Development of an automated counting method to evaluate SARS-CoV-2 positive nasal mucosa epithelial cells in SARS-CoV-2 infected Syrian hamsters

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LIÈGE université
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Travail de fin d'études
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AIM OF THE WORK

The COVID-19 pandemic caused by SARS-CoV-2 requires effective strategies to prevent viral transmission. The potential of nasal vaccination as a mean of inhibiting viral entry and replication in the nasal mucosa, which serves as the primary site of SARS-CoV-2 infection, needs to be investigated. This research aims to develop a new method which would allow for an automated cell counting in order to compare the results obtained in SARS-CoV-2 infected an unvaccinated Syrian hamster to SARS-CoV-2 infected Syrian hamsters vaccinated either by the nasal or the systemic route, in order to assess the efficacy of the vaccines.

SUMMARY

Immunostaining combined with image analysis using QuPath software was used to count the number of SARS-CoV-2 infected cells. A novel method of digesting whole nasal mucosa was developed to obtain a cell suspension for flow cytometry, the results of which will be used to validate the QuPath results. Ten Syrian hamsters were used as positive controls to aid method development. Results highlight the importance of repeatable sampling techniques, mucosal dissection and staining techniques. This research shows that the QuPath open source image analysis software using a trained cell classifier is promising for automating the counting of SARS-CoV-2 infected cells. Furthermore, the new method for whole nasal mucosa dissection worked, suggesting that our new counting method would allow the establishment of a baseline number of SARS-CoV-2 infected cells in the nasal mucosa of SARS-CoV-2 infected Syrian hamsters, which could be compared to hamsters vaccinated by the nasal and systemic routes in order to assess their efficacy.

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OBJECTIF DU TRAVAIL

La pandémie de COVID-19 causée par le SARS-CoV-2 nécessite des stratégies efficaces pour prévenir la transmission virale. Le potentiel de la vaccination nasale comme moyen d'inhiber l'entrée et la réplication du virus dans la muqueuse nasale, qui est le principal site d'infection par le SARS-CoV-2, doit être étudié. Cette recherche vise à développer une nouvelle méthode automatisée pour le comptage de cellules épithéliales afin de comparer les résultats obtenus chez des hamsters syriens infectés par le SARS-CoV-2 non vaccinés, vaccinés par voie nasale ou encore par voie systémique, afin d'évaluer l'efficacité des vaccins.

RESUME

L'immunomarquage combiné à l'analyse d'images à l'aide du logiciel QuPath a été utilisé pour compter le nombre de cellules infectées par le SARS-CoV-2. Une nouvelle méthode de digestion de la muqueuse nasale a été mise au point pour obtenir une suspension cellulaire pour la cytométrie en flux, dont les résultats seront utilisés pour valider les résultats de QuPath. Dix hamsters syriens ont été utilisés comme témoins positifs pour faciliter la mise au point de la méthode. Les résultats soulignent l'importance de la reproductibilité des techniques d'échantillonnage, de la dissection des muqueuses et des techniques de coloration. Cette recherche montre que le logiciel d'analyse d'images QuPath, et son outil classificateur de cellules, une fois entraîné, est prometteur pour automatiser le comptage des cellules infectées par le SARS-CoV-2 sur une lame histologique de cornets nasaux de hamster infecté. En outre, la nouvelle méthode de dissection de la muqueuse nasale a fonctionné, ce qui suggère que notre nouvelle méthode de comptage permettrait d'obtenir le nombre de cellules infectées par le SARS-CoV-2 dans la muqueuse nasale de hamsters syriens non vaccinés infectés par le SARS-CoV-2. Ce résultat pourrait être comparé à celui chez des hamsters vaccinés par voie nasale et systémique afin d'en évaluer l'efficacité.

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Contents

1. Introduction

1.1. Context

In December 2019, a cluster of patients presenting symptoms of viral pneumonia began in Wuhan, China. Patients were presented with high fever, cough and fatigue. While most of the patients recovered without any complication, some of them developed severe dyspnoea (Huang et al., 2020). Viral particles isolated from the inferior respiratory tract and PCR sequencing of the virus revealed it to be a betacoronavirus genetically close to the severe acute respiratory syndrome coronavirus (SARS-CoV) and the Middle East respiratory syndrome coronavirus (MERS-CoV). On the 11th of March 2020, the International Committee on Taxonomy of Viruses (ICTV) named this new virus SARS-CoV-2, for severe acute respiratory syndrome coronavirus-2. The same day, the disease was named COVID-19, for coronavirus disease 2019, and declared as a pandemic by the World Health Organisation (Ciotti et al., 2020; WHO, 2023). COVID-19 showed many waves, supposedly due to the emergence of different variants of the virus, presence of asymptomatic carriers, high transmissibility of the virus (Teijaro and Farber, 2021), lack of individual and herd immunity, climatic conditions, governmental protection measures, comorbidities… (She et al., 2022), causing over 750 million human cases and nearly 7 million deaths (6 938 353 deaths) worldwide as reported by the World Health Organization (WHO) on the 30th of May 2023 (WHO, 2023).

Fortunately, the most common form of COVID-19 is similar to the flu, with fever, cough and dyspnoea (Anka et al., 2021; Harrison et al., 2020). Nevertheless, SARS-CoV-2 is amongst the most virulent coronaviruses: it causes severe symptoms that can lead to ARDS (acute respiratory distress syndrome), pneumonia, renal failure, cardiac and kidney damage, and even death in human patients. Critically ill patients share a common characteristic that is an inappropriate overactivation of the immune system, which is in itself deadlier than the SARS-CoV-2 infection (Anka et al., 2021; Harrison et al., 2020; Mistry et al., 2022; Zanza et al., 2022).

1.2. Transmission, immunity and existing vaccines

The primary route of transmission of coronaviruses is by respiratory droplets, but they can also spread by aerosol, direct contact with contaminated surfaces and oro-fecal transmission (Harrison et al., 2020). Therefore, the upper respiratory tract is the main entry port for SARS-CoV-2 and a better understanding of the mechanisms by which the virus binds and replicates in the epithelial cells, in particular those of the nasal mucosa, is key to develop strategies to prevent entry and replication of viral particles.

There is now evidence that after infection there is a presymptomatic period (lasting on average 5 days) during which patients are highly contagious without showing any symptoms. This phase is the incubation period between infectious exposure to SARS-CoV-2 and the first clinical signs and accounts for the majority of SARS-CoV-2 infections. However, it is difficult to estimate this proportion accurately, for example because patients don't seek medical help, and the range of estimates varies widely between studies (table I) (Asghar et al., 2022; Gao et al., 2021). Some, but not all, patients then enter the symptomatic phase, during which viral expression declines. Patients then either recover or enter a more critical phase due to their inappropriate immune response (Anka et al., 2021; Harrison et al., 2020; Mistry et al., 2022; Zanza et al., 2022). The way out of the pandemic is collective immunisation, which is achieved when a large proportion of the population is immune. This will drastically reduce viral transmission and prevent the spread of the disease.

Table I. The incidence of asymptomatic SARS-CoV-2 infections in different studies (Gao et al., 2021).

	China[8] (n = 72,314)	Japan[9] (n = 565)	Diamond Princess[10] (n = 3711)	Korea[11] (n = 28)	Washington[12] (n = 76)	Wuhan Children[13] (n = 1391)
RT-PCR positive cases	56,128	13	634	28	23	171
Asymptomatic cases	889	4	328	3	13	27
Incidence rate (%)[a]	1.6	30.8	51.7	10.7	56.5	15.8

[a] As of the data published in the literature, the proportion of asymptomatic infections in the population with positive nucleic acid test.

This table shows the estimated incidence rate of asymptomatic SARS-CoV-2 infection according to different studies. Results vary greatly in between studies, depending on location and methods.

1.3 Immunity and vaccination (systemic and nasal route)

The scientific community started searching for a vaccine, which was the most promising strategy to reach collective immunity in a short time lapse and protect the most people possible, along with nonpharmaceutical interventions such as isolation, hands washing and masks (Bushman et al., 2021). COVID-19 vaccines were developed at an unprecedented speed, and a vaccination campaign was performed on a large part of the population in the hopes to achieve herd immunity (Randolph and Barreiro, 2020; She et al., 2022). Indeed, in order to decrease the infectious rate of a contagious pathogen, herd immunity has to be attained. The infectious rate is defined by R_0, or basic reproduction number, which is the average number of secondary cases caused by one infectious individual introduced within a susceptible population. In order to stop the spread of an infectious pathogen, this number should be <1 (i.e. one person infects less than one person) (Anderson and May, 1985; Randolph and Barreiro, 2020).

The vast majority of COVID-19 vaccines (intramuscular and intranasal) mimic the SARS-CoV-2 spike protein in order to trigger the immune system to create memory immune cells ready to neutralise a subsequent infection (Alu et al., 2022). Indeed, the spike protein is the primary target for antibodies when a natural infection occurs *in vivo* (Baden et al., 2021; Teijaro and Farber, 2021). To this end, two main technologies are used: mRNA vaccines and adenovirus (AdV) vector vaccines. These vaccines technologies are safe: mRNA vaccines for instance are intrinsically safe because they contain only the elements necessary for the expression of the protein of interest (spike), and they don't interact with the genome (Schlake et al., 2012; Yuan et al., 2022). The SARS-CoV-2 spike protein provided by vaccines induces an immune reaction consisting of B and T lymphocytes, balanced in favour of cytotoxic T lymphocytes ($CD8^+$) rather than helper T lymphocyte ($CD4^+$). Cytotoxic T lymphocytes account for about 80% of the total inflammatory cells that infiltrate the pulmonary interstitium in COVID-19 patients (Li et al., 2020). The role of cytotoxic T lymphocytes is to kill viral infected cells. As for the role of B memory lymphocytes, it is to produce spike-specific IgG in case of an ulterior SARS-CoV-2 infection. IgG protect the patient of the disease, but do not prevent infection, since it happens by

the nasal route, and local immunity consisting of IgA would be necessary in order to avoid infection (Mistry et al., 2022; Teijaro and Farber, 2021).

