 4 viral variants and 12 experimental batches, we analyzed the mutations compared to the references from GISAID.

We found a total of 34 mutations (GR: 8; Alpha: 10; Delta: 5; Omicron: 11) compared to the GISAID reference (**Figure S1A**). 18 mutations (GR: 3; Alpha: 5; Delta: 2; Omicron: 8) are highly likely to be accumulated between passage 3 and passage 4 since the VAF difference between the GISAID and viral stocks (passage 4) is low (< 0.5). On the other hand, 16 mutations (GR: 5; Alpha: 5; Delta: 3; Omicron: 3) might be acquired before passage 3 because the VAF difference between the GISAID and viral stocks (passage 4) is high (> 0.8).

Of note, since the experimental batches were aliquots of the viral stock (passage 4), the VAFs across batches are similar. However, in the Omicron variant, some mutations had heterogeneous VAFs among batches. The viruses with these mutations might result from the subclonal viral population of the passage 3 viral stock, considering the relatively lower number of the Omicron for sequencing compared to other variants.

Discussion

In this study, we developed a new system to measure and directly compare the relative infectivity

of SARS-CoV-2 variants. Our system suggested that the Omicron (BA.1) variant is 5-to 7- times more infectious than the other viral variants, including the GR (B.1.1.119), Alpha (B.1.1.7), and Delta (B.1.617.2), against human alveolar cells.

Our system evaluates the infectivity of respiratory viruses directly against their target cell types, hAT2 cells in contrast with other approaches based on animal models ¹⁵ or 2D cell lines ²³. Using human alveolar organoids, our assay is free from the issue of viral tropism and recapitulates normal human tissue physiology. Furthermore, this strongpoint allows our system to be expanded to any virus if organoids have the main target cell of the virus.

Moreover, this system also has technical advantages. The system can trace viral variants with high sensitivity since full-length single-cell RNA sequencing captures much information, which is viral genomic mutations, compared to 3' enriched single-cell RNA sequencing. In addition, the relative infectivity of viral variants can be quickly determined in a fully controlled condition because the turn-around time of our assay is ~3 days.

Although our study has many advantages, the data should be interpreted carefully since the experimental condition is different from the condition of epidemiological studies. An epidemiology study showed that the Delta transmits faster than the Alpha ⁹. However, in our results and spike binding affinity study ²⁴, the Alpha is more infectious than Delta at the initial stage of infection. Presumably, the Alpha is more infective at the early phase of infection, but the Delta has replicated more in the late stage and/or during the clinical course. Likewise, our data should be interpreted cautiously because four different viral variants competed in the series of experiments, and the small number of infected cells in experimental batches. To accurately measure the relative infectivity of the two variants specifically, another set of experiments, using the Alpha and Delta only, may be necessary.

Of note, recent studies showed that the Omicron depends more on an endocytic pathway for cellular entry rather than membrane fusion entry by TMPRSS2 in the alveolar cells ^{12,14}, speculating the Omicron variant is less infective than the other variant in alveolar cells. Though our alveolar organoids express TMPRSS2 robustly as in the research by Meng et al. (**Figure 1B**), our data indicates that the Omicron variant much more rapidly infected hAT2 cells than the other variants.

We expect that this system could further investigate transcriptome changes of human genes at the single-cell resolution level. To do so, infected cells should be incubated for a longer time, ideally for at least 24 hours, as such a duration is necessary for alveolar cells to reprogram their transcription against viral infection ^{21,22}. Furthermore, this assay can also be applied to an organoid co-culture infection model, which may allow us to investigate the response of immune cells to different viral variants. In the future, together with other complementary approaches, our method will help to reveal the functional characteristics of emerging viral variants, especially for comparison

among variants.

Limitations of study

Technically, calculating the number of infective viruses before mixing each viral variant is required, because the fraction of infective viruses can differ among viral variants (**Figure S1C**). Moreover, since the fraction of defective viruses are imbalanced, infected cells should be sufficiently washed to remove floating viral RNA transcripts. Furthermore, the manual selection of the infected single cells is the rate-limiting step in our assay. However, the process can be readily scalable by using a fluorescence-activated cell sorter ²⁵.

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Author Contributions

Conceptualization, Y.S.J.; Infection, T.K., K.I.M.; Virus preparation J.W.K., J.C.; Virus decomposition, T.K., K.I.M.; Specimen preparation K.J.N., Y.H.K.; Writing, T.K., K.I.M., Y.S.J; Manuscript finalization, all authors; Supervision Y.S.J., J.-Y.L., K.J.N.

Declaration of Interests

Young Seok Ju is a co-founder and Chairperson of Genome Insight. Jeong Seok Lee is a co-founder and chief executive officer of Genome Insight. Kwon Joong Na is a co-founder and chief medical officer of Portrai, Inc..

Inclusion and Diversity

We support inclusive, diverse, and equitable conduct of research

Figure legends

Figure 1. Integration of alveolar organoids and full-length single-cell RNA-seq techniques to understand the relative infectivity of SARS-CoV-2 variants. See also Figure S1 and Table S1.

(A) Schematic diagram of the experimental procedures of the virus competition assay with the expected experimental time shown in parentheses.

(B) Clonal viral genomic mutations (VAF > 99%) in the four viral variants that are used for viral tracing with the viral genomic mutations from GISAID reference shown in parentheses.

(C) Normalized expression levels of host genes (UMI counts) in the infected single-cells (Y-axis values of dots are normalized expression + 1).

(D) The number of reads (1 sequencing read is 150 base pairs) from full-length single-cell transcriptome sequencing for each infected cell. Dark blue, the number of total reads; orange, the number of viral reads.

(E) The genomic location of the viral genomic mutations (the top four panels). The normalized coverage of viral transcripts in infected cells by two different single-cell transcriptomic methods (the bottom panel).

(F) Comparison of two different viral variants decomposition methods (average VAF method and non-negative matrix factorization method). The dashed line represents the median (0.99).

(G) Number of unique viral mutations covered by transcriptome sequencing and the accuracy of viral variant decomposition.

Figure 2. A higher infectivity of the Omicron variant in human type-2 alveolar cells

(A) The proportion of each viral variant in each infected cell. Experimental conditions (post infection time and viral incubation time) are shown at the bottom. The order of infected cells (X-axis) is identical to the one in Fig 1D. The distance between two adjacent big ticks is 10 cells. The distance between one big tick and one small tick is 5 cells.

(B) The proportion of each viral variant over 12 different experimental batches in this study. Only cells with a viral incubation time of 5 minutes are shown.

(C) The proportion of the Omicron variant increases in the batch with a longer viral incubation time.

STAR Methods

KEY RESOURCES TABLE

RESOURCE AVAILABILITY

Lead Contact

Materials Availability

Data and Code Availability

EXPERIMENT MODEL AND SUBJECT DETAILS

Alveolar organoids establishment from human lung tissues

Virus stock preparation of each viral variant used for the competition assay

METHOD DETAILS

Virus competition system among VOCs: single cell infection with multiple VOCs

Virus competition system among VOCs: single-cell isolation

Virus competition system among VOCs: library preparation for isolated single cells.

