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LETTER TO THE EDITOR

# FISHing for COVID-19: Studying SARS-CoV-2 with single-molecule FISH and flow-FISH

For more than 2 years, the COVID-19 pandemic has plagued the world. The causative agent, SARS-CoV-2, is a single-stranded RNA virus that is easily transmitted, resulting in >603 million confirmed infections worldwide (https://covid19.who.int/) at the time of writing. Many efforts have been undertaken to unravel SARS-CoV-2 biology, epidemiology and pathology, but also the immune responses directed against the virus has been thoroughly investigated. To do so, new reagents were required to study this newly emerged virus, and novel assays were developed, ranging from diagnostic PCR tests, antibody ELISAs and at-home test kits, all with the aim to study and combat the COVID-19 pandemic.

However, many of these assays make use of, or detect, isolated viral genetic material or viral proteins, thus not allowing to study infected cells at a single cell level. One of the assays that has been developed and allows for studying SARS-CoV-2 infection at the single cell level makes use of single-molecule fluorescence *in situ* hybridization (smFISH) [1]. By employing probe sets that target the conserved regions of the SARS-CoV-2 genome, viral RNA can be visualized at the single molecule level. Authors showed that in Vero cells, a primate cell-line, the FISH assay was suitable to detect the level of SARS-CoV-2 infection. Also human cell lines, human post-mortem patient tissue samples, and also cells isolated from a nasal swab typically used for SARS-CoV-2 diagnostic purposes were tested. While preliminary, as authors only tested one nasal swab, the assay was able to distinguish between a PCR positive and PCR negative sample with the SARS-CoV-2 smFISH assay.

This knowledge was replicated and expanded upon by other groups that also employed smFISH to study SARS-CoV-2 infection in cell lines [2, 3], tissue sections of infected Syrian hamsters [2], and human lung, lymph node and placental tissue obtained from autopsies [3]. In one of these reports, authors also used the smFISH assay to investigate cellular localization and viral replication, comparing different viral strains [2]. Interestingly, the Victoria strain, an early Wuhan-related isolate, and the Alpha-variant B.1.1.7 strain behave very differently upon infection. While cellular infection is similar (as indicated by the amount of SARS-CoV-2 $RNA^+$ cells), the replicative capacity of the Victoria strain is much higher compared to the Alpha variant (as indicated by the amount of RNA per $RNA^+$ cell), indicating delayed replication by the Alpha variant. Based on their findings and other published work, authors speculate that lower amounts of RNA could be beneficial for the virus in terms of immune escape, thus increasing transmissibility.

The other report used multiplex smFISH by combining the SARS-CoV-2 probe set with other RNA target species to identify different cell types [3]. Specifically, they used *AGER* as a marker for alveolar type 1 cells, and *SFTPC* as a marker for alveolar type 2 cells in a lung sample from a SARS-CoV-2 infected individual [3]. They showed that, in this patient, only type 2 cells are SARS-CoV-2 $RNA^+$. Furthermore, authors used a similar approach to identify alveolar macrophages by employing a *MARCO* probe set. They could identify alveolar macrophages infected with SARS-CoV-2, and interestingly, upon comparing alveolar type 2 cells with alveolar macrophages, the distribution of viral RNA is different in these two cell types. While in alveolar type 2 cells, the RNA is distributed throughout the cytoplasm, in the alveolar macrophages, the staining seems to be more restricted. Based on their results, authors speculate that this might be indicative of restriction of viral RNA to a subcellular compartment in alveolar macrophages.

While the smFISH approach is interesting and potentially has merit to e.g. investigate subcellular localization [2, 3] and (new) antiviral drugs [1, 2], microscopic techniques are labor intensive, and require a high level of expertise. Furthermore, data analysis of microscopic readouts are laborious and can be influenced by investigator bias upon manual counting. This could be mitigated by, for example, a flow-cytometric RNA readout. Indeed, the use of flow cytometry-based fluorescence in situ hybridization (Flow-FISH), as also employed to study other microorganisms such as EBV and HIV-1 via flow cytometry [4–6], would be of interest here. Like smFISH, Flow-FISH makes use of labeled probe sets [4], and often allows for the use of smFISH probe sets. However, unlike smFISH, Flow-FISH can be performed in a high-throughput fashion due to the use of flow cytometry as a read-out, and, akin to smFISH [3], is suitable for multiplexing to simultaneously assess multiple RNA species [7]. Furthermore, Flow-FISH also allows for in-depth phenotyping of cells by combining RNA measurements, with (labeled) probes or probe sets, with protein measurements, through the use of (labeled) antibodies suitable for flow cytometry [8, 9]. While, depending on the protocol, there are some limitations to the fluorescent dyes conjugated to antibodies that are suitable for Flow-FISH [10], multi-color flow cytometry would allow for a more in-depth characterization by allowing a higher degree of multiplexing compared to smFISH.