Systemic vaccines (given by an intramuscular injection) showed their efficacy to attain individual immunity, as vaccinated patients show a less severe form of COVID-19, or no disease at all (Huang and Kuan, 2022; Kuodi et al., 2022). Moreover, systemic COVID-19 vaccines are shown to decrease viral load in the nasal mucosa, which is linked to a decrease of the transmission rate (Vitiello et al., 2021). However, transmission still happens, thus the virus can circulate in the population, therefore they will not be enough to end the pandemic. Indeed, it was found that elevated nasal titres of SARS-CoV-2 could still be detected in patients effectively vaccinated by the systemic route. This leads to believe that vaccinated individuals, presenting a milder form of the disease or even no symptom, could still spread the virus, primarily by the nasal route (Bleier et al., 2021). Indeed, injectable vaccines provide patients with little added mucosal protection as the local immunological reaction they induce is mainly transudation of IgG at the surface of the airways. In order to drastically decrease or ideally stop viral transmission, an effective vaccine would require preventing viral entry and/or replication in the nasal mucosa primary target cells (Bleier et al., 2021; Russell et al., 2020; Zhou et al., 2022). Most of the approved and in trial vaccines (phase 3) are to be administered by intramuscular injection (systemic route), as of July 2021, which does not prevent the virus from entering the nasal mucosa (Fan et al., 2021).

Nasal vaccination inducing local immunity would prevent viral entry in the cell, thus viral replication, and therefore transmission. Indeed, the transmission route of SARS-CoV-2 implies that its first interactions with the immune system occur in the nasal and buccal mucosae. Mucosal immunity occurs in the mucosa-associated lymphoid tissue (MALT), which generates mainly immunoglobulins A (IgA)-producing B-cells (Russell et al., 2020; Smith et al., 2021). Bleier et al. (2021) claimed in Nature that ‘protection in both the upper and lower respiratory tracts will be required to prevent transmission and disease in humans’ (Bleier et al., 2021).

1.4. The Syrian hamster as a model

To test vaccines, one needs a model, which ideally mimics the disease in humans, making results easily transposable to humans. To this end, the Syrian hamster was proposed as a model, as it had already been proven to be a good model for the understanding of the pathogenesis, and testing of antiviral therapies and vaccines against SARS-CoV (Roberts et al., 2005). The Syrian hamster was demonstrated to be an adequate model not only for SARS-CoV-2 pathogenesis, replication, organ tropism and transmission (Chan et al., 2020; Imai et al., 2020; Sia et al., 2020) but also for testing vaccines and antiviral drugs (Imai et al., 2020; Schrage et al., 2022). Indeed, SARS-CoV-2 was shown to replicate efficiently in the lower and upper respiratory tracts after intranasal infection (Imai et al., 2020). Moreover, CT scans and histopathology realised on the lungs show lesions comparable to those observed in humans (Chan et al., 2020; Imai et al., 2020), thus making the Syrian hamster an adequate model for our experiment. In addition, Syrian hamsters are naturally susceptible to SARS-CoV-2 infection and show moderate and transient clinical signs such as body weight loss, lethargy and tachypnoea that last up to 14 days after infection, with a peak of symptoms at 6 days post-infection. Pneumocytes hyperplasia is observed at 7 days post-infection, but surprisingly no mortality is seen in these animals after SARS-CoV-2 infection (Chan et al., 2020; Imai et al., 2020; Reyna et al., 2022; Sia et al., 2020).

1.5. Virology and tropism

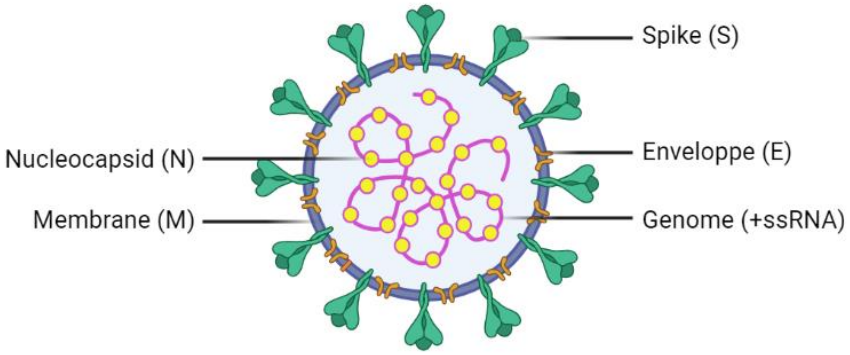


Figure 1: Representation of SARS-CoV-2 (Quertain E., created with BioRender.com, 2023).

SARS-CoV-2 (severe acute respiratory syndrome coronavirus 2) is an enveloped, positive-sense, single-stranded betacoronavirus, a genus that also includes SARS-CoV and MERS-CoV. The virion is composed of four structural proteins: nucleocapsid (N), envelope (E), spike (S), and membrane (M) (figure 1).

The spike protein is a homotrimeric glycoprotein that gives the virus its crown-like aspect. It is the key to the cell entry since its receptor-binding domain (RBD) binds to the virus receptor at the cell's surface. The ACE2 (angiotensin converting enzyme 2) protein plays an important role in the host-viral interaction as it was first identified in 2003 as the receptor for SARS-CoV (Li et al., 2003) and other coronaviruses, such as HCoV-NL63, HCoV-229E, HCoV-OC43 and HCoV-HKU1, which cause mild upper respiratory tract infections and flu-like symptoms (Jackson et al., 2022). It was then believed that ACE2 was the obligate receptor for SARS-CoV-2, since it binds to its receptor-binding domain (Asghar et al., 2022; Baggen et al., 2021; Jackson et al., 2022). Indeed, it was shown that ACE2's overexpression in human poorly susceptible cells and in mice (naturally non-susceptible) enabled infection, and that ACE2-knock-out VERO E6 cells showed no infection for SARS-CoV-2. However, it was since highlighted that SARS-CoV-2 could infect ACE2 deficient cells, thus leaving an interrogation on the precise role of ACE2 and a place for finding other SARS-CoV-2 receptors (Baggen et al., 2021). Moreover, it was shown that SARS-CoV-2 can replicate in ACE2 and TMPRSS2 (another factor helping viral entry) deficient cells (Puray-Chavez et al., 2021). Later, it was shown that ACE2 is an interferon-stimulated gene (ISG) (Baggen et al., 2021; Ziegler et al., 2020). An interferon is an antiviral cellular defence, which means that in case of viral infection, interferon would increase in the cell, thus stimulating production of ACE2, which would be expected to protect the tissue (Ziegler et al., 2020). Indeed, ACE2 was shown to have a critical function in acute respiratory distress syndrome (ARDS) (Imai et al., 2005; Verdecchia et al., 2020), since its expression is seemingly upregulated in SARS-CoV-2 infected airway epithelial cells (Chua et al., 2020; Ziegler et al., 2020). However, it disputed that SARS-CoV-2 could use upregulation of ACE2 caused by interferon to its advantage, enhancing susceptibility of the tissue to the infection (Chua et al., 2020). Cells expressing more than one receptor or co-receptor are at a greater risk of viral infection. For instance, ACE2+/TMPRSS2+ cells are for the

majority type 2 pneumocytes (Ziegler et al., 2020), which are one of the main targets for SARS-CoV-2 infection. Recently, other SARS-CoV-2 receptors than ACE2 and TMPRSS2, such as NRP1 (McGill et al., 2021; Zhu et al., 2022), CD147 (McGill et al., 2021), KREMEN 1 (Lim et al., 2022), ASGR1 (Gu et al., 2022) LDLRAD3, TMEM30A, and CLEC4G (Zhu et al., 2022). However, the precise role of these receptors is still unclear, but they seem to allow SARS-CoV-2 entry in the cells, even in cells where ACE2 is absent (Gu et al., 2022; Lim et al., 2022; Zhu et al., 2022).

The binding between the receptor-binding site and the receptor on the cell's surface then allows fusion of the virus to the cell membrane. The S protein is cleaved in two (S1 and S2) by furin, a proprotein convertase. The two subunits are non-covalently linked on the surface of the then mature virion: S1 is the one that binds the receptor and S2 fixes the S protein to the virion membrane thanks to a fusion peptide. The S2 domain is then cut by TMPRSS2 (transmembrane serine protease 2), exposing the S2' site and allowing the formation of a fusion pore whereby viral RNA can enter the host cell. Cathepsin L (CTSL) plays the same role in the endolysosomes. The nucleocapsid protein surrounds the positive single-strand RNA which that constitutes the genome of SARS-CoV-2. Once this RNA is released in the host cytosol, replication can start (Asghar et al., 2022; Harrison et al., 2020; Jackson et al., 2022).