The fraction of infective viruses and viral transcripts in a virus mixture

Viral genomic mutations

Data processing of the full-length single-cell transcriptome sequencing

Decomposition of the viral variants that infected a single-cell

Criteria for infection at a single cell

Normalized coverage of virus transcripts

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical Analysis

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-human CD31 APC	Biologend	Cat#303116; RRID:AB_187751
Anti-human CD45 APC	Biologend	Cat#368512; RRID:AB_2566372
Anti-human EpCAM FITC	Biologend	Cat#324204; RRID:AB_756078
Mouse anti-HTII-280 IgM	Terrace Biotech	Cat#TB-27AHT2-280; RRID:AB_2832931_
PE goat anti-mouse IgM	Thermo-Fisher	Cat#12-5790-81; RRID:AB_465939
Bacterial and virus strains		
SARS-CoV-2 GR clade (B.1.1.119)	KCDC	GISAID Number: EPI_ISL_812963
SARS-CoV-2 Alpha variant (B.1.1.7)	KCDC	GISAID Number: EPI_ISL_738139
SARS-CoV-2 Delta variant (B.1.617.2)	KCDC	GISAID Number: EPI_ISL_2887353
SARS-CoV-2 Omicron variant (B.1.1.529)	KCDC	GISAID Number: EPI_ISL_6959993
Biological samples		
Human lung tissue samples	This paper	N/A
Chemicals, peptides, and recombinant proteins		
Collagenase/Dispase	Sigma-Aldrich	Cat#10269638001
Dispase II	Sigma-Aldrich	Cat#4942078001
DNase I	Sigma-Aldrich	Cat#D4527-10KU
RBC lysis solution	Roche	Cat#11814389001
Accutase	STEMCELL Technologies	Cat#07920
Modified Eagle Medium	Gibco	Cat#11935-046
HI FBS	Gibco	Cat#10082-147
Serum-free DMEM	Gibco	Cat#41966-029
FBS	Gibco	Cat#16000-044
Growth Factor Reduced Matrigel	CORNING	Cat#356231
Y-27632	Sigma-Aldrich	Cat#Y0503
Advanced DMEM/F12	Thermo-Fisher	Cat#12634010
B27 supplement	Thermo-Fisher	Cat#17504044
FGF 7	PEPROTECH	Cat#100-19
FGF 10	PEPROTECH	Cat#100-26
Noggin	PEPROTECH	Cat#120-10C
EGF	PEPROTECH	Cat#100-15
N-Acetylcysteine	Sigma-Aldrich	Cat#A9165
Nicotinamide	Sigma-Aldrich	Cat#N0636
SB431542	Calbiochem	Cat#616461
CHIR99021	TOCRIS	Cat#4423
Recombinant Human R-Spondin 1 protein	R&D Systems	Cat#4645-RS
HEPES	Gibco	Cat#15140-122
Penicillin / Streptomycin	Gibco	Cat#15630-080
Glutamax-I	Gibco	Cat#35050-061
Amphotericin B	Sigma-Aldrich	Cat#A2942

Gentamicin	Sigma-Aldrich	Cat#G1397
Recovery solution	Corning	Cat#354253
CellTracker Green CMFDA Dye	Thermo-Fisher	Cat#C2925
Bovine Serum Albumin solution	Sigma-Aldrich	Cat#A8412s
Recombinant RNase Inhibitor	Takarabio	Cat#2313A
dNTP Set. 100mM Solutions	Thermo-Fisher	Cat#R0181
RIGAKU REAGENTS PEG 8000, 50%(w/v)	Rigaku reagents	Cat#25322-68-3
Maxima H Minus Reverse Transcriptase	Thermo-Fisher	Cat#EP0753
GTP(Tris buffered solution 100mM)	Thermo-Fisher	Cat#R1461
KAPA HiFi Hotstart PCR Kit	Roche	Cat#KK2502
Triton™ X-100 solution 10%	Sigma-Aldrich	Cat#93443-100ML
Sodium Chloride (5M)	Invitrogen	Cat#AM9760G
Magnesium Chloride (1M)	Invitrogen	Cat#AM9530G
NN-Dimethylformamide	Sigma-Aldrich	Cat#D4551
Tris-HCl pH 7.5(1M)	Tech & Innovation	Cat#BTH-9183-001L
Tris-HCl pH 8.3(1M)	Tech & Innovation	Cat#BTH-9175-001L
Phusion™ High-Fidelity DNA Polymerase	Thermo-Fisher	Cat#F-530S
Nextera XT DNA library prep kit	Illumina	Cat#20015963
Critical commercial assays		
C-chip Neubauer improved	iNCYTO	Cat#DHC-01
QIAamp viral mini kit	Qiagen	Cat#52904
NEBNext ARTIC SARS-CoV-2 FS kit	NEB	Cat#E7658S
Smart aliquotor CE chip	iBioChip	Cat#H2-SACE-5PK
Deposited data		
Single-cell bam files of infected alveolar cells aligned by BWA and RNA sequencing of viral variants stock	EGA	EGAS00001006730
All scripts for calculating fraction of each SARS-CoV-2 variant	Zenodo	DOI: 10.5281/zenodo.7275406
Additional metadata for analysis	Mendeley	DOI: 10.17632/j26ht6sy3p.1
Experimental models: Cell lines		
VeroE6 cell	21	RRID:CVCL_0059
Experimental models: Organisms/strains		
Oligonucleotides		
OligoT30VN /5Biosg/ACGAGCATCAGCAGCATACGAT TTTTTTTTTTTTTTTTTTTTTTTTTTTTTVN	26	IDT
TSO /5Biosg/AGAGACAGATTGCGCAATGNN NNNNNNrGrGrG	26	IDT
Forward primer TCGTCGGCAGCGTCAGATGTGTATAAGA GACAGATTGCGCAA*T*G	26	IDT
Reverse primer ACGAGCATCAGCAGCATAC*G*A	26	IDT
Recombinant DNA		
Software and algorithms		
FACSDiva software version (ver. 6.1.3)	BD Biosciences	
R (ver. 4.0.3)	Comprehensive R Archive Network	https://cran.r-project.org

Python (ver. 2.7.16)	Python Software Foundation	https://www.python.org/
Samtools (ver. 1.9)	27	http://www.htslib.org/ ; RRID:SCR_002105
Varscan2 (ver. 2.4.2)	28	http://dkoboldt.github.io/vars-can/ ; RRID:SCR_006849
HaplotypeCaller	29	https://gatk.broadinstitute.org/hc/en-us/articles/360037225632-HaplotypeCaller RRID:SCR_001876
Integrated Genomics Viewer	30	http://software.broadinstitute.org/software/igv/ RRID:SCR_011793
STAR (ver. 2.6.4)	31	https://github.com/alexdobin/STAR ;RRID:SCR_015899 RRID:SCR_004463
zUMIs	32	https://github.com/sdparekh/zUMIs RRID:SCR_016139
BWA-MEM	33	http://bio-bwa.sourceforge.net/bwa.shtml RRID:SCR_010910
iVar	34	https://github.com/andersen-lab/ivar
Fastp	35	https://github.com/OpenGenome/fastp RRID:SCR_016962
Kraken	36	https://github.com/DerrickWood/kraken
Seurat	37	https://github.com/satijalab/seurat RRID:SCR_007322
Scran	38	https://github.com/elswob/SCRAN RRID:SCR_016944
Scanpy	39	https://github.com/scverse/scanpy RRID:SCR_018139
Gggenes		https://github.com/wilkox/gggenes
Other		

RESOURCE AVAILABILITY

Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Young Seok Ju (ysju@kaist.ac.kr).

Materials Availability

All 3D models generated in this study are available from the Lead Contact with a completed Materials Transfer Agreement.

Data and Code Availability

Single-cell bam files of SMART-seq3 are uploaded to the European Genome-Phenome Archive. Furthermore, metadata of infected alveolar cells, expression of alveolar cell markers, normalized depth of viral transcripts by two different methods, and raw data of the number of viruses by two different methods are uploaded on Mendeley. Accession numbers are listed in the key resources table. The data will be fully available as soon as the administration process completes.

All original code and additional files have been deposited at Zenodo is publicly available. DOIs are listed in the key resources table.

Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENT MODEL AND SUBJECT DETAILS

Alveolar organoids establishment from human lung tissues

Human normal lung tissues were acquired from lung cancer patients with lobectomy surgery at SNUH with informed consent (IRB approval no. C-1809-137-975). From the human lung surgical samples, alveolar organoids were established as previously described²². To remove cell-free RNA from RSPO-1 conditioned media, we used lyophilized RSPO-1 (80ng/ml) (R&D systems 4645-RS) instead of the RSPO-1 conditioned media.

Virus stock preparation of each viral variant used for the competition assay

VeroE6 cells were infected with a 0.01 MOI and grown in DMEM with 2% FBS and 1% P/S for 48 hours at 37°C with 5% CO₂ as previously described for the virus stock preparation²². A purified viral stock was used to calculate the number of live viruses by plaque assay. The passage of all the viral stocks we used for the competition assay was 4. We counted passage 1 as the virus

acquired after the first infection of the patient sample to VeroE6.

METHOD DETAILS

Virus competition system among VOCs: single cell infection with multiple VOCs

First, human alveolar organoids were recovered by depolymerizing the Matrigel (Corning 354230) with Recovery solution (Corning 354253) at 4°C for 20 minutes. Furthermore, to remove the remaining Matrigel and dissociate the organoids into single cells, the organoids were incubated in Accutase (Stem Cell Technologies 07920) at 37°C for 5 minutes with additional mechanical pipetting. After washing, the cells were manually counted with the iNCYTO chip (iNCYTO DHC-N01). The cells were resuspended in Advanced DMEM/F12 (Thermo-fisher 12634010) with 1 U/ml Penicillin/Streptomycin (Gibco 15630-080), 10mM HEPES (Gibco 15140-122), and 1% Glutamax (Gibco 35050-061) (v/v) (hereafter referred to as ADF+++). 5,000 cells in 175 ul ADF +++ were aliquoted into Protein LoBind® tubes (Eppendorf 0030108116).

For the preparation of the viral variants mixture, based on the PFU concentration of each viral variant, we diluted each viral variant stock to contain equal amounts of viable viruses before mixing viral variants (MOI ~ 2.5; 12,500 PFU each respectively). Then each diluted viral variants were mixed in a Protein LoBind® tube with a final volume of 175 ul. After adding the viral variants mixture viruses to alveolar cells, the cell-virus solution was thoroughly mixed by pipetting by 20 times. The tubes were then incubated for 5 minutes at 37°C and then, the cell-virus mix was washed for a total of 40,000X to remove the viable virus at the cell surface. Washed cells were embedded in Matrigel and cells were incubated for different post-infection times. Post-infection times were divided into 5 minutes, 4 hours, 24 hours, 48 hours, and 72 hours. Without batch O, the viral incubation time of all batches is 5 minutes. Two populations of infected cells with different viral incubation times of 5 and 60 minutes consist of batch O. This information is shown as viral incubation time with a heatmap (**Figure 2A**).

Virus competition system among VOCs: single-cell isolation

Fluorescence microscopy was used to increase the contrast of the live cells and decrease the single-cell capture time. Furthermore, a microchip was used to isolate a single cell, and pictures were taken to store the cell status (**Figure S1D**).

After various post-infection times, infected alveolar cells were recovered from Matrigel same as above. Recovered alveolar cells were washed at least 40,000X to reduce the number of free-floating viral RNA transcripts. The cells were incubated with a 1,000X CMFDA cell tracker (Thermo-Fisher C2925) for 10 minutes at 37°C. Next, the cells were loaded with 0.5% BSA (Sigma-Aldrich A8412) to decrease cell attachment on the smart aliquotor CE chip (iBioChip H2-SACE-5PK), and a single cell was picked up using 1 ul pipette after manual curation of the cell number using a

fluorescence and phase contrast microscope.

Virus competition system among VOCs: library preparation for isolated single cells.

For the SMART-seq3 library preparation, the minimal lysis buffer amount was first optimized for the cell in 1 ul PBS. It was determined that a 4 times volume in every step before the 1st bead cleanup, compared to the original SMART-seq3 paper, was enough to successfully amplify the cDNA from the RNA.

The lysis buffer was added to a captured cell, snap frozen, and stored at -80°C until lysis and reverse transcription. Then the cells were thawed with the lysis buffer followed by the SMART-seq3 library protocol ²⁰. The pooled library was sequenced by Illumina NovaSeq by paired-end sequencing with an average of 314 Mb per cell.

The fraction of infective viruses and viral transcripts in a virus mixture

We conducted a plaque assay to count the number of infective (viable) viruses at each diluted viral variant and amplicon sequencing to calculate the fraction of viral RNA transcripts, at the viral mixture over experimental batches H, I J, and K (**Figure S1B**). For plaque assay, diluted viruses were used to calculate the PFU of each viral variant from each experimental batch. Ideally, each viral variant has an equal 12,500 PFU. For viral RNA transcripts, we extracted RNA from the viral variant mixture and conducted RNA sequencing of the amplicon library with the same method in each viral variant's RNA sequencing above. After the same alignment method with the viral genomic mutations, we calculated the fraction of viral RNA transcript from each viral variant. Ideally, the viral RNA transcript of each viral variant consists of 25%.

Viral genomic mutations

To confirm the mutations of each virus that were used for the viral barcodes, we first conducted RNA sequencing of the viral stocks of each variant. Viral RNA was extracted by the QIAamp viral mini kit (Qiagen 52904) from a virus stock containing >10,000 viral PFU. Then, a sequencing library was constructed with the NEBNext ARTIC SARS-CoV-2 FS kit (NEB E7658S).

For each viral variant, paired-end sequencing was conducted by Illumina NovaSeq. Adapter sequences were removed with fastp ³⁵. Then, the adapter-removed fastq files were aligned to the SARS-CoV-2 reference genome sequence, wuhCor1.fa (NC_045512v2) with BWA-MEM³³. Primer sequences in the amplicons were removed using iVar ³⁴. Any human contamination reads were removed by Kraken ³⁶.

The mutation sites were called using VarScan pileup2snp and pileup2indel ⁴⁰ and GATK HaplotypeCaller ²⁹. Candidate calls were manually inspected using Integrative Genomic Viewer ³⁰

and in total, 118 clonal mutations (VAF>99.5%) were finally obtained (**Supplementary Table 1**) and compared among the four viral variants (**Figure 1B, Figures S1A**). For indel, we didn't count the reads which can't span the homopolymer region right next to the indel position.

Since some loci were stochastically not covered by RNA sequencing of viral stock, we rescued the mutations. For example, the mutation with the genomic position 6,954 from the Alpha variant batch B and the mutation with the genomic position 23,948 from the Omicron variant batch D were manually rescued (**Figure S1A**).

Data processing of the full-length single-cell transcriptome sequencing

From the pooled fastq file, we counted reads and UMIs of gene expression using zUMIs³². This condition is the same as the SMART-seq3 paper²⁰. To align single-cell transcriptome sequences from infected cells, a joint reference genome sequence was established by concatenating the human genome (GRCh38.p13) and SARS-CoV-2 genome (wuhCor1.fa). A joint gene annotation file (gtf) was also generated by merging the primary annotation gtf of GRCh38.p13 and ncbiGenes.gtf downloaded from the human GENCODE site and SARS-CoV-2 UCSC site, respectively. From the gtf file, we removed the nested exons of ORF1ab for calculating the gene expression of the viral transcripts. The UMI count matrix for exons was used for the transcriptome analysis.

