

Bioimage informatics

# An open-source high-content analysis workflow for CFTR function measurements using the forskolin-induced swelling assay

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## Abstract

**Motivation:** The forskolin-induced swelling (FIS) assay has become the preferential assay to predict the efficacy of approved and investigational CFTR-modulating drugs for individuals with cystic fibrosis (CF). Currently, no standardized quantification method of FIS data exists thereby hampering inter-laboratory reproducibility.

**Results:** We developed a complete open-source workflow for standardized high-content analysis of CFTR function measurements in intestinal organoids using raw microscopy images as input. The workflow includes tools for (i) file and metadata handling; (ii) image quantification and (iii) statistical analysis. Our workflow reproduced results generated by published proprietary analysis protocols and enables standardized CFTR function measurements in CF organoids.

**Availability and implementation:** All workflow components are open-source and freely available: the htmrenamer R package for file handling <https://github.com/hmbotelho/htmrenamer>; CellProfiler and ImageJ analysis scripts/pipelines [https://github.com/hmbotelho/FIS\\_image\\_analysis](https://github.com/hmbotelho/FIS_image_analysis); the Organoid Analyst application for statistical analysis [https://github.com/hmbotelho/organoid\\_analyst](https://github.com/hmbotelho/organoid_analyst); detailed usage instructions and a demonstration dataset [https://github.com/hmbotelho/FIS\\_analysis](https://github.com/hmbotelho/FIS_analysis). Distributed under GPL v3.0.

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**Supplementary information:** [Supplementary data](#) are available at *Bioinformatics* online.

## 1 Introduction

Cystic Fibrosis (CF) is a life-threatening multi-organ disease caused by mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR) protein (Riordan *et al.*, 1989). Mutations can be classified into seven classes according to their effect on CFTR synthesis, processing and function (De Boeck and Amaral, 2016). Disease-causing mutations impair chloride and

bicarbonate permeation through CFTR and dysregulate fluid homeostasis across mucosal surfaces (O'Sullivan and Freedman, 2009). In the respiratory tract, CFTR dysfunction results in mucus obstruction in the lungs, pulmonary exacerbations and progressive lung disease. No lifelong cure exists for individuals with CF.

Currently, four CFTR-targeting FDA-approved drugs are available for individuals with CF: ivacaftor (VX-770) (Ramsey *et al.*, 2011), lumacaftor (VX-809)/ivacaftor (Wainwright *et al.*, 2015),

tezacaftor (VX-661)/ivacaftor (Taylor-Cousar *et al.*, 2017) and elxacaftor (VX-445)/tezacaftor/ivacaftor (Heijerman *et al.*, 2019; Middleton *et al.*, 2019). Except for VX-770 itself, all drugs are combinations of this potentiator with one or two corrector molecules (VX-809, VX-661 or VX-445) that act upon the trafficking defect of e.g. F508del-CFTR (the most common CFTR mutation) and a functional potentiator molecule (VX-770).

Organoid technology is a valuable tool for CF personalized medicine, diagnostics and therapy (Takahashi, 2018). The forskolin-induced swelling (FIS) assay utilizes stem cell-derived intestinal organoids to measure (residual) CFTR function in a patient-specific manner (Dekkers *et al.*, 2013). In FIS experiments intestinal organoids are cultured in multiwell plates in the presence of compounds expected to restore CFTR function, which is measured by live-cell fluorescence microscopy as time-dependent organoid swelling due to ion and water transport into the lumen (Boj *et al.*, 2017; Dekkers *et al.*, 2013). FIS responses are proportional to CFTR function (Dekkers *et al.*, 2013), recapitulate the individual genetic background (Dekkers *et al.*, 2016) and might serve as a biomarker of CF disease severity (de Winter-de Groot *et al.*, 2018). The FIS assay has aided in the identification of ivacaftor responders with rare CFTR mutations for which this modulator was initially not registered (Dekkers *et al.*, 2016). It also enables classifying CFTR genotypes according to the response to existing CFTR modulators (theratyping) *in vitro* thereby potentially identifying rare CFTR genotypes that might benefit from existing and experimental CFTR modulators (Clancy *et al.*, 2019).