1.6. Pathogenesis and immune response

Most of COVID-19 research concerns the pulmonary tissue. Indeed, patients mainly showed pneumonia-like symptoms during the pandemic, which lead to believe that the lungs are a major target for SARS-CoV-2. However, the major entry route is the nose and nasopharynx, and therefore, a better comprehension of the pathogenesis of COVID 19 at this level is key. Viral tropism relies on the repertoire of receptors that the receptor-binding domain can attach to, as well as two factors in the host cells: their susceptibility and their permissiveness (Baggen et al., 2021; Harrison et al., 2020). Little research has been done so far to understand what cells allow SARS-CoV-2 entry and replication in the nasal mucosa. It has now been shown that the distribution of infected cells types is uneven in the nasal mucosa. SARS-CoV-2 enters mainly non-neuronal cells of the olfactory epithelium (such as ciliated cells),

even if neuronal cells can be targeted as well, which explains neurological symptoms such as anosmia (Iadecola et al., 2020; Pipolo et al., 2022; Saussez et al., 2021). Moreover, SARS-CoV-2 shows a broad organ tropism, and seems to replicate easily in airways epithelial cells and in enterocytes of the small intestine, but in virtually all organs (Hamming et al., 2004; Liu et al., 2021), causing various clinical signs such as acute cardiac, liver and kidney injuries (Anka et al., 2021; Harrison et al., 2020).

After entry in the respiratory tract, alveolar macrophages and vascular endothelial cells account for some of the first targets of SARS-CoV-2 for viral entry (Harrison et al., 2020). The severity of COVID-19 is most likely linked to an excessive inflammatory response rather than an insufficient one in patients presenting a severe form of COVID-19 (Misset et al., 2022). Indeed, compared to patients exhibiting milder symptoms, those that have a severe form of the disease present, on the one hand, high levels of circulating antiviral cytokines. The level of humoral cytokines, including IL-6, IL-10, CCL20, VEGF, FGF, PD-L1, TNF, IL-1β and lL-1RA, correlates positively with the severity of the disease, inducing a ‘cytokine storm’, or cytokine releasing syndrome (CRS). These cytokines stimulate natural killer (NK) cells, whose role is to kill virus-infected cells. Moreover, SARS-CoV-2 induces excessive proinflammatory macrophage activation. On the other hand, interferon levels (whose role is to protect the cells in case of a viral infection) are negatively correlated to the disease severity. Another important difference between patients lies in white blood cells, neutrophils, lymphocytes, basophils and platelets counts: patients with a more severe form of COVID-19 show a decreased total number of these cells (Bao et al., 2021; Mistry et al., 2022; Smith et al., 2021).

2. Study aims and significance

Systemic vaccines have been show to provide individual immunity, as vaccinated patients show a less severe form of COVID-19 (Huang and Kuan, 2022). However, transmission can still occur, for example there were 219 183 confirmed cases worldwide for the first week of June 2023 showing that SARS-CoV-2 is still in circulation and thus can affect more vulnerable or unvaccinated people. Rather than using systemic vaccines to prevent severe forms of the disease, the hypothesis of this research is that nasal vaccines would be used to stop SARS-CoV-2 from circulating and prevent the virus from entering the nasal mucosal cells, which is the main route of SARS-CoV-2 infection. The R0 would therefore be 0 because the virus could not enter its target cells.

The aim of this research is to develop and test a method that allows quantification the number of SARS-CoV-2 infected cells in the nasal mucosa of SARS-CoV-2 infected Syrian hamsters. This will allow us to set a baseline and thus to test in the future different vaccines and determine their efficacy to reduce viral particles in the nasal mucosa. The aim is to compare in the future amount of viral particles in nasal mucosa after intranasal vaccination versus systemic vaccination. In addition, this method would allow observing which cells populations are SARS-COV-2 permissive, whether their distribution is uneven, and if so, what the distribution pattern is.

To this end, an automated counting method using QuPath on histological slides stained with immunohistochemistry and immunofluorescence was developped. This method is semi-quantitative and allows us to observe the morphology of the tissue, i.e. the anatomical distribution of the cells, the identification of the populations of infected cells and their distribution.

To confirm these results, the second method is flow cytometry, an accepted cell counting method on a cell suspension of digested nasal mucosa cells. To do this, we established a new method for digesting whole nasal mucosa. Flow cytometry on SARS-CoV-2 infected Syrian hamster nasal mucosa cells will be compared with the

percentage of SARS-CoV-2 infected cells obtained by the first histological method, in order to validate this number.

Ultimately, this new automated method will be used to test intranasal SARS-CoV-2 vaccines, in order to assess their efficacy in preventing viral entry in the cells, and comparing them to systemic vaccines. Indeed, if the virus cannot enter the cell, it cannot replicate and dies, ultimately causing the end of the pandemic.

3. Materials and Methods

3.1. Virus

SARS-CoV-2 Wuhan strain isolated from a Belgian woman coming back from Lombardy in March 2020 was used (Garigliany et al., 2020). The virus clade 20A, lineage B.1, spike S614G was at its sixth passage in VERO E6 cells. All experiments with SARS-CoV-2 were performed in enhanced biosafety level 3 (BSL3) containment laboratories at the University of Liège, Belgium.

3.2. Animals and housing

All experiments were performed in compliance with national and international animal welfare regulations. The local ethics committee of the University of Liège has given its consent.

Female Syrian hamsters were acquired at 5 weeks of age and acclimatised in the BSL-3 facility during 6 days before being inoculated with SARS-CoV-2. They were housed by two in a cage. The animals were inoculated by the intranasal route. Two days after infection, they were euthanized under general gas anaesthesia by bleeding in order to avoid blood contamination of the nasal turbinates samples. Animals underwent a tracheal wash with PBS in order to remove nasal discharge. Nasal turbinates were collected and dissected right away. Samples were then either decalcified or digested, depending on the method used, respectively for histological slides (figure 2) or flow cytometry.

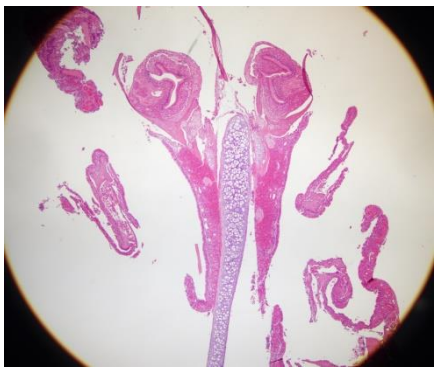
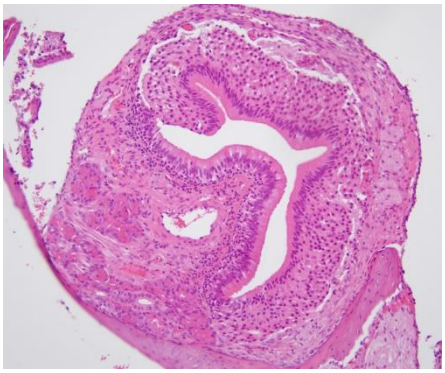
(A)  (B) 

Figure 2: Histological slides, nasal turbinates of Syrian hamsters, haematoxylin and eosin stain. A) 50x B) 100x (Photos: Quertain E., 2023).

3.3. Histopathology and QuPath

Prior to digital cell counting using the QuPath software, tissues (nasal turbinates) underwent decalcification, dehydration, paraffinization and sectioning in order to produce histological slides.

After deparaffinization, histological slides were colored, either by immunohistochemistry (IHC) or by immunofluorescence (IF).

Then, images were analysed using QuPath**,** an open source software for digital pathology slides analysis, in order to count the total number of cells and classify it depending of their SARS-CoV-2 infection status.

3.3.1. Immunostaining (IHC and IF)

Immunohistochemistry and immunofluorescence are staining methods based on the reaction between an antigen and one or several antibodies. The antigen used here is the viral nucleocapsid protein, present in the cytoplasm of SARS-CoV-2 infected cells. Antigen marking can be done by either a direct or an indirect method. In the direct method, the coloured signal is directly linked to the antibody, which saves time in the experiment. Here the indirect method was used (figure 3), where a a marked secondary antibody links to the primary antibody: this allows for a better sensitivity by amplifying the signal (Chen et al., 2010).

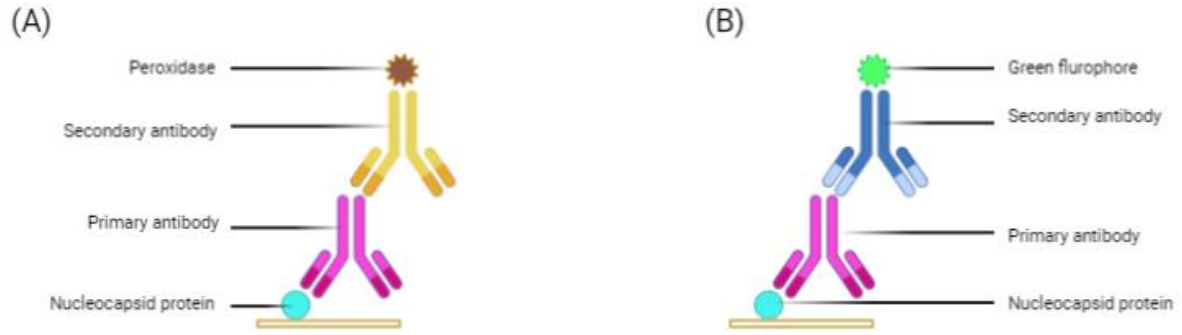


Figure 3: Principle of the indirect method for immunostaining, antigens, antibodies and stain used (A) Immunohistochemistry; (B) Immunofluorescence (Quertain E., created using BioRender, 2023).

An antibody is composed of two fractions: one variable (Fv) that attaches to the antigen and one constant (Fc). Our primary antibody is a SARS-CoV/SARS-CoV-2 nucleocapsid monoclonal antibody, produced in rabbit. The secondary antibody has a Fv specific to the primary antibody's Fc specie, and a Fc of a third specie. It is attached to the colouring agent: either peroxidase (for IHC) or a fluorophore (for IF) (Ryu, 2017; The Ahmed Lab: Northwestern Neurosurgery, 2020). Here, the secondary antibody used is an anti-rabbit goat peroxidase-conjugated antibody.