For the quality control of the single-cell RNA sequencing data, any cells with a mitochondria percentage above 40, the number of genes expressed below 1,250, or a total UMI count below 1,250 were excluded. To normalize expression levels of infected alveolar cells, we divided the UMI count of each gene by the total UMI count of a cell, multiply 10,000 and add 1 for plotting as a log scale (**Figure 1C**). Single-cell RNA sequencing analysis was done using Scanpy, if not otherwise stated³⁹.

$$\text{Normalized expression level} = \left(\frac{\text{UMI count of a gene}}{\text{UMI count of a cell}} * 10000 \right) + 1$$

Decomposition of the viral variants that infected a single-cell

We used the variant allele fraction (VAF) of each variant locus to calculate the proportion of the viral variant that infected a specific cell. Because not all 118 loci were covered by single-cell transcriptome sequencing, specific consideration was necessary for an accurate decomposition. To this end, we used two different methods which were finally proven to be concordant with each other.

The first method is to utilize the average VAF of the multiple mutation loci. For each of the four viral variants, we calculated the average VAF of all covered unique mutation sites of a viral variant

as a proxy of their fraction in a cell (F_{α} , F_{δ} , F_{\omicron} , and F_{GR}). For cells with the sum of the average VAFs (F_{sum} ; $F_{\alpha}+F_{\delta}+F_{\omicron}+F_{GR}$) smaller than 1, cellular infection was explained by an average of ~ 0.95 quite well. For most cells with $F_{sum} > 1$, we normalized each F value with the F_{sum} value. For cells explicitly infected but having $F_{\alpha}+F_{\delta}+F_{\omicron}=0$, F_{GR} was explicitly assigned to 1 because the GR variant has only 3 specific mutations (**Figure 1B**), and the viral variant is more likely to be unexposed in the transcriptome sequencing by chance. Lastly, for cells with more than two viral variants which are not covered for every unique mutation of that (NA), $1 - (F_{sum} \text{ without NA})$ is assigned as unknown

The second method is using nonnegative matrix factorization (NMF). Given a sample VAF matrix of size $118 \times \text{number of samples}$, the linear combination of the VOC genotypes (0 or 1) that best explains each VAF column of the sample matrix was calculated. This process can be summarized as follows where the VOC matrix has a size of 118×4 , and the weight matrix has a size of $4 \times \text{number of samples}$:

$$VAF \text{ matrix} = Genotype \text{ matrix} \times Weight \text{ matrix}$$

Because the two methods showed a high linear correlation, we used and showed the results obtained from the first method in (**Figure 1F**). Cosine similarity was calculated after NA converting to zero. Even if we removed infected cells with NA fraction, the median and pattern of the cosine similarity were similar to converting the NA to 0 in infected alveolar cells.

Criteria for infection at a single cell

In practice, some extracellular virus RNA may contribute to the viral reads in single-cell transcriptome sequencing although a cell is not infected. From empty wells in the single-cell transcriptome sequencing, we set a threshold for the viral infection as 4 or more viral UMI in more than 2 different viral genes except for the N gene. Although alveolar organoids were washed at least 40,000, loading washed cell solution multiple times into a microchip leads to a higher number of free viral RNA transcripts.

Normalized coverage of virus transcripts

We drew the gene plot above the coverage plot by gggenes. For two single-cell transcriptome methods, we used the current paper's data using the full-length transcriptome method and the previous paper's data, infected alveolar cells at 3 days post-infection with MOI 1, using 3' enriched method²². The read-depth of deduplicated bam files was analyzed by SAMtools²⁷ and normalized for each infected cell.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical Analysis

To calculate the Omicron's dominance for single and total infected cells, we adopted the proportion test with a random expectation of 0.25. Furthermore, to compare each variant's infectivity, we calculated the odds ratios for each pair of viruses.