Human CF organoid culturing (Sato *et al.*, 2011; Vonk *et al.*, 2020) and the FIS assay have been standardized (Boj *et al.*, 2017; Dekkers *et al.*, 2013). However, no standard protocols exist for FIS image processing and data analysis, with each individual laboratory relying on proprietary or custom solutions. This might limit inter-laboratory reproducibility of CFTR function measurements in organoids and limit future diagnostic/theranostic applications of FIS measurements.

We present a cross-platform FIS data analysis workflow composed of free and open-source software. This workflow was benchmarked against part of a published high-quality dataset of FIS measurements that covered a range of mild-to-severe CFTR genotypes (Dekkers *et al.*, 2016) and produced nearly identical results, both in terms of CFTR function and response-to-therapy measurements. We anticipate that this workflow will fulfill the urgent need in the CF community for FIS data analysis reproducibility, standardization and assay dissemination.

## 2 FIS analysis workflow

The readout of the FIS assay is a normalized quantification of relative organoid swelling as a function of compound treatment (Fig. 1A). The FIS analysis workflow is composed of a suite of open-source tools that allow users to perform all steps involved in converting raw microscopy images into publication-ready results (Fig. 1B). The workflow integrates imaging data produced from different microscopy systems (Leica Microsystems and ZEISS data are currently supported) into a single workflow thereby rendering image and data analysis microscope brand agnostic. Tools were implemented in R (R Core Team, 2013), CellProfiler (Carpenter *et al.*, 2006) and Fiji/ImageJ (Schindelin *et al.*, 2012), and run in Windows and macOS environments. The workflow comprises the following steps, which are further described in the following sections:

1. *Image file conversion*: conversion of microscopy images from native proprietary formats into TIF;
2. *Image file renaming*: TIF files are renamed and structured into a folder tree where file and folder names contain relevant experimental metadata;
3. *Image analysis*: a high-content image analysis pipeline measures per-organoid cross-sectional area across the entire dataset.

Aberrant organoids can be excluded from the analysis using morphological or intensity features;

4. *Statistical analysis*: A web application—*Organoid Analyst*—allows exploratory data analysis, image visualization, segmentation quality assessment, object- and well-level quality control and data normalization. Summary statistics and plots are also created.

### 2.1 Image file conversion

The raw data for the FIS assay is a collection of time-lapse fluorescence microscopy images. Typically, each well is imaged at a single position with a low magnification objective and a time resolution of 10 min for a total observation time of 60 min. Our FIS analysis workflow requires that microscopy images be exported from their native formats into TIF using the corresponding microscope controller software (Supplementary File S1). This provides compatibility with file format and metadata storage schemes from different manufacturers.

### 2.2 Image file renaming

To enable analysis of image files acquired by microscopes of different manufacturers we developed the *htmrenamer* R package, which recreates a file renaming strategy initially used in a publication by (Neumann *et al.*, 2006). *htmrenamer* provides functions (e.g. `rename_zeiss()`) and graphical user interfaces (e.g. `rename_zeiss_gui()`) to adapt microscope TIF files into a common file and folder structure (Fig. 2A). As such, downstream image data processing is effectively rendered microscope brand agnostic. The hierarchical folder structure minimizes the number of files in a single folder and optimizes the performance in some file-systems.

Analysis of the FIS assay data requires robust methods for annotating imaging data with relevant experimental information (e.g. plate name, well identifiers, time information, experimental treatment). *htmrenamer* includes this information in TIF file names—and in the folders where they are stored—in a standardized format inspired by OME-TIFF (Linkert *et al.*, 2010). This approach provides enough information to interpret the biological content, requires minimal computational overhead and provides easily extractable metadata for image and statistical analysis. Since the full metadata is included in each file and folder name experimental information is always available. It can also be combined—as in the case of Leica LAS X MatrixScreener exports—with OME-TIFF, which includes additional experimental metadata in the file header.

The file renaming process requires a systematic description of the experimental treatment in each well (compound and concentration). Such well descriptors are provided as a plain text file that we and others (Neumann *et al.*, 2006) call ‘microscope infile’ (Table 1), which can be generated within *htmrenamer* [e.g. `newinfile.char()`, Supplementary Dataset S1]. Renamed files are assigned a prefix which identifies the experiment (or plate) and are placed in a hierarchical folder tree consisting of three levels (plate/well/subposition). The renaming process preserves the original/raw TIF files and offers optional lossless compression.