For immunohistochemistry (figure 4), sections were first stained with haematoxylin and eosin. The primary antibody used, which marks SARS-CoV-2 nucleocapsid protein, is AB 40143-R001 (Sino Biological Inc), 1 hour at room temperature. After a PBS wash, the secondary antibody used is Dako ENVISION+ anti-rabbit, applied 30 minutes at room temperature (figure 3A). AEC VEKTOR 5K-4205 was then applied for 4 minutes at room temperature. Slides were finally stained with haematoxylin, and mounted with glycerogen.

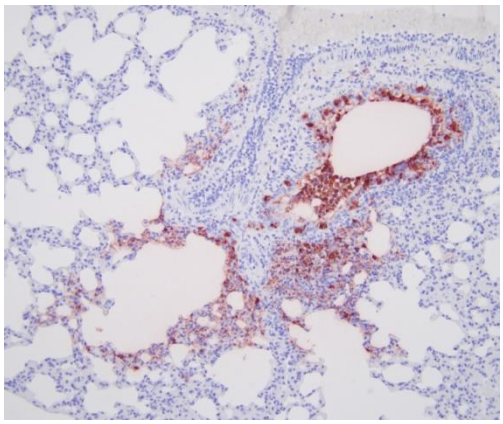


Figure 4: Immunohistochemistry (DAB) and haematoxylin staining of the lungs of a Syrian hamster infected with SARS-CoV (positive control) (Photo: Quertain E., 2023).

For immunofluorescence (figure 5), sections were first stained with haematoxylin and eosin. The primary antibody used, which marks SARS-CoV-2 nucleocapsid protein, is AB 40143-R001 (Sino Biological Inc), 1 hour at room temperature. After a PBS wash, the secondary antibody used is ob150077 1/500 (already attached to a green fluorophore), applied 30 minutes at room temperature (figure 3B). Slides were finally mounted on Antifade Mountant.

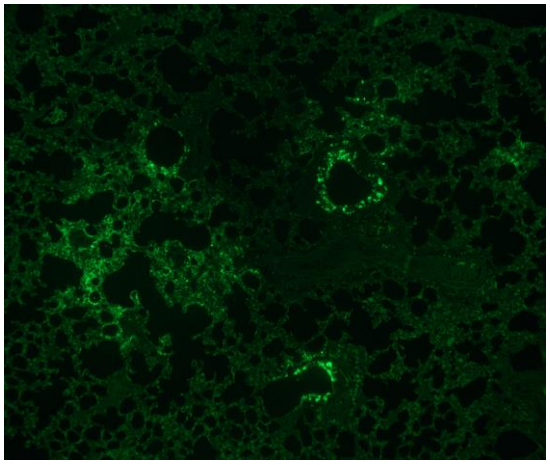


Figure 5: Immunofluorescence staining of the lungs of a Syrian hamster infected with SARS-CoV-2 (positive control) (Photo: Quertain E., 2023).

This staining method was developed with a positive control on SARS-CoV-2 infected Syrian hamster lungs, but was not used in the subsequent analysis using QuPath. In order to count the number of cells, it is necessary to stain the nuclei. However, in this preliminary study to develop the counting procedure, we used the IHC staining for the subsequent stages.

3.3.2. Image analysis: QuPath

Histological slides of SARS-CoV-2 infected Syrian hamster nasal turbinates underwent an immunohistochemistry stain, which is DAB, a chromogen used in immunohistochemistry, and haematoxylin (figure 6). Images were obtained using a ToupCam camera, via the ToupView software.

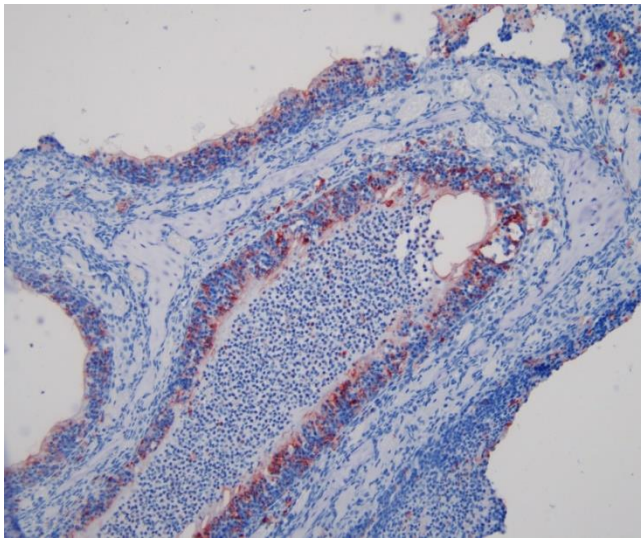


Figure 6: Histological slide of nasal turbinate of SARS-CoV-2 infected Syrian hamster, haematoxylin and DAB IHC. The SARS-CoV-2 nucleocapsid protein is stained in brown, indicating SARS-CoV-2 infected cells (Photo: Quertain E., 2023).

In the QuPath open source software, this script (figure 7) was developed to recognise objects (cells), and classify it in 3 categories: negative (blue), positive (red) and false positive (pink). False positive objects are cells first detected as positive by the QuPath open source software, then determined as negative by the operator. The object classifier was then trained to differentiate better these different classes. After applying the object classifier to the image, the QuPath software gives a percentage of SARS-CoV-2 infected cells in the selected area, which corresponds to the percentage of positive cells among the total number of cells detected (figure 8).

```
1. setImageType('BRIGHTFIELD_H_DAB');
2. setColorDeconvolutionStains('{"Name" : "H-DAB estimated", "Stain 1" : "Hematoxylin", "Values 1" : "0.83333 0.54032 0.11669",
"Stain 2" : "DAB", "Values 2" : "0.20864 0.78402 0.58463", "Background" : " 201 206 200"}');
3. runPlugin('qupath.imagej.detect.cells.PositiveCellDetection', '{"detectionImageBrightfield":"Optical density
sum","backgroundByReconstruction":true,"backgroundRadius":15.0,"medianRadius":0.0,"sigma":3.0,"minArea":10.0,"maxArea":1000.0,"
threshold":0.1,"maxBackground":2.0,"watershedPostProcess":true,"excludeDAB":false,"cellExpansion":5.0,"includeNuclei":true,"smooth
Boundaries":true,"makeMeasurements":true,"thresholdCompartment":"Nucleus: DAB OD
mean","thresholdPositive1":0.2,"thresholdPositive2":0.4,"thresholdPositive3":0.6000000000000001,"singleThreshold":true}')
4. runObjectClassifier("test 5");
```

Figure 7: QuPath generated script used for total and SARS-CoV-2 infected cell counting (Quertain E., 2023).

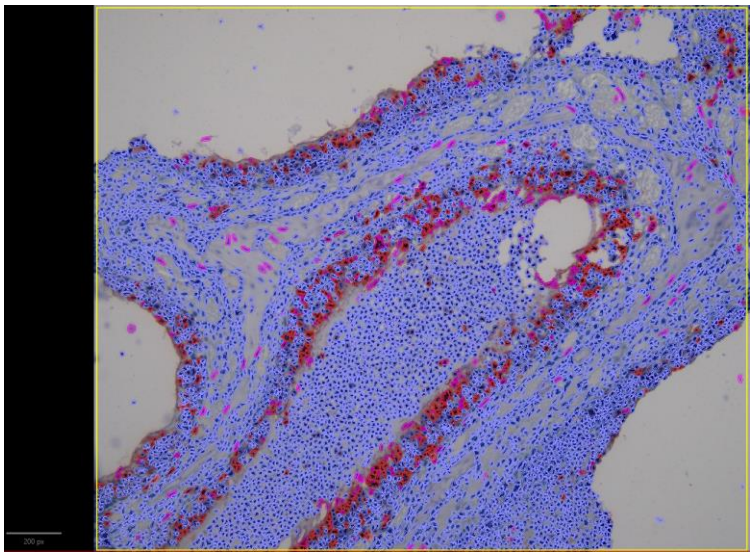


Figure 8: Application of the QuPath script showed in figure 7 on figure 6 (Photo: Quertain E., 2023).

3.4. Flow cytometry on cell suspension (FACS)

Flow cytometry is a method analysing cells in suspension in order to obtain several parameters, such as their size, granularity and fluorescent features (Adan et

al., 2017). Fluorescence is obtained with the same principle as immunofluorescence for histological slides, with antibodies attached to a fluorophore linking to an antigen.

Fluorescent activated cell sorters (FACS) are flow cytometers that can differentiate populations of cells in a suspension based on their fluorescence labels (Wilkerson, 2012). Moreover, this method not only counts the number of marked cells but also measures the intensity of the fluorescence, thus giving a quantitative measure of the marked protein/antigen (McKinnon, 2018).

Flow cytometry was used to count the absolute number of infected cells in the tissue amongst the total number of cells. In addition, the size and fluorescent marking of the cells allows to know which cell populations are represented.

3.4.1. Whole nose digestion and cell suspension

Flow cytometry was done on cell suspension obtained after whole nose digestion of the SARS-CoV-2 infected Syrian hamsters.

In order to digest the nasal mucosa and recover cells, the nasal turbinates were incubated in PBS containing an enzyme mix constituted of 220U/ml type IV collagenase, 1.4 mg/ml pronase and 0.1 mg/ml DNase. They were incubated at 37°C on a rotating plate for 15, 45 or 90 minutes, in order to assess whether the duration of digestion plays a role in the number of cells harvested, and thus optimise it. Nasal conchae were then removed of the solution. A single cell suspension was obtained from passing the suspension through a Falcon® 70 µm nylon cell strainer. The supernatant was removed after centrifugation of the suspension at 300 g for 10 min. After using BD Pharm Lyse™ on the supernatant following manufacturer instructions, then the pellets were resuspended in flow cytometry buffer.