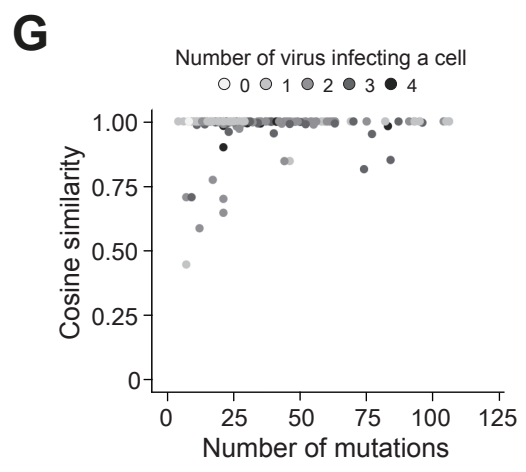
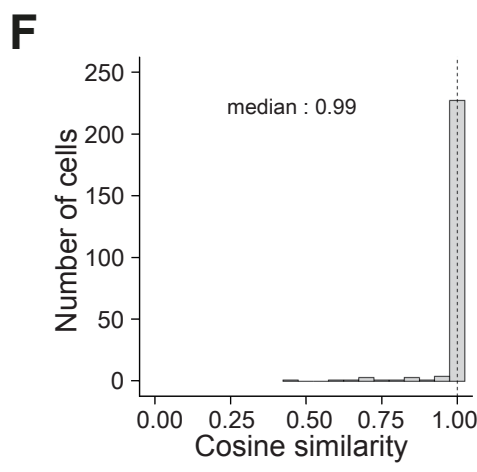
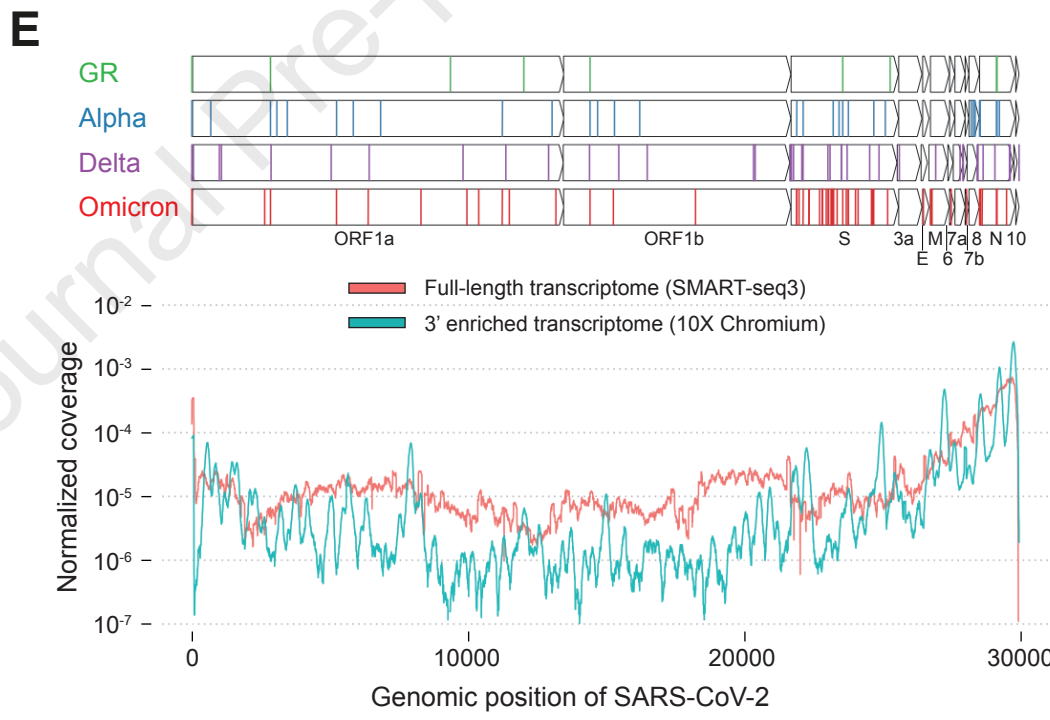
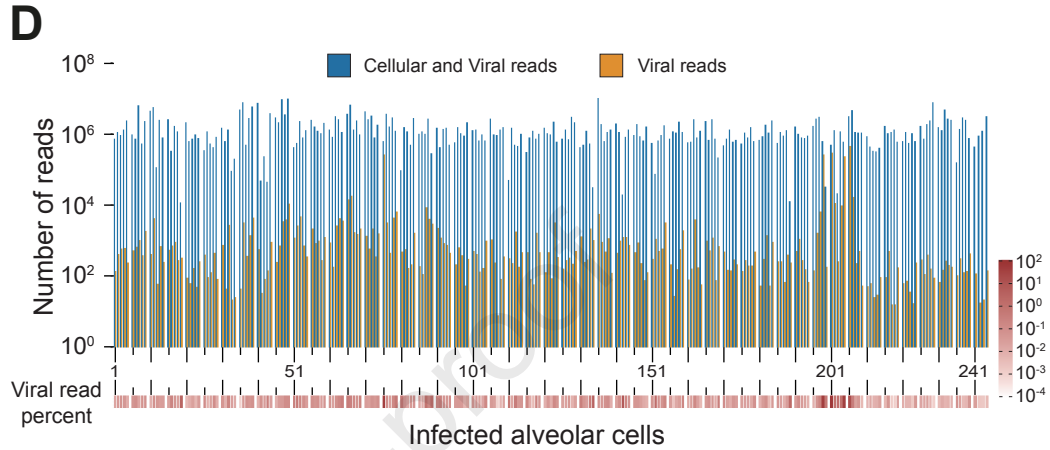
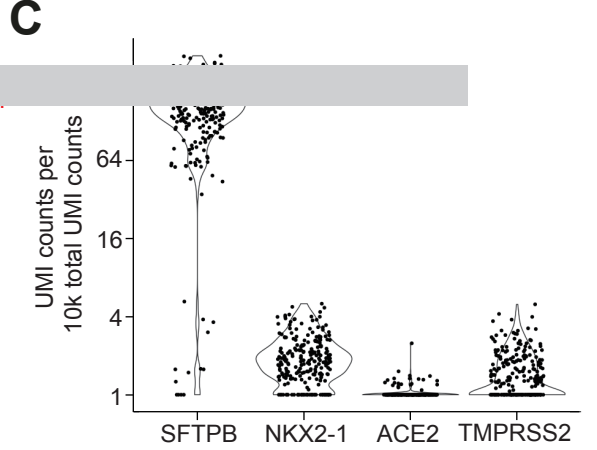
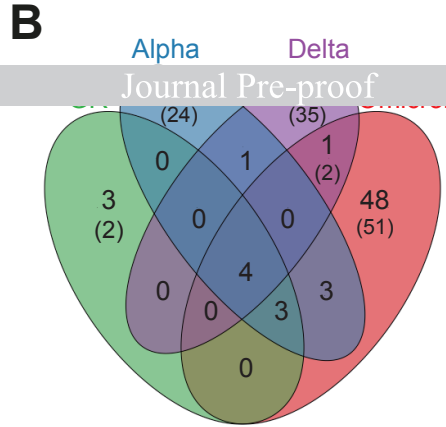
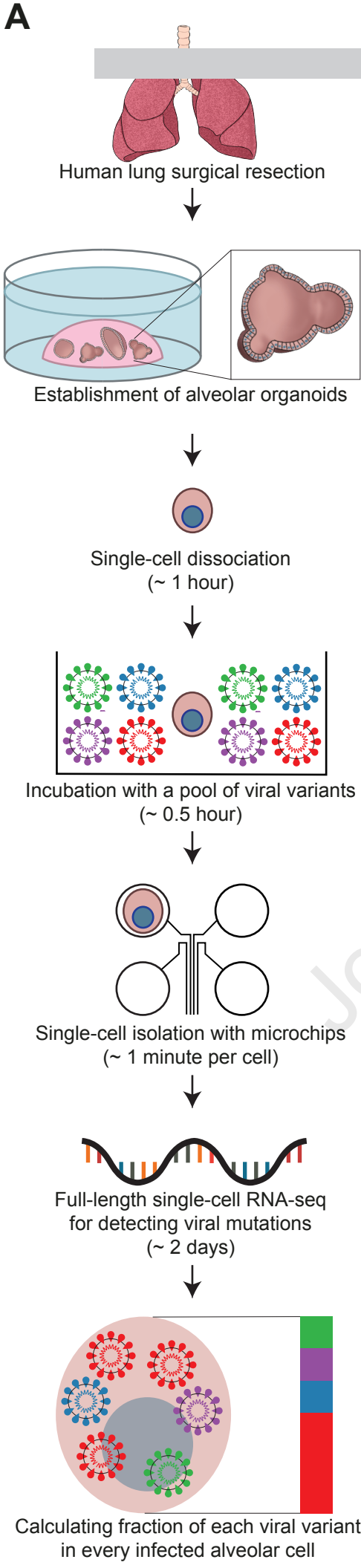
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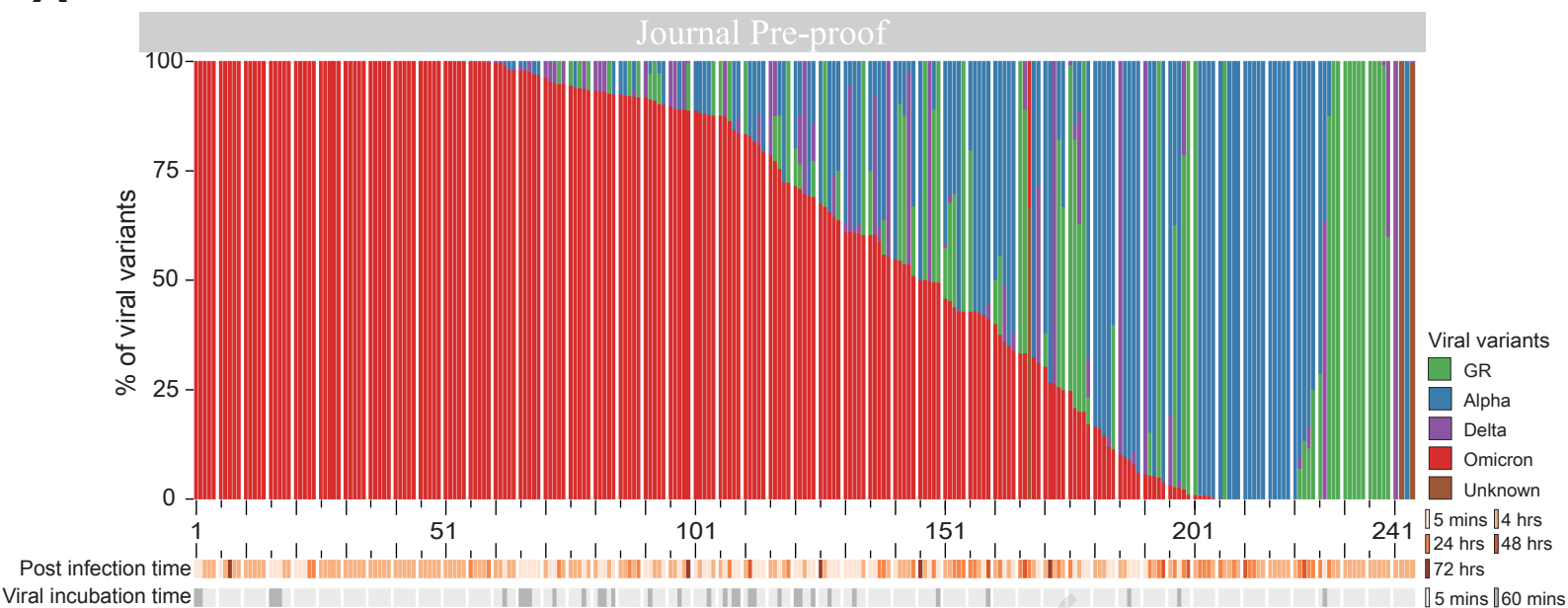
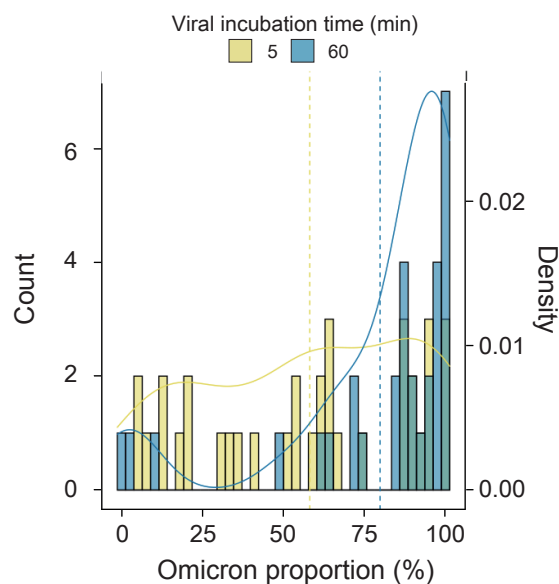
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A**B****C**