A SARS-CoV-2 Flow-FISH assay using smFISH probes has already been developed [11]. This Flow-FISH assay was used to investigate a potentially druggable host process. As SARS-CoV-2 hijacks the host cell machinery to induce RNA production in order to facilitate virion generation, the authors suspected that metabolic changes occur to facilitate this. RNA synthesis requires glucose as a ribose source, but

  

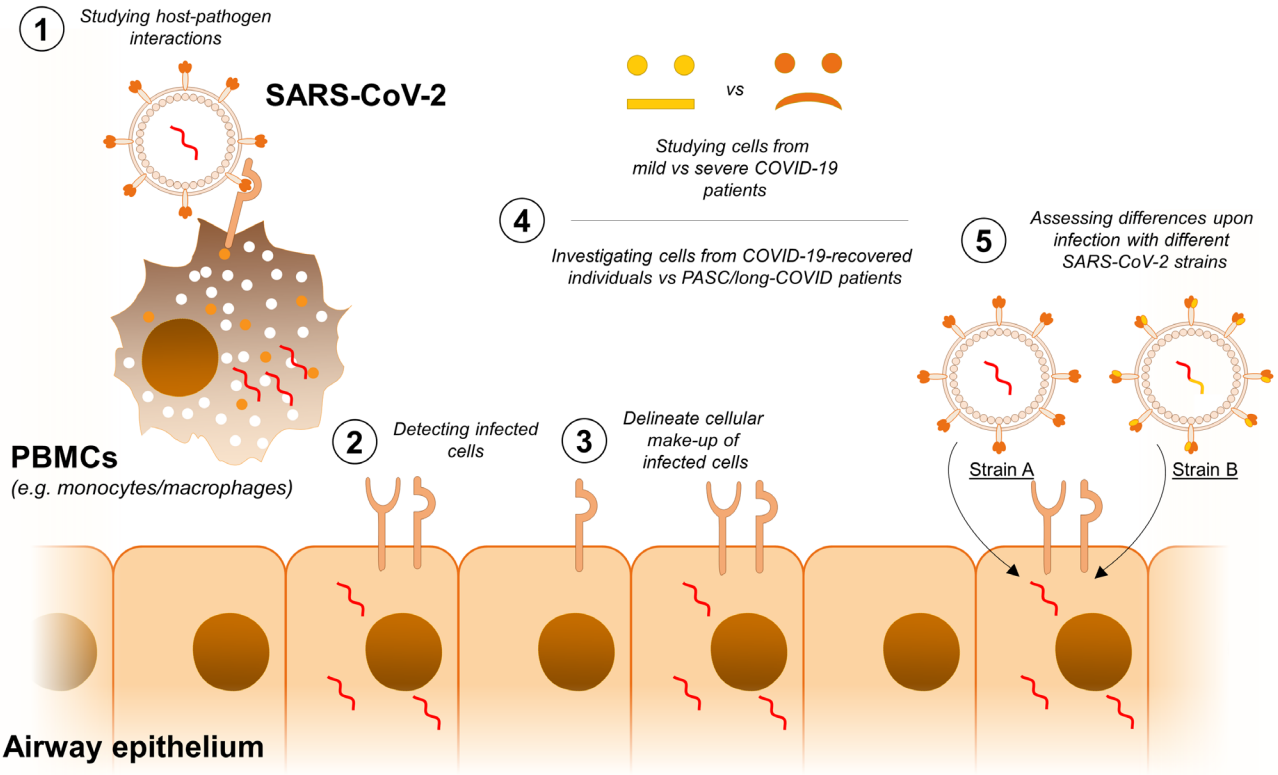


**FIGURE 1** Potential future applications of SARS-CoV-2 flow-FISH. Due to the flow cytometric approach, SARS-CoV-2 Flow-FISH can be used to further unravel SARS-CoV-2 biology to advance scientific knowledge and design new therapeutic applications. Potential applications include 1) studying host-pathogen interactions, 2) detecting and 3) characterizing infected cells, e.g. to delineate the cellular make-up of infected cells and investigate immune escape, 4) studying (molecular) mechanisms mediating disease outcome or longevity and 5) investigating differences upon infection with different SARS-CoV-2 strains. Please note that due to the customizability of Flow-FISH, markers can be added/exchanged depending on researcher needs

also one-carbon units derived from folate species. Indeed, glucose and folate were significantly depleted in SARS-CoV-2 infected cells. Upon treatment of cells with methotrexate (a drug that, amongst other targets, blocks one-carbon metabolism and nucleotide synthesis), SARS-CoV-2 nucleocapsid RNA expression in infected cells was significantly reduced as measured by SARS-CoV-2 Flow-FISH. To further unravel which enzymes in the folate generation pathway are exploited by SARS-CoV-2, authors probed serine hydroxymethyltransferase 1 and 2 (SHMT1 and SHMT2), metabolic enzymes that play a role in one-carbon folate species generation. Similarly, to treatment with methotrexate, treating SARS-CoV-2 infected cells with an SHMT1/2-specific dual inhibitor significantly diminished SARS-CoV-2 Nucleocapsid RNA expression in infected cells as determined with SARS-CoV-2 Flow-FISH. Together, these data show that the folate pathway is important for virion production, and could potentially be a druggable target for inhibiting viral replication in infected cells.