Two individuals are used in each tube for the five parameters observed: negative control, 15 minutes digestion, 45 minutes digestion, 90 minutes digestion, and secondary antibody only control.

3.4.2. Immunostaining

For immunostaining, after resuspension of cells in PBS supplemented with 0.5% of BSA and 0.1% of sodium azide, and cells permabilisation with Triton, a primary antibody (SARS-CoV/SARS-CoV-2 nucleocapsid monoclonal antibody) and a secondary antibody green 488 were added in the suspension.

The collected cells underwent flow cytometry at the GIGA Laboratory (CHU, Liège).

4. Results

Flow cytometry showed a small number of infected cells throughout the nasal mucosa of Syrian hamsters. This number varied from 0.6% to 1.6% depending on the duration of mucosal digestion.

The QuPath software object classifier showed a percentage of SARS-CoV-2 infected cells ranging from 1.15% to 6.89%.

Since the purpose of flow cytometry is to validate our counts on histological slides, the results of the two methods should be interpreted together. Indeed, if there is no significant difference between the percentage of infected cells obtained by the two methods, then the result of flow cytometry, which is a recognised cell counting method, would validate the results of the QuPath method.

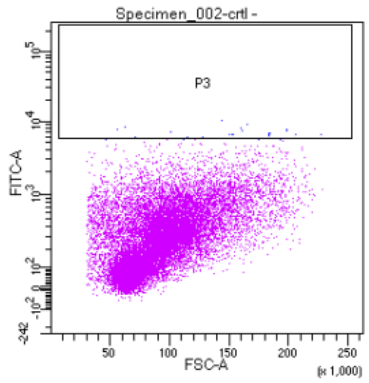
4.1. Flow cytometry

After analysis of the cell suspension, flow cytometry provides results in the form of scatter plots (figure 9). The y-axis (FITC-A) corresponds to the fluorescence intensity of the cell and the x-axis (FSC-A) corresponds to the cell size. By combining these two data we get a picture of the cell type. After eliminating the background events, the total cells are called Population 2 (P2) and the SARS-CoV-2 positive cells are called Population 3 (P3).

Here the total number of events (cells) is approximately 20,000. This indicates that the method for digesting the mucosa worked, as we obtained around 20,000 cells in the different samples, even though the ideal would have been around 100,000 cells. The control sample shows a result of 18,959 cells. 26 of these were detected as positive. This represents 0.1% of the total number of cells. In comparison, the sample digested for 15 minutes has a total of 21,142 cells. 132 of these were detected as positive, representing 0.6% of the total number of cells. Interestingly, the total number of cells detected decreased as the length of mucosa digestion increased. In the different samples, the percentage of positive cells in the total number of cells ranged from 0.6% to 1.6%.

The results of the control manipulation (figure 9, B) using only the secondary antibody show that there are many background cells that are false positive cells (for the SARS-CoV-2 nucleocapsid protein).

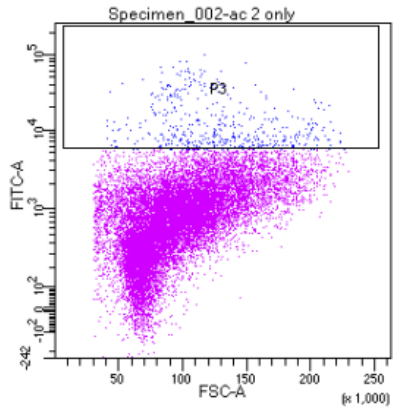
A. Control tube



Tube: crtl -

Population	#Events	%Parent	%Total
All Events	25,119	####	100.0
P1	20,356	81.0	81.0
P2	18,959	93.1	75.5
P3	26	0.1	0.1

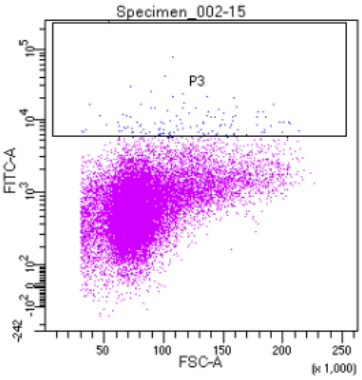
B. Secondary antibody only control tube



Tube: ac 2 only

Population	#Events	%Parent	%Total
All Events	24,546	####	100.0
P1	20,734	84.5	84.5
P2	19,121	92.2	77.9
P3	489	2.6	2.0

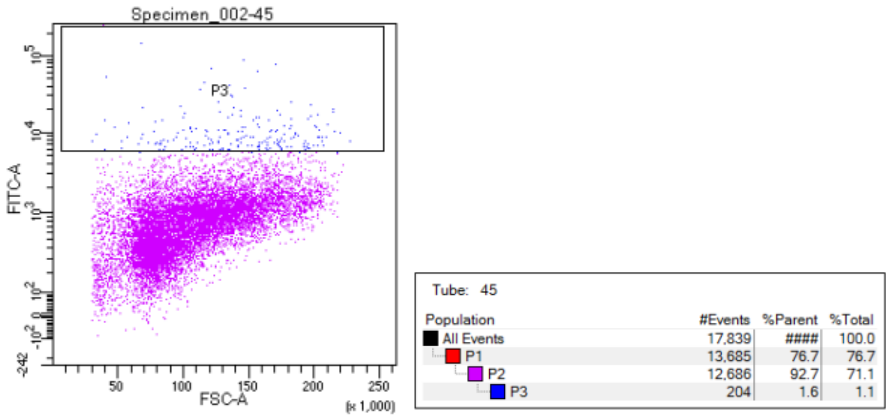
C. 15 minutes digestion



Tube: 15

Population	#Events	%Parent	%Total
All Events	29,579	####	100.0
P1	22,168	74.9	74.9
P2	21,142	95.4	71.5
P3	132	0.6	0.4

D. 45 minutes digestion



E. 90 minutes digestion

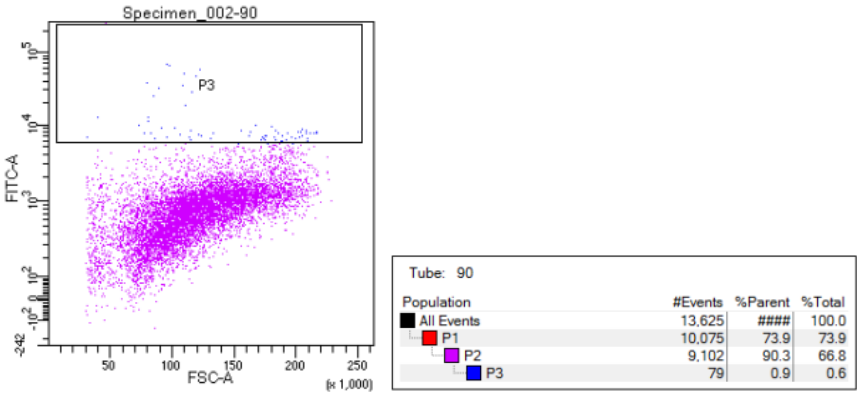


Figure 9: Results of the flow cytometry method on cell suspension obtained from digested nasal mucosa of SARS-CoV-2 infected cells (GIGA, Liège CHU, Belgium, 2023).

4.2. QuPath analysis of histological slides

After applying the object classifier on 3 different slides, QuPath gives the total number of detected cells, the number of positive cells, of false positive cells (therefore counted as negative), and the percentage of infected cells in the total number of cells (table II).

Table II. Results of the QuPath object classifier applied to histological slides stained with DAB immunohistochemistry (Quertain E., 2023).

	Positive samples slides		Negative control
	Slide 1	Slide 2	Slide 3
Number of total cells	9,382	6,718	2094
Number of positive cells	647	77	0
Percentage of positive cells	6.89%	1.15%	0%

Results from two slides of nasal turbinates from a SARS-CoV-2 infected Syrian hamster and a negative control (nasal turbinates from a non-SARS-CoV-2 infected Syrian hamster) using the QuPath open source software object classifier.

These results show the percentage of infected cells on a picture of a region on a histological slide done on a small portion of the nasal turbinates, thus are not comparable with each other. In order to be able to compare these numbers, this method should be applied to whole slides scanned.

5. Conclusion

The aim of this research was to develop a new cell counting method for SARS-CoV-2 infected cells in the nasal mucosa of the Syrian hamster, as it has been shown that intranasal vaccination would prevent the virus from entering the nasal epithelial cells, thereby preventing the replication and ultimately the transmission of the virus, thus providing herd immunity. The systemic vaccines (intramuscular injections) used today mainly provide individual immunity -helping to prevent vaccinated individuals from developing a severe form of COVID-19-, and have been shown to be poorly effective in providing herd immunity. It would be possible to assess the efficacy of these vaccines in preventing viral entry into the nasal mucosa by determining the number of infected cells in the nasal mucosa of SARS-CoV-2 infected Syrian hamsters.

In this research, two methods have been developed in parallel. The first involved semi-automated counting of cells on immunohistochemically stained histological slides using QuPath software and its cell classifier tool trained to detect SARS-CoV-2 positive cells. The second was a validation study of the QuPath cell count using flow cytometry. A new protocol was developed to digest the nasal mucosa to obtain a cell suspension. Both methods are designed to count SARS-CoV-2 infected cells among the total cells counted.