Highlights of the paper titled “Relative infectivity of the SARS-CoV-2 Omicron variant in human alveolar cells

Highlights

1. An efficient system for evaluating the relative infectivity of SARS-CoV-2 variants
2. Human alveolar organoids are host cells for comparing viral infectivity against lung
3. Full-length single-cell RNA-seq identifies viral variants in each infected cell
4. The Omicron variant shows 5-7-times higher infectivity in human alveolar cells

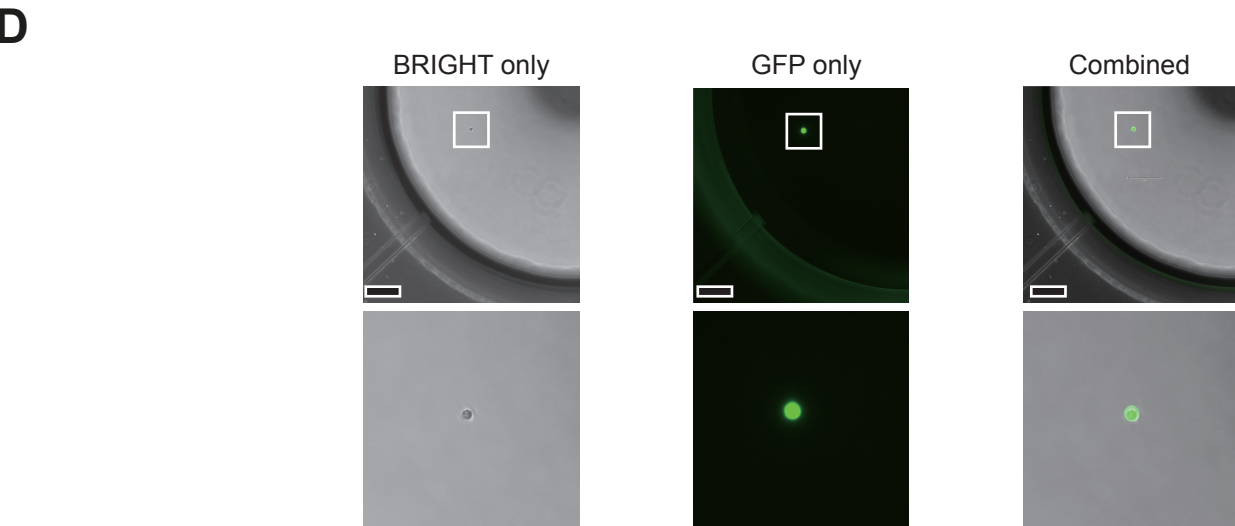
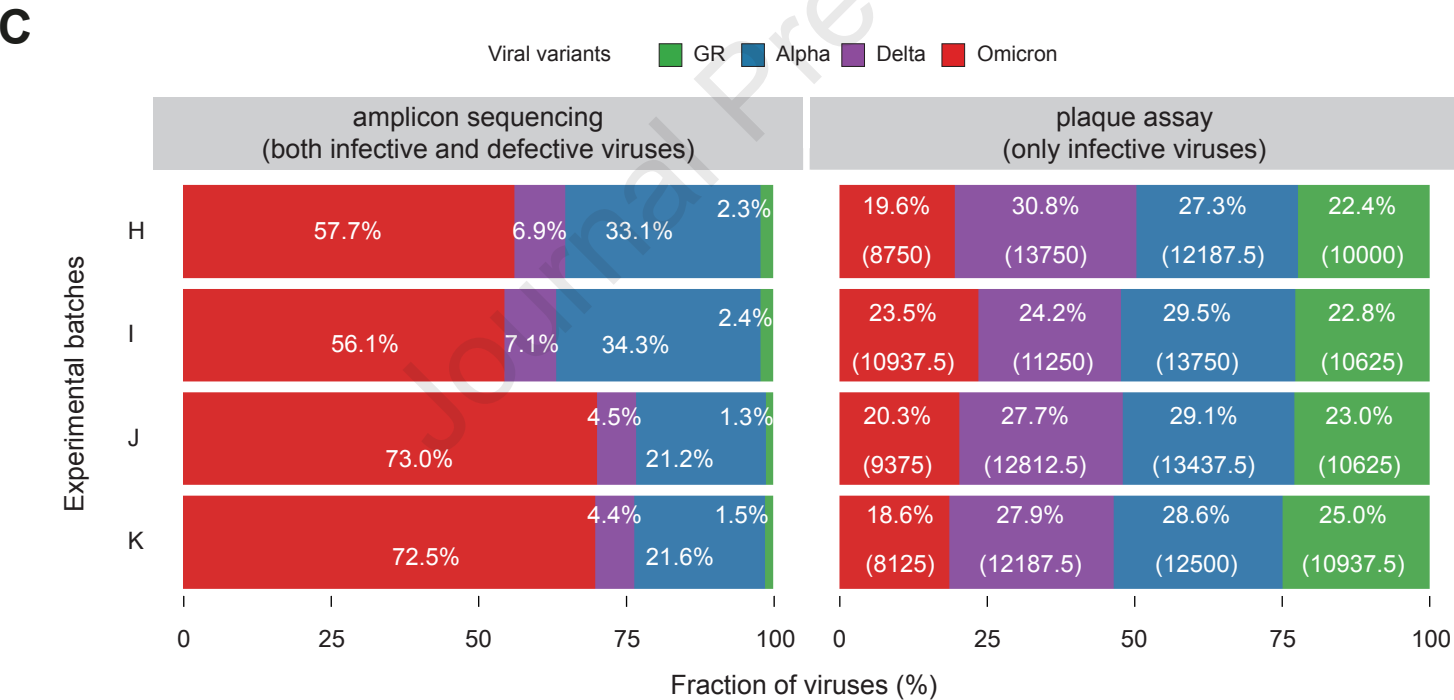
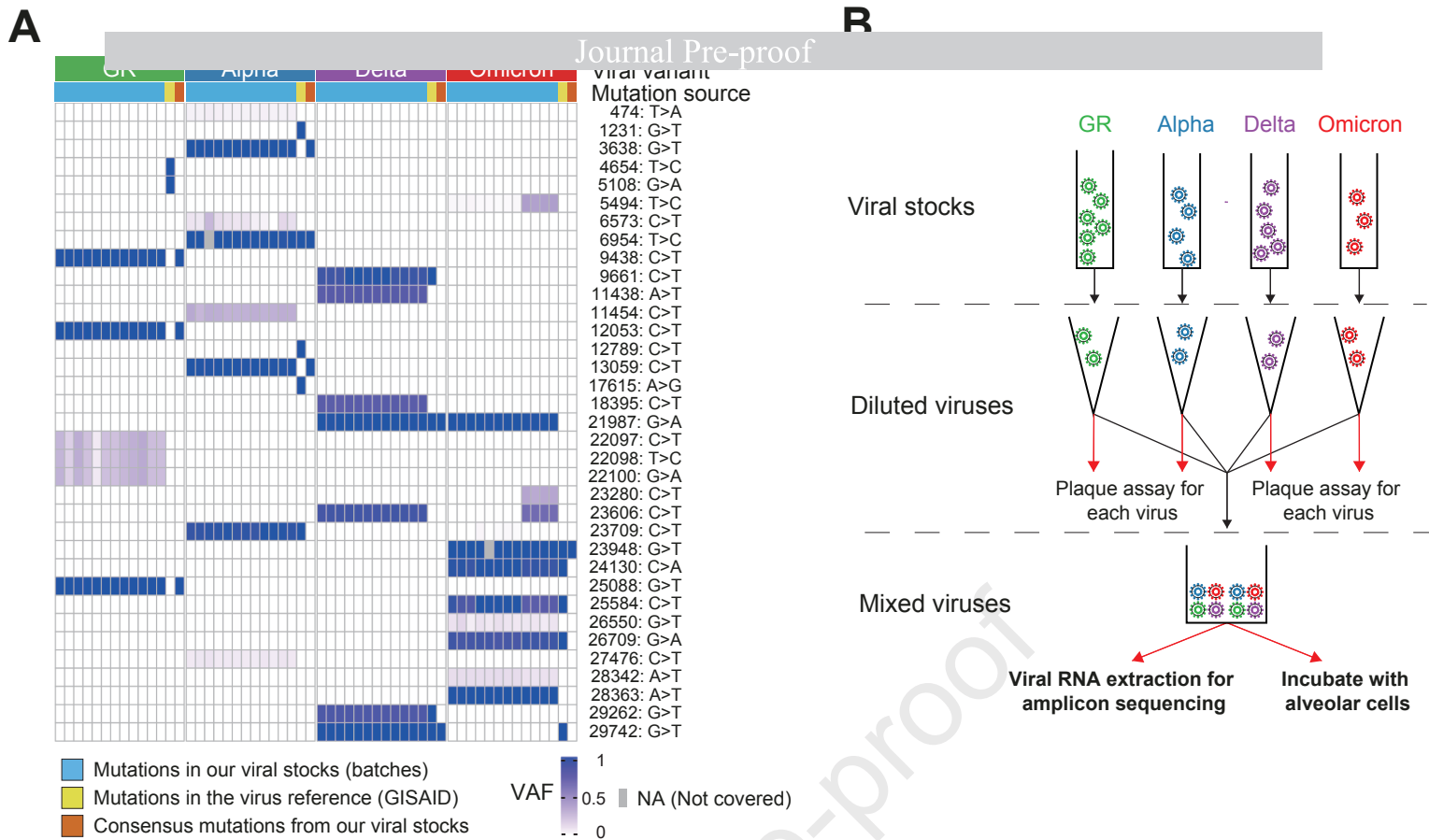