### 2.3 Image analysis

Image analysis pipelines have been implemented in CellProfiler and Fiji/ImageJ (Fig. 2B and 2C). The cross-sectional area of each organoid at each time point is exported in tabular plain text format (CSV) along with experimental metadata. The pipelines implemented in either tool share the same steps, except for pre-processing and fill holes strategy (see below), with output data being stored in the same format. This means that imaging datasets can be analyzed with either approach without compromising compatibility with downstream statistical analysis (see Section 5). Single channel or RGB TIF images generated with *htmrenamer* are supported.

CellProfiler is the recommended tool for the analysis of most FIS datasets due to its parallelized batch image analysis capabilities, interactive test mode and easy customization of the analysis process. In the image analysis pipeline implemented in CellProfiler (Fig. 2B)

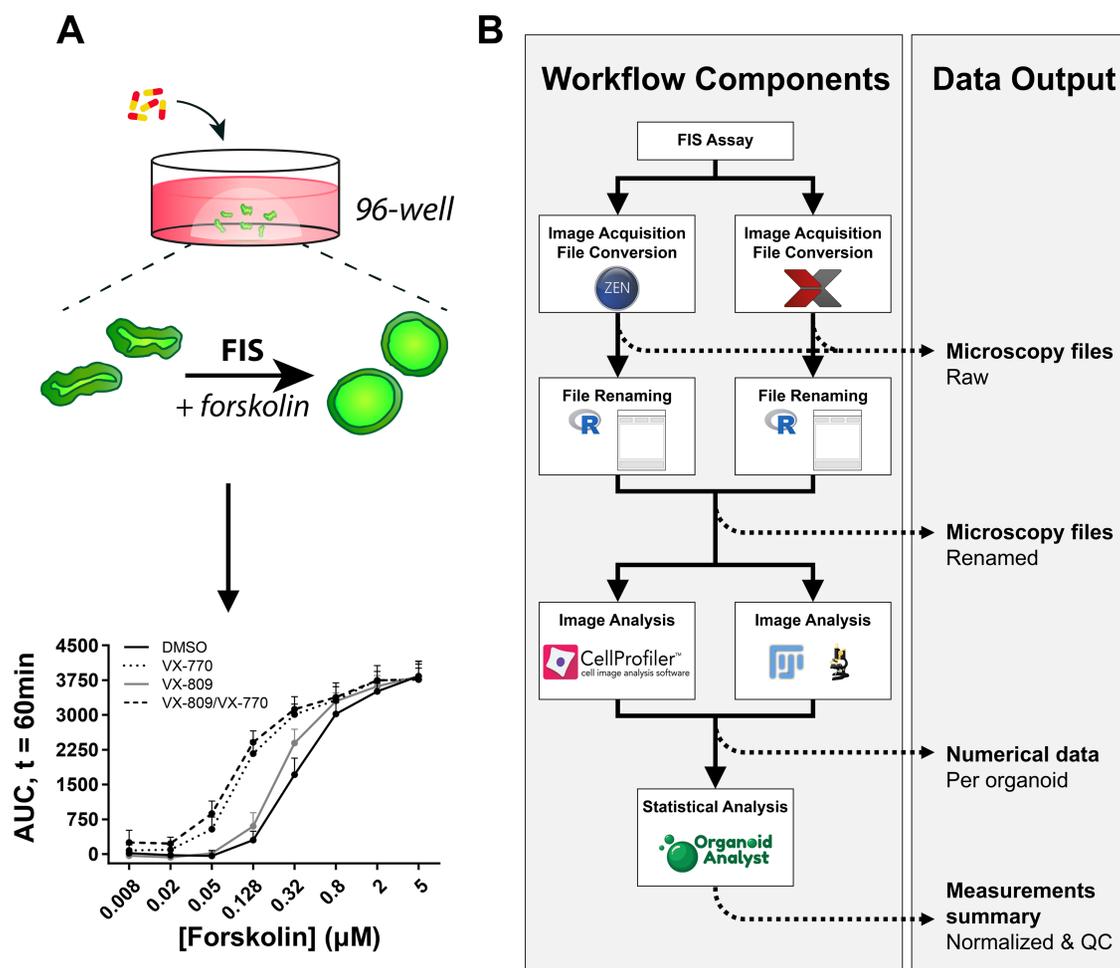


Fig. 1. Overview of the FIS analysis workflow. (A) Schematic representation of the FIS assay (top) with a typical representation of an organoid swelling curve (below). (B) Flowchart of the entire workflow: (i) image export by the microscope controller software, (ii) renaming of raw images with the *btmrenamer* R package, (iii) image analysis with CellProfiler or Fiji/ImageJ and (iv) statistical data analysis with *Organoid Analyst*

images are loaded and metadata is extracted from file and folder names. The fluorescence channel is selected and gray values are rescaled to the full intensity range to compensate for fluorescence bleaching and dye dilution. Image segmentation is complemented by object level quality control, where any quantitative feature can be used to discard aberrant organoids (Fig. 3C). The object tracking feature identifies and labels organoids that are not found in all time points (e.g. due to swelling outside image borders or bleached fluorescence at later time points) with 'nan'. Organoids with 'nan' labels can be discarded during statistical analysis (see below). However, this feature is only relevant in sparsely seeded wells because organoid swelling can result in objects coalescing that may not be easily declumped (Fig. 3B). Finally, object area is measured, converted into  $\mu\text{m}^2$  and exported (CSV) along with segmentation masks (PNG).

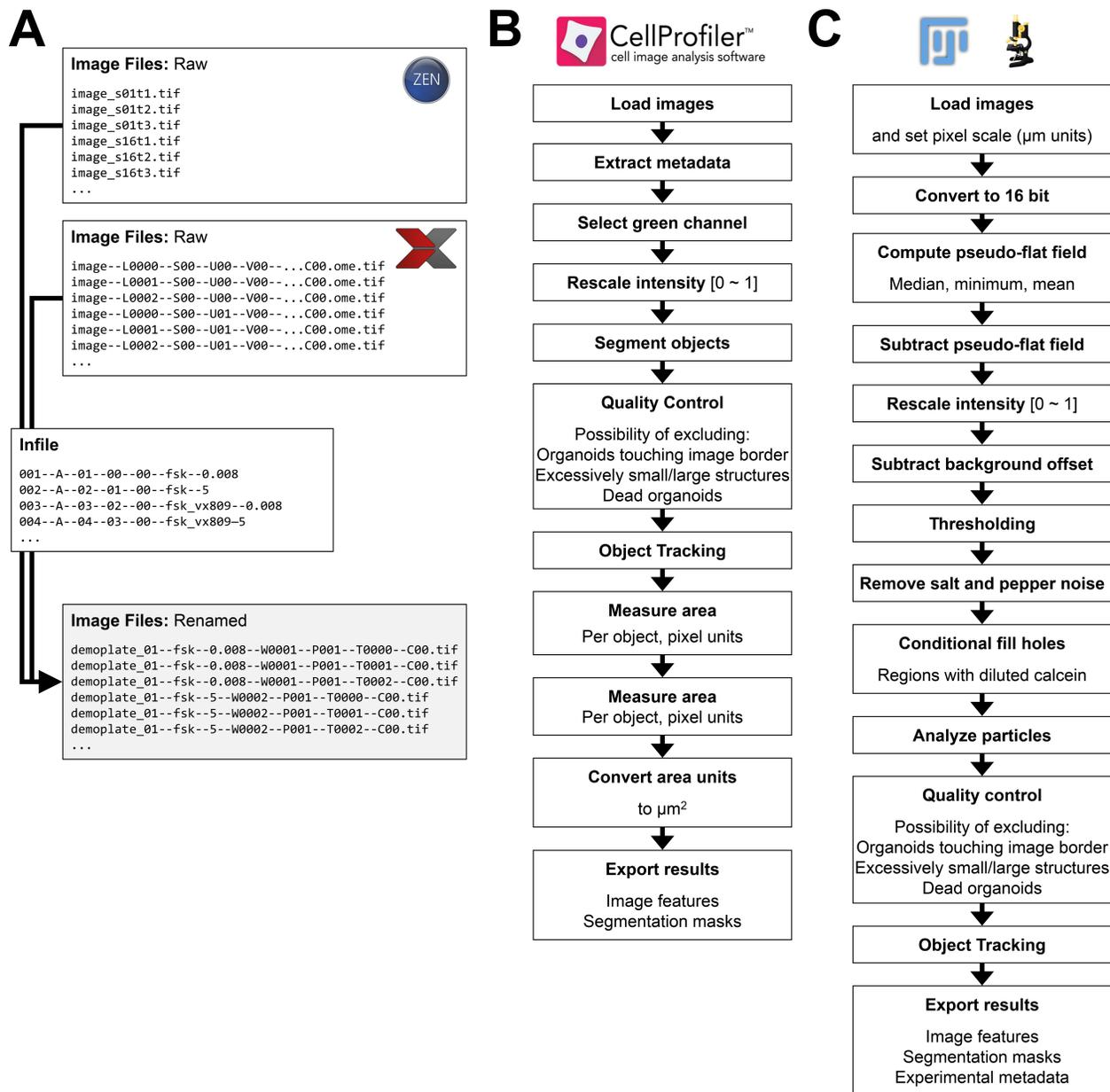
The Fiji/ImageJ approach (Fig. 2C) is best suited for datasets containing images with uneven background fluorescence (e.g. illumination defects in widefield microscopy; Fig. 3A) or in cases where organoid segmentation is hampered by dye dilution due to extensive organoid swelling (Fig. 3B). In these cases, fluorescence intensity at the center of organoids may be so low that global thresholding produces ring-shaped objects at late time points, which may not be adequately addressed with the 'fill holes' operation. To enhance segmentation accuracy, we developed a 'conditional fill holes' operation which is implemented in ImageJ (see Fig. 3B and Section 4). The Fiji/ImageJ image analysis includes two scripts. The 'FIS test' script provides a graphical user interface (GUI) where image analysis and quality control parameters can be iteratively adjusted with the effect on image segmentation and feature extraction being displayed

(similar to CellProfiler's Test Mode). After determining all analysis parameters, the 'FIS analysis' script can be used for batch analysis of the data (Fig. 2C). First, images are loaded and the pixel size is set. Next, a pseudo-flat field background correction is applied (usually by subtraction of a median filtered image) to address any illumination defects. Gray values are rescaled and objects are segmented using auto- or manual thresholding. Object declumping can be performed with an optional watershed operation. The conditional fill holes algorithm is applied and per-object measurements are computed. Finally, object-level quality control is applied (Fig. 3C), objects are tracked and image features, metadata (CSV) and segmentation masks (TIF, PNG or both) are exported.

#### 2.4 Statistical analysis

*Organoid Analyst* is a web application for the statistical analysis of FIS data based on the R *shiny* package (Chang et al., 2018). The application runs locally on Windows and macOS personal computers. It processes the features obtained in CellProfiler or Fiji/ImageJ, provides an interactive interface for exploratory data analysis, image visualization and object-level quality control, and generates summarized publication-ready datasets. *Organoid Analyst* implements the following workflow (Fig. 4A):

1. *Load data*: The user is prompted to select the results folder from the CellProfiler or Fiji/ImageJ analysis;
2. *Settings*: The user is prompted for the names of relevant columns in CSV tables, the type of multiwell plate used in the experiment,



**Fig. 2.** File management and image analysis workflow. (A) Examples of the renaming process of ZEISS ZEN Blue TIF exports and Leica LAS X MatrixScreener OME-TIFF exports. Leica LAS X Navigator TIF files are also supported. Renaming rules are provided through a plain text microscope infile as described in Table 1. Overview of the image analysis pipelines implemented in CellProfiler (B) and ImageJ (C)

**Table 1.** File renaming strategy of ZEISS and Leica TIF/OME-TIFF exports

Files	Formats	Examples
ZEISS	File name	image_s01t1.tif
	Structure	prefix_swelltime.tif
Leica MatrixScreener	File name	image--L0000--S00--U00--V00--...C00.ome.tif
	Structure	prefix--Ltime--Splate--Ucolumn--Vrow--...Cchannel.ome.tif
Leica Navigator	File name	image_A1-1_t0_z0_ch00.tif
	Structure	prefix_rowcolumn-position_ttime_zslice_chchannel.tif
Infile	Well description	001--A--01--00--00--fsk--0.008
	Structure	well--row--column--column--row--compound--concentration
Renamed files	File name	demoplate_01--fsk--0.008--W0001--P001--T0000--C00.tif
	Structure	plate--compound--concentration--Wwell--Psubposition--Ttime--Cchannel.tif

Note: File and folder names are given the same structure. Here, the infile was stored as a file named 'demoplate\_01.txt'.

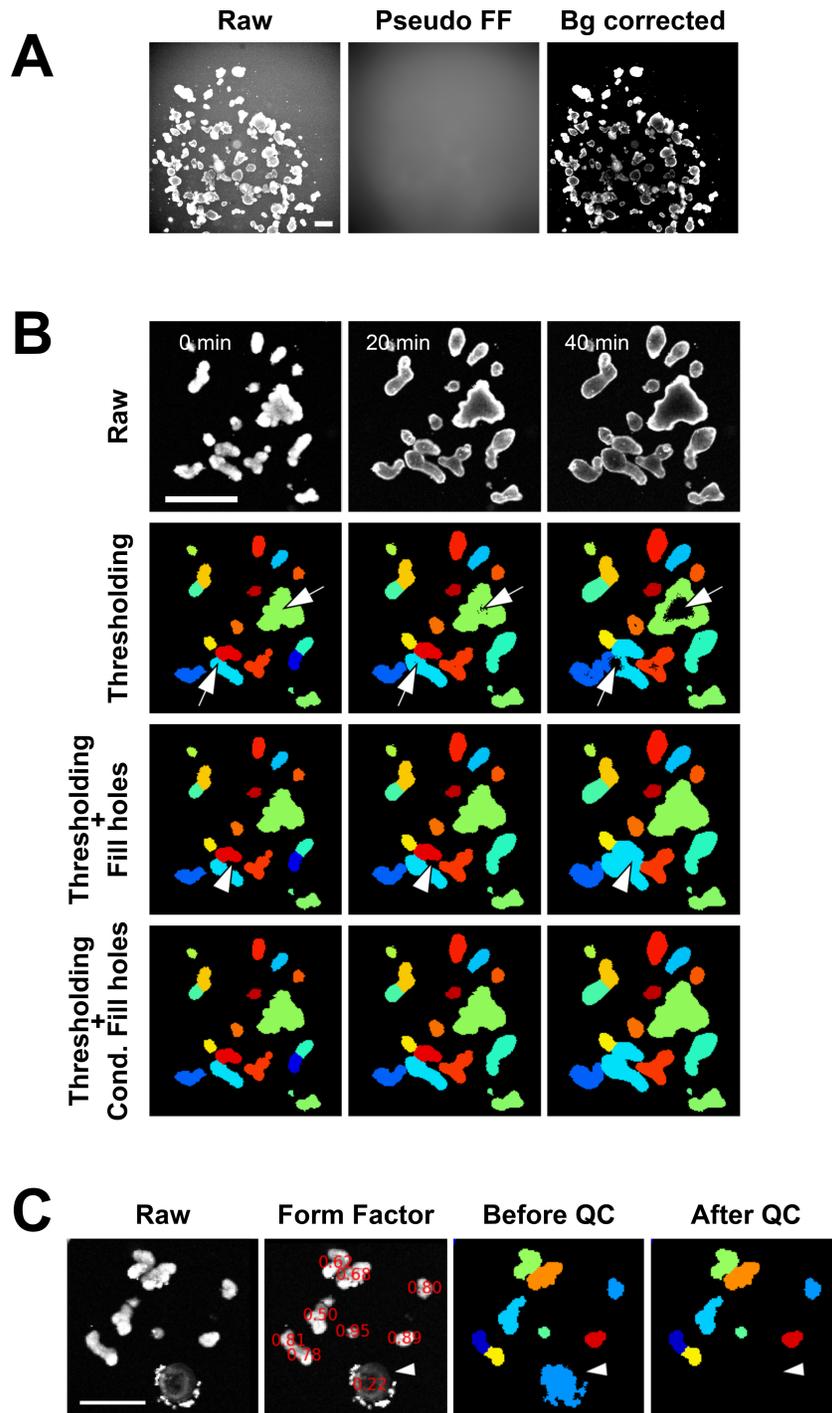