This manipulation has shown that the new method holds great promise. Indeed, it would allow effective SARS-CoV-2 positive cell counting and comparison of results in groups of unvaccinated, systemically vaccinated and intranasally vaccinated Syrian hamsters, with certain improvements (see discussion). This would actually be the next step for this research work carried out at the ULiège Pathology and Morphology laboratory.

6. Discussion

The hypothesis of this research is that nasal vaccines against SARS-CoV-2 will provide local immunity at the site of virus transmission, preventing entry into the nasal mucosa, infection and ultimately transmission of the virus in the population. This would be an advantage over currently used COVID-19 vaccines, which are systemic (*via* an intramuscular injection) and mainly provide individual immunity to the vaccinated person, resulting in a less severe form of COVID-19, but do not prevent transmission.

The results of cell counting using the open source QuPath cell classifier and flow cytometry are not yet comparable to assess whether there is a significant difference between the results and therefore to validate the QuPath cell counts with the flow cytometry counts. For the results to be comparable, samples must be collected under similar and reproducible conditions. Indeed, the anatomical region from which the samples are taken should be the same. A shortcoming of histology is that it relies on very small slides (4 µm) of the anatomical region of interest. It has been suggested that the distribution of SARS-CoV-2 infected cells in the nose is not uniform (Pipolo et al., 2022), so the development of a standardised nasal dissection procedure would make it possible to assess whether there is a preferential anatomical distribution for SARS-CoV-2 infection in the nasal mucosa. In addition, nasal mucosa samples prepared for flow cytometry should be smaller to be more comparable to the sample size of histological slides. In addition, using smaller sections of nasal turbinates for digestion cytometry would allow for a less diluted FACS result, thus increasing the accuracy of the flow cytometry cell counting method.

The nucleocapsid protein was used as the antigen for staining histological slides and cell suspensions because it is a highly conserved protein in SARS-CoV-2 and other coronaviruses compared to the spike protein, which has a higher mutation rate (Bai et al., 2021; Feng et al., 2022; Forcelloni et al., 2021). The nucleocapsid protein is also a good therapeutic target and an efficient tool for the detection of SARS-CoV-2 infection (Bai et al., 2021).

Other antigens can be labelled to identify the cell type and aid interpretation of the results. For example, pancytokeratin (panCK) labels epithelial cells and can be used to confirm their identification in flow cytometry. In addition, we would know whether neuroepithelial cells (then labelled panCK+/NRP1+) are infected by using neuropilin 1 (NRP1), which is a neuronal (but not only) marker (Schwarz and Ruhrberg, 2010). These markers could also be used for histopathological examination. However, not many immunomarkers have been validated for the hamster model.

The immunohistochemistry method for QuPath image analysis provides a semi-qualitative assessment of the slide. The pathologist can answer the question 'is the stain there?'. However, for a more accurate and reproducible result, the question 'how much stain is there?' should be asked. Using the pathologist's experience as a reference point, the answer to this question can be subjective. However, as this method depends on the experimenter, it would not be reproducible. Therefore, a more quantitative method should be used (Taylor and Levenson, 2006). The College of American Pathologists (Chicago, IL, USA) and the UK National External Quality Assessment Immunocytochemistry have stated that the interpretation of IHC stains depends not only on the observer but also on the integrity of the staining process (Taylor, 1994). Reproducible immunohistochemistry is difficult to achieve as intensity is difficult to control from cell to cell, slide to slide and even more so from section to section. This is why standardisation of the entire protocol is a must. Table III is a list of parameters that need to be considered to standardise an immunohistochemical staining protocol. Computer-assisted image analysis provides a quantifiable result that is superior to image analysis performed by most observers (Taylor and Levenson, 2006).

Table III: The different steps that need to be standardized in order to attain repeatability and quantitativity of the IHC method (after Taylor and Levenson, 2006).

Pre-analytical	□ Specimen handling □ Decalcification □ Fixation □ Paraffin embedding and sectioning □ Deparaffinization
Analytical	□ Staining method and protocol □ Negative and positive controls
Post-analytical	□ Scoring/quantification □ Report

In order to obtain quantitative results from an immunostaining image analysis, these steps need to be standardised, i.e. repeatable for every sample.

The QuPath cell counting procedure is therefore semi-quantitative. An immunofluorescent stain should be used with this counting method to obtain a quantitative result. In addition, a nuclear labelling dye should be used for the cell count, such as DAPI (4′,6-diamidino-2-phenylindole), a blue fluorescent DNA dye that binds to the thymine and adenosine of DNA to label the nuclei. Immunofluorescence can also be used to measure the intensity of fluorescence, making it possible to assess the intensity of viral replication. If, in the end, the vaccine does not completely prevent the virus from entering the cells, we would be able to objectively measure a reduction in the level of infection, which would be an interesting result in itself. This phenomenon could then also be observed in the flow cytometric immunostaining.

Histopathology offers a great advantage, which is that the morphology of the tissue can be observed. This makes it possible to see if there is a pattern in the distribution of SARS-CoV-2 infected cells, and if so, what that pattern is. The QuPath image analysis method has been developed using images taken from sections of histological slides. However, whole slide scans would be required for reproducibility of the method. This would make it possible to compare the percentage of infected cells out of the total number of cells on different slides.

In the end, after some modifications, this new automated counting method looks very promising for testing nasal vaccines against SARSCOV2. It has also allowed the development of an innovative method for digesting the nasal mucosa.

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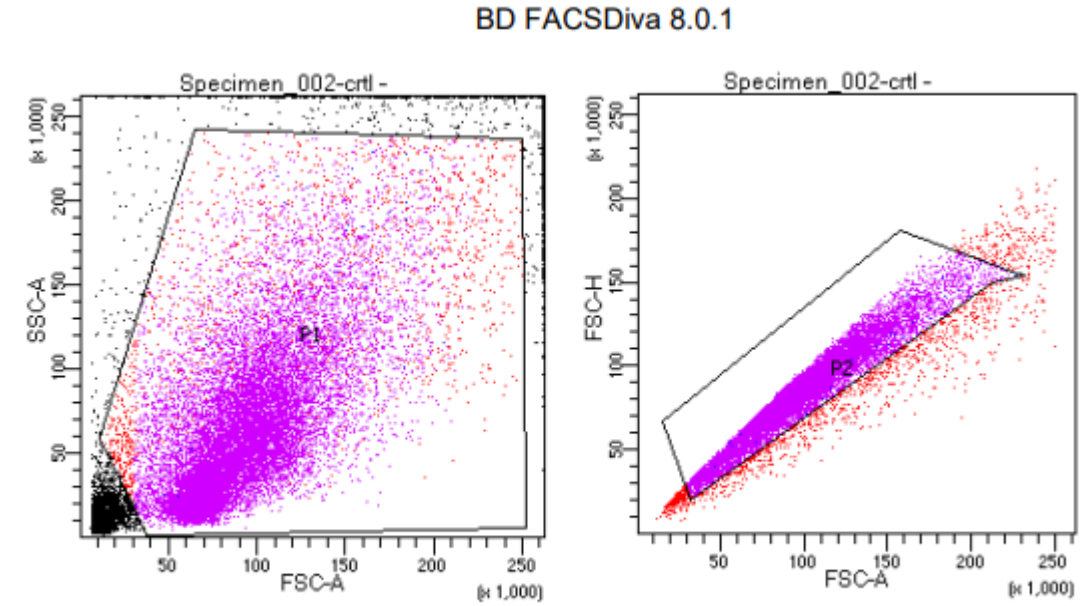
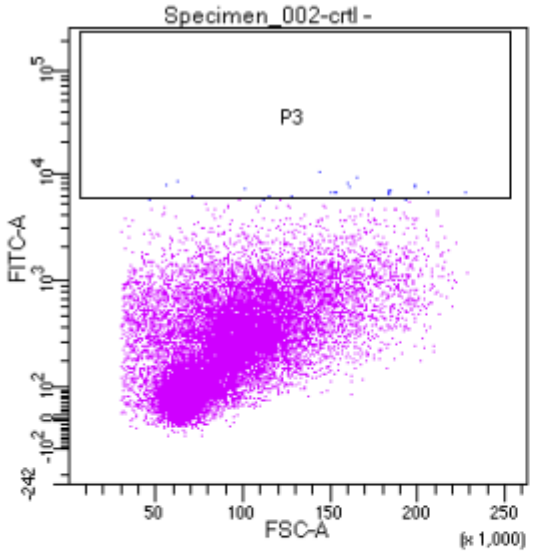
Appendix

Annex 1: FACS results (GIGA, CHU Liège, Belgium).