Figure S1. Detailed information of competition assay. Related to Figure 1.

- (A) M [REDACTED] and the mutation position of the SARS-CoV-2 genome is written in a row. The order of viral stocks is the same as in Figure 2B.
- (B) Schematic diagram of calculating the fraction of viral variants in the competition assay. Infective (viable) viruses were counted by plaque assay of each viral variant and both infectious and defective were by RNA sequencing of the viral mixture. The fraction is written in white with the number of PFU in parentheses.
- (C) Fraction of viral variants by two different methods, including amplicon sequencing and plaque assay.
- (D) Manual picking of single cells in the biosafety level 3 laboratory.

Table S1. A full list of clonal genomic mutations of SARS-CoV-2 variants. Related to Figure 1

118 clonal viral genomic mutations were listed in this file.

A full list of genomic mutations of the Alpha, Delta, Omicron, and GR

Alpha, Delta, GR, and Omicron has REF-POS-ALT if they have mutation in that site

vocs means that the number of viruses which have specific mutation

unique_voc means the name of virus with unique mutation at specific mutation

for INDELs, if ALT is AA and REF is A, it was written as ALT = + A

CHROM	POS	REF	ALT	alpha	delta	gr	omicron	vocs	unique_voc
NC_045512v2	210	G	T	NA	G210T	NA	NA		1 delta
NC_045512v2	241	C	T	C241T	C241T	C241T	C241T		4 multi
NC_045512v2	913	C	T	C913T	NA	NA	NA		1 alpha
NC_045512v2	1191	C	T	NA	C1191T	NA	NA		1 delta
NC_045512v2	1267	C	T	NA	C1267T	NA	NA		1 delta
NC_045512v2	2832	A	G	NA	NA	NA	A2832G		1 omicron
NC_045512v2	3037	C	T	C3037T	C3037T	C3037T	C3037T		4 multi
NC_045512v2	3267	C	T	C3267T	NA	NA	NA		1 alpha
NC_045512v2	3638	G	T	G3638T	NA	NA	NA		1 alpha
NC_045512v2	5184	C	T	NA	C5184T	NA	NA		1 delta
NC_045512v2	5386	T	G	NA	NA	NA	T5386G		1 omicron
NC_045512v2	5388	C	A	C5388A	NA	NA	NA		1 alpha
NC_045512v2	5986	C	T	C5986T	NA	NA	NA		1 alpha
NC_045512v2	6512	A	#NAME?	NA	NA	NA	AGTT6512A		1 omicron
NC_045512v2	6539	C	T	NA	C6539T	NA	NA		1 delta
NC_045512v2	6954	T	C	T6954C	NA	NA	NA		1 alpha
NC_045512v2	8393	G	A	NA	NA	NA	G8393A		1 omicron
NC_045512v2	9438	C	T	NA	NA	C9438T	NA		1 gr
NC_045512v2	9891	C	T	NA	C9891T	NA	NA		1 delta
NC_045512v2	10029	C	T	NA	NA	NA	C10029T		1 omicron
NC_045512v2	10449	C	A	NA	NA	NA	C10449A		1 omicron
NC_045512v2	11282	A	#NAME?	NA	NA	NA	AGTTTGTCTG		1 omicron
NC_045512v2	11287	G	#NAME?	GTCTGGTTTT	NA	NA	NA		1 alpha
NC_045512v2	11418	T	C	NA	T11418C	NA	NA		1 delta
NC_045512v2	11537	A	G	NA	NA	NA	A11537G		1 omicron
NC_045512v2	12053	C	T	NA	NA	C12053T	NA		1 gr
NC_045512v2	12946	T	C	NA	T12946C	NA	NA		1 delta
NC_045512v2	13059	C	T	C13059T	NA	NA	NA		1 alpha
NC_045512v2	13195	T	C	NA	NA	NA	T13195C		1 omicron
NC_045512v2	14408	C	T	C14408T	C14408T	C14408T	C14408T		4 multi
NC_045512v2	14676	C	T	C14676T	NA	NA	NA		1 alpha
NC_045512v2	15240	C	T	NA	NA	NA	C15240T		1 omicron
NC_045512v2	15279	C	T	C15279T	NA	NA	NA		1 alpha
NC_045512v2	15451	G	A	NA	G15451A	NA	NA		1 delta
NC_045512v2	16176	T	C	T16176C	NA	NA	NA		1 alpha
NC_045512v2	16466	C	T	NA	C16466T	NA	NA		1 delta
NC_045512v2	18163	A	G	NA	NA	NA	A18163G		1 omicron
NC_045512v2	20262	A	G	NA	A20262G	NA	NA		1 delta
NC_045512v2	20320	C	T	NA	C20320T	NA	NA		1 delta
NC_045512v2	21575	C	T	NA	C21575T	NA	NA		1 delta
NC_045512v2	21618	C	G	NA	C21618G	NA	NA		1 delta
NC_045512v2	21691	C	T	NA	C21691T	NA	NA		1 delta
NC_045512v2	21762	C	T	NA	NA	NA	C21762T		1 omicron
NC_045512v2	21764	A	#NAME?	ATACATG217	NA	NA	ATACATG217		2 multi
NC_045512v2	21846	C	T	NA	NA	NA	C21846T		1 omicron
NC_045512v2	21986	G	#NAME?	NA	NA	NA	GGTGTTTATT		1 omicron
NC_045512v2	21987	G	A	NA	G21987A	NA	NA		1 delta
NC_045512v2	21990	T	#NAME?	TTTA21990T	NA	NA	NA		1 alpha