A commercial SARS-CoV-2 Flow-FISH kit is also available. This kit as also been used to study potential antiviral drugs. In a recent unpublished pre-print [12], authors postulated that any therapeutic strategy that targeted both inflammatory processes, to limit immunopathology, and inhibited viral replication, to inhibit viral spread, would be superior compared to targeting either pathway separately. One receptor that is involved in both processes is the peroxisome proliferator receptor alpha (PPAR-α). Activation of PPAR-α leads to dismantling of lipid droplets, a prime source for Corona-virus replication. One of the activating ligands of PPAR-α is palmitoylethanolamide, a lipid that has anti-inflammatory properties. For palmitoylethanolamide synthesis, the enzyme N-acylethanolamine acid amide hydrolase (NAAA) is critical. Authors show that pharmacological inhibition of NAAA results in a reduction in SARS-CoV-2 replication in various cell lines, while simultaneously inducing autophagy in infected cells. In *ex vivo* experiments, authors show that, also in SARS-CoV-2 infected human peripheral blood mononuclear cells, NAAA inhibitors reduce viral replication as measured by SARS-CoV-2 Flow-FISH. Furthermore, NAAA inhibitors also inhibit the production of tumor necrosis factor alpha (TNF-α). TNF-α is an inflammatory cytokine that is produced by a myriad of cells, and in the context of SARS-CoV-2 infection, plays an important role in the pathogenesis of severe COVID-19 [13]. Furthermore, TNF-α was shown to be increased in patients that had passed away after experiencing acute respiratory distress syndrome compared to those that have survived [14], and high TNF-α also correlated with post-acute sequelae of COVID-19 (PASC) [15], which poses a significant health risk post-recovery. While this is data from an unreviewed pre-print, these results are encouraging and indicate that NAAA inhibition might provide a two-pronged therapeutic benefit that not only inhibits viral replication, but also mitigates pathogenic inflammatory processes.

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As discussed, over the past 2 years, novel assays have been developed to study SARS-CoV-2. In emerging diseases, traditionally available reagents such as antibodies are often not yet available. However, the probe sets employed in FISH and Flow-FISH assays can be rapidly designed. Especially in situations with rapidly mutating viruses, such as SARS-CoV-2, this adaptability is a major benefit, and the few reports that have employed this assay have shown promising results in the context of potential druggable targets. Indeed, as a research tool, smFISH and Flow-FISH both have merit. While both smFISH and Flow-FISH are useful as tools to investigate host-pathogen interactions and to unravel viral biology, the high-throughput advantages and the option for a higher degree of multiplexing highlight the potential advantages of Flow-FISH. However, current literature has not yet (fully) embraced this assay in the field of studying SARS-CoV-2, but in the future, it has several interesting applications (Figure 1). For instance, SARS-CoV-2 Flow-FISH could be used to assess infected cells, for example, to compare the extracellular make-up of infected cells versus non-infected cells. This could for instance provide information regarding whether for example, inhibitory receptor expression or do-not-eat-me-signals are altered in infected cells as a strategy to escape immune recognition. It would be especially interesting to investigate this when comparing cells infected with different SARS-CoV-2 strains. Furthermore, phenotypic or functional differences of infected cells in patients with mild versus severe COVID-19 could be investigated to further pinpoint potential host factors involved in the pathogenesis process. Lastly, this cellular phenotyping could be used to investigate whether (a)typical markers are expressed by infected cells that could be used to more easily identify cells harboring SARS-CoV-2, similarly as to what HIV-1 Flow-FISH has been used for [16, 17].

Of note, a rapid (~4 h) protocol for smFISH-mediated viral quantification has already been developed [18]. However, the current diagnostic tools available for SARS-CoV-2 diagnostics are sufficient and can be effectively employed by a broader set of laboratories compared to a typical Flow-FISH assay. Indeed, these factors need to be carefully considered before considering to implement novel assays in a diagnostic space just for the sake of novelty [5].

## AUTHOR CONTRIBUTIONS

Julian J. Freen-van Heeren conceived the manuscript topic, performed literature research, and wrote the manuscript.

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## CONFLICT OF INTEREST

The author declare no conflicts of interest.

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