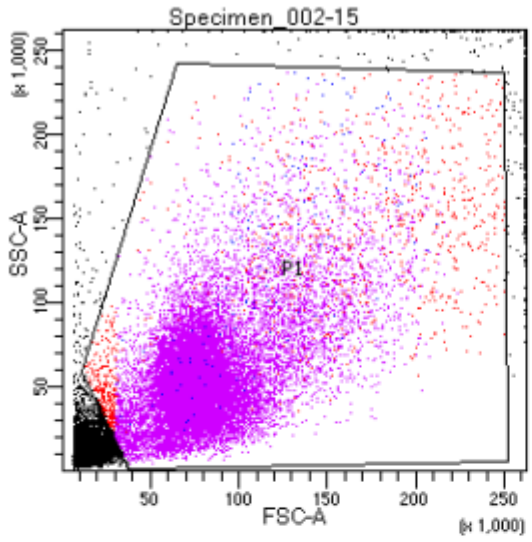


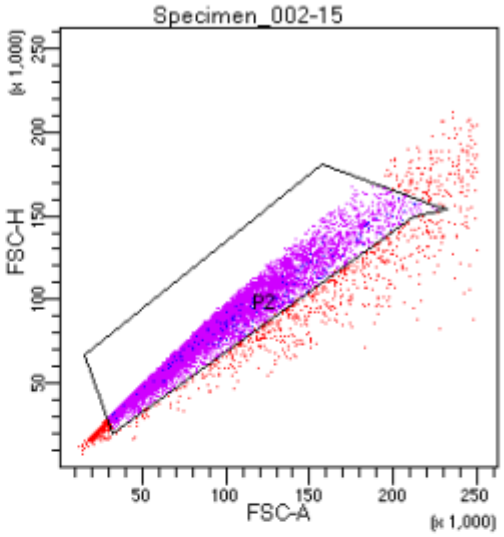


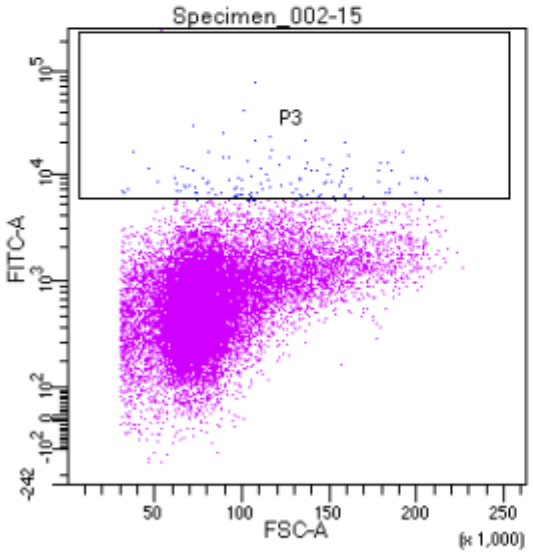
Tube: crtl -

Population	#Events	%Parent	%Total
All Events	25,119	####	100.0
P1	20,356	81.0	81.0
P2	18,959	93.1	75.5
P3	26	0.1	0.1

BD FACSDiva 8.0.1



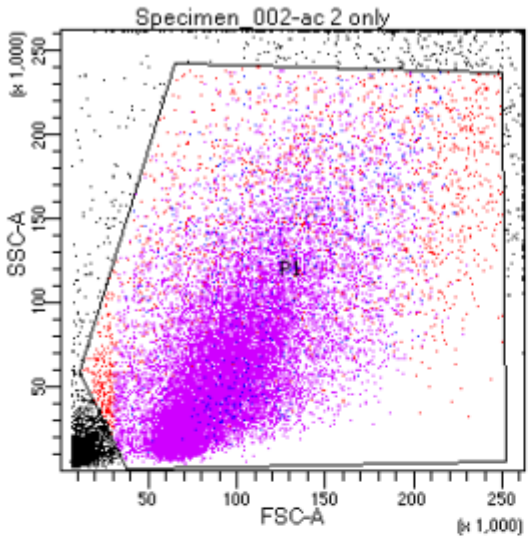


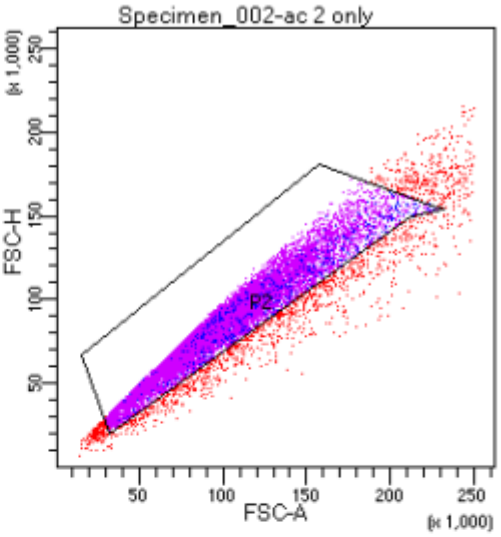


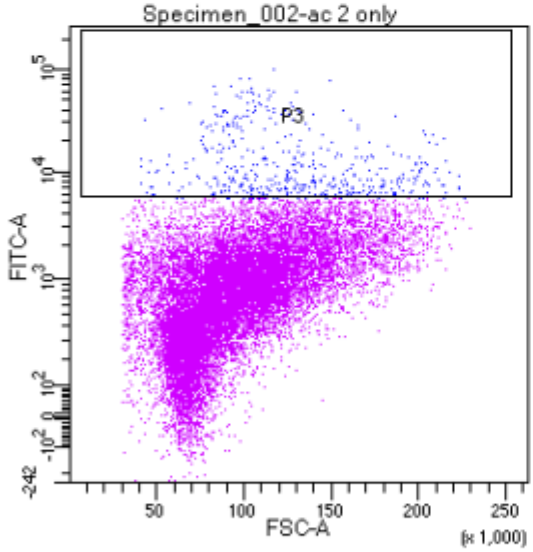
Tube: 15

Population	#Events	%Parent	%Total
All Events	29,579	####	100.0
P1	22,168	74.9	74.9
P2	21,142	95.4	71.5
P3	132	0.6	0.4

BD FACSDiva 8.0.1



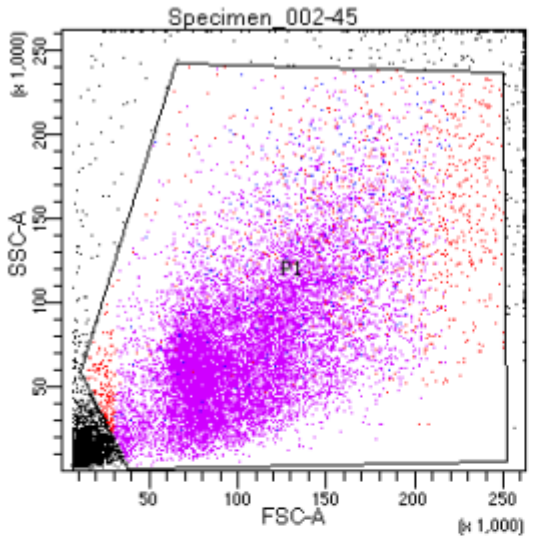


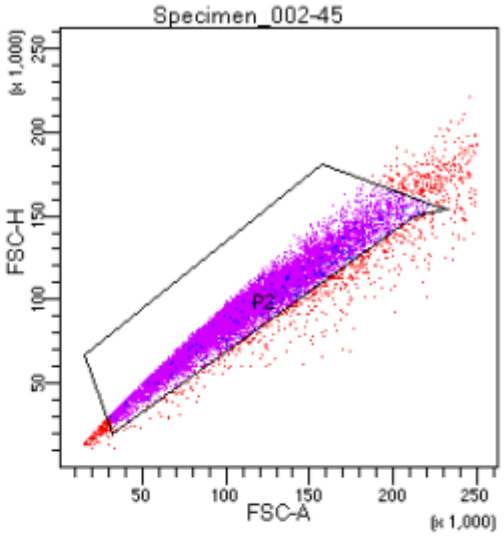


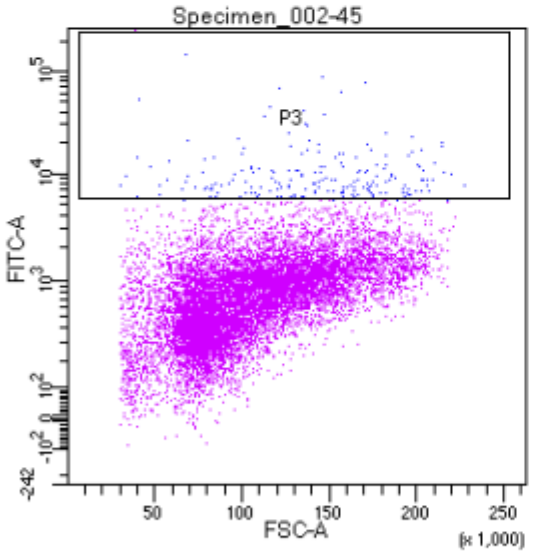
Tube: ac 2 only

Population	#Events	%Parent	%Total
All Events	24,546	####	100.0
P1	20,734	84.5	84.5
P2	19,121	92.2	77.9
P3	489	2.6	2.0

BD FACSDiva 8.0.1



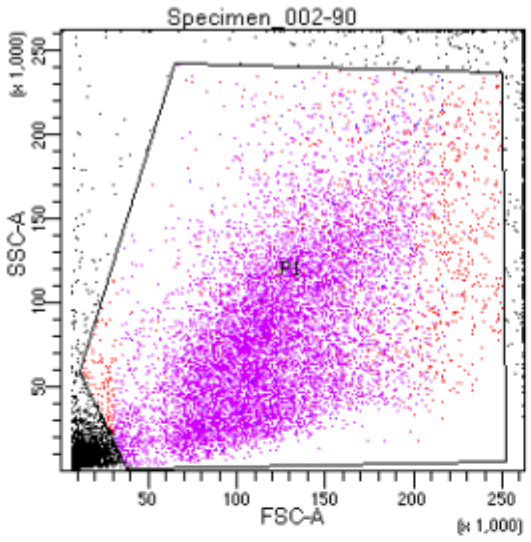


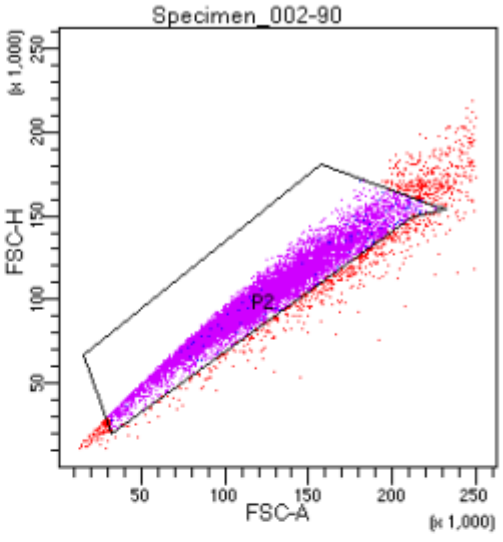


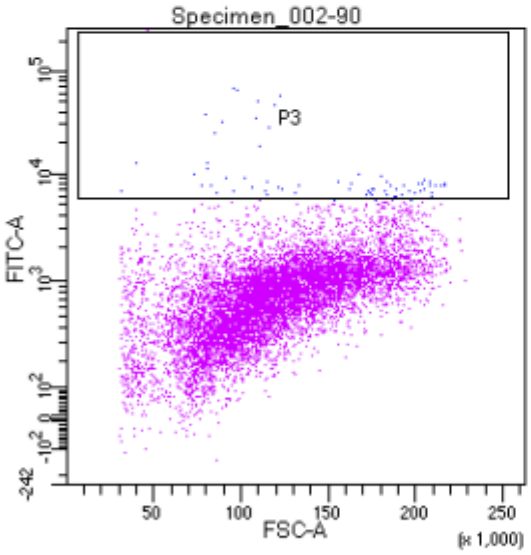
Tube: 45

Population	#Events	%Parent	%Total
All Events	17,839	####	100.0
P1	13,685	76.7	76.7
P2	12,686	92.7	71.1
P3	204	1.6	1.1

BD FACSDiva 8.0.1





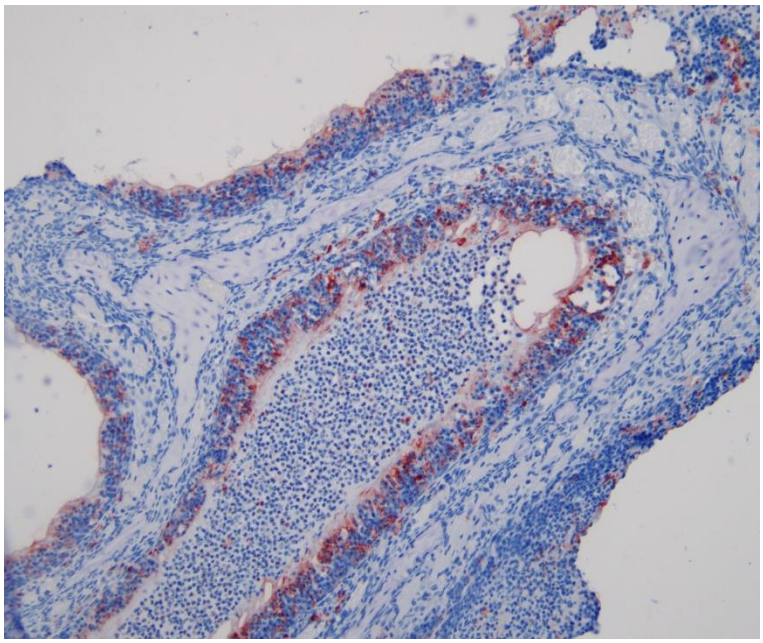


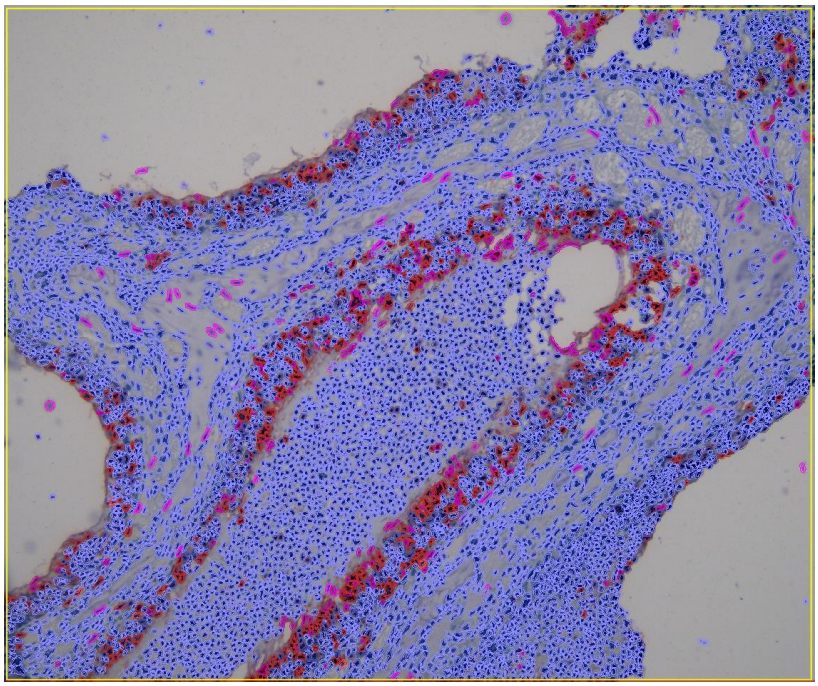
Tube: 90

Population	#Events	%Parent	%Total
All Events	13,625	####	100.0
P1	10,075	73.9	73.9
P2	9,102	90.3	66.8
P3	79	0.9	0.6

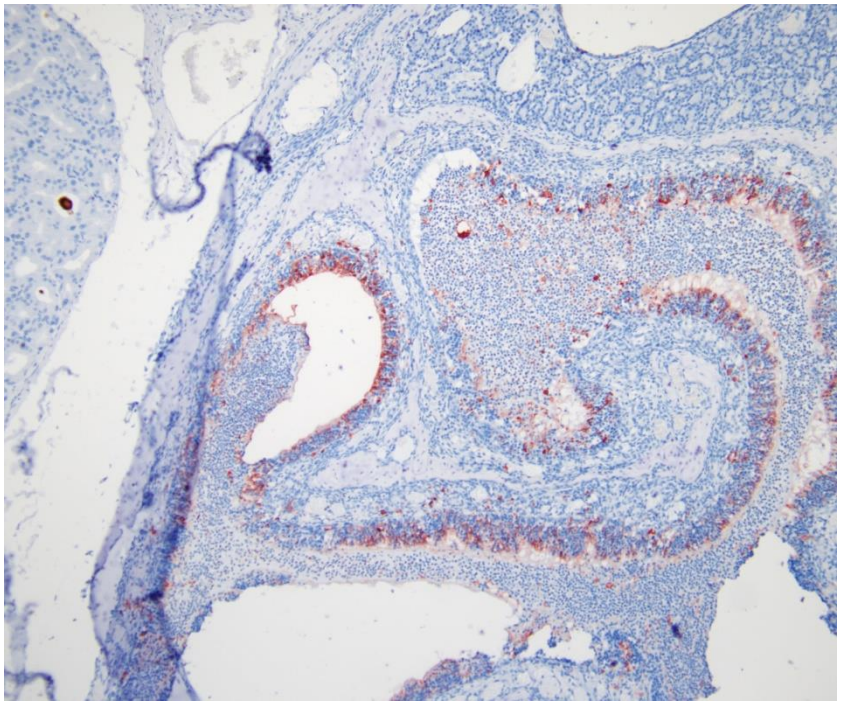
Annex 2: Microscopic pictures of histological slides used for QuPath image analysis, DAB and haematoxylin stain (Photos: Quertain E., 2023).

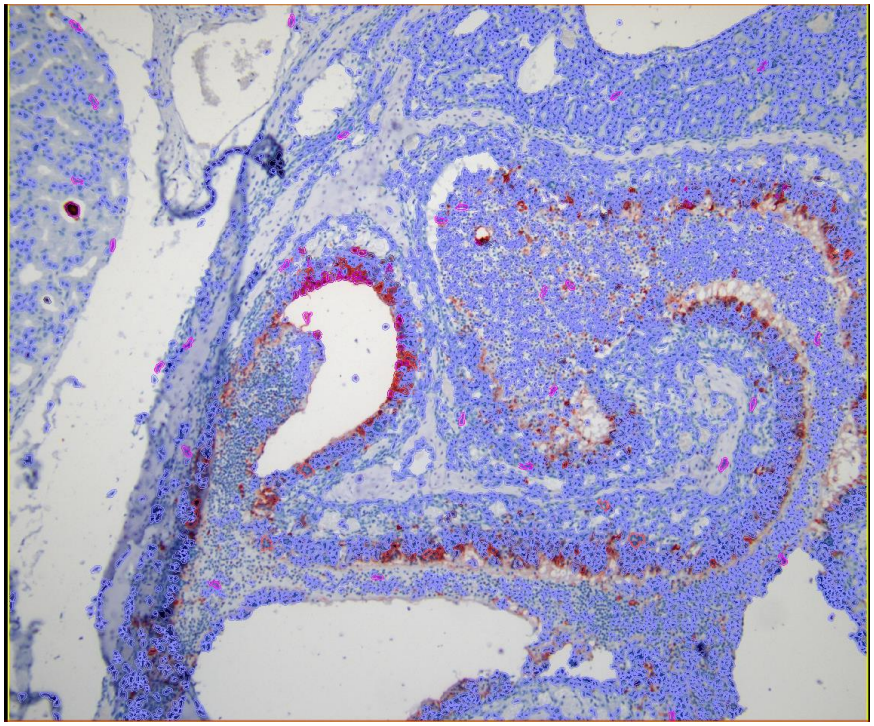
- Slide 1: nasal turbinates





- Slide 2: nasal turbinates





- Slide 3: negative control