NC_045512v2	22028 G	#NAME?	NA	GAGTTCA220	NA	NA	1 delta
NC_045512v2	22193 A	#NAME?	NA	NA	NA	AATT22193A	1 omicron
NC_045512v2	22204 T	#NAME?	NA	NA	NA	T22204TGAG	1 omicron
NC_045512v2	22578 G	A	NA	NA	NA	G22578A	1 omicron
NC_045512v2	22673 T	C	NA	NA	NA	T22673C	1 omicron
NC_045512v2	22674 C	T	NA	NA	NA	C22674T	1 omicron
NC_045512v2	22679 T	C	NA	NA	NA	T22679C	1 omicron
NC_045512v2	22686 C	T	NA	NA	NA	C22686T	1 omicron
NC_045512v2	22813 G	T	NA	NA	NA	G22813T	1 omicron
NC_045512v2	22882 T	G	NA	NA	NA	T22882G	1 omicron
NC_045512v2	22898 G	A	NA	NA	NA	G22898A	1 omicron
NC_045512v2	22917 T	G	NA	T22917G	NA	NA	1 delta
NC_045512v2	22992 G	A	NA	NA	NA	G22992A	1 omicron
NC_045512v2	22995 C	A	NA	C22995A	NA	C22995A	2 multi
NC_045512v2	23013 A	C	NA	NA	NA	A23013C	1 omicron
NC_045512v2	23040 A	G	NA	NA	NA	A23040G	1 omicron
NC_045512v2	23048 G	A	NA	NA	NA	G23048A	1 omicron
NC_045512v2	23055 A	G	NA	NA	NA	A23055G	1 omicron
NC_045512v2	23063 A	T	A23063T	NA	NA	A23063T	2 multi
NC_045512v2	23075 T	C	NA	NA	NA	T23075C	1 omicron
NC_045512v2	23202 C	A	NA	NA	NA	C23202A	1 omicron
NC_045512v2	23271 C	A	C23271A	NA	NA	NA	1 alpha
NC_045512v2	23401 G	T	NA	G23401T	NA	NA	1 delta
NC_045512v2	23403 A	G	A23403G	A23403G	A23403G	A23403G	4 multi
NC_045512v2	23525 C	T	NA	NA	NA	C23525T	1 omicron
NC_045512v2	23599 T	G	NA	NA	NA	T23599G	1 omicron
NC_045512v2	23604 C	A	C23604A	NA	NA	C23604A	2 multi
NC_045512v2	23604 C	G	NA	C23604G	NA	NA	1 delta
NC_045512v2	23854 C	A	NA	NA	NA	C23854A	1 omicron
NC_045512v2	23948 G	T	NA	NA	NA	G23948T	1 omicron
NC_045512v2	24410 G	A	NA	G24410A	NA	NA	1 delta
NC_045512v2	24424 A	T	NA	NA	NA	A24424T	1 omicron
NC_045512v2	24469 T	A	NA	NA	NA	T24469A	1 omicron
NC_045512v2	24503 C	T	NA	NA	NA	C24503T	1 omicron
NC_045512v2	24506 T	G	T24506G	NA	NA	NA	1 alpha
NC_045512v2	24745 C	T	NA	C24745T	NA	NA	1 delta
NC_045512v2	24914 G	C	G24914C	NA	NA	NA	1 alpha
NC_045512v2	25000 C	T	NA	NA	NA	C25000T	1 omicron
NC_045512v2	25088 G	T	NA	NA	G25088T	NA	1 gr
NC_045512v2	25469 C	T	NA	C25469T	NA	NA	1 delta
NC_045512v2	26270 C	T	NA	NA	NA	C26270T	1 omicron
NC_045512v2	26530 A	G	NA	NA	NA	A26530G	1 omicron
NC_045512v2	26577 C	G	NA	NA	NA	C26577G	1 omicron
NC_045512v2	26767 T	C	NA	T26767C	NA	NA	1 delta
NC_045512v2	27259 A	C	NA	NA	NA	A27259C	1 omicron
NC_045512v2	27638 T	C	NA	T27638C	NA	NA	1 delta
NC_045512v2	27739 C	T	NA	C27739T	NA	NA	1 delta
NC_045512v2	27752 C	T	NA	C27752T	NA	NA	1 delta
NC_045512v2	27807 C	T	NA	NA	NA	C27807T	1 omicron
NC_045512v2	27972 C	T	C27972T	NA	NA	NA	1 alpha
NC_045512v2	28048 G	T	G28048T	NA	NA	NA	1 alpha
NC_045512v2	28111 A	G	A28111G	NA	NA	NA	1 alpha
NC_045512v2	28247 A	#NAME?	NA	AGATTTC282	NA	NA	1 delta
NC_045512v2	28270 T	#NAME?	TA28270T	TA28270T	NA	NA	2 multi
NC_045512v2	28271 A	T	NA	NA	NA	A28271T	1 omicron
NC_045512v2	28280 G	C	G28280C	NA	NA	NA	1 alpha

NC_045512v2	28281 A	T	A28281T	NA	NA	NA	1 alpha
NC_045512v2	28282 T	A	T28282A	NA	NA	NA	1 alpha
NC_045512v2	28311 C	T	NA	NA	NA	C28311T	1 omicron
NC_045512v2	28361 G	#NAME?	NA	NA	NA	GGAGAACGC	1 omicron
NC_045512v2	28461 A	G	NA	A28461G	NA	NA	1 delta
NC_045512v2	28881 G	A	G28881A	NA	G28881A	G28881A	3 multi
NC_045512v2	28881 G	T	NA	G28881T	NA	NA	1 delta
NC_045512v2	28882 G	A	G28882A	NA	G28882A	G28882A	3 multi
NC_045512v2	28883 G	C	G28883C	NA	G28883C	G28883C	3 multi
NC_045512v2	28977 C	T	C28977T	NA	NA	NA	1 alpha
NC_045512v2	29229 G	A	NA	NA	NA	G29229A	1 omicron
NC_045512v2	29402 G	T	NA	G29402T	NA	NA	1 delta
NC_045512v2	29427 G	A	NA	G29427A	NA	NA	1 delta
NC_045512v2	29742 G	T	NA	G29742T	NA	NA	1 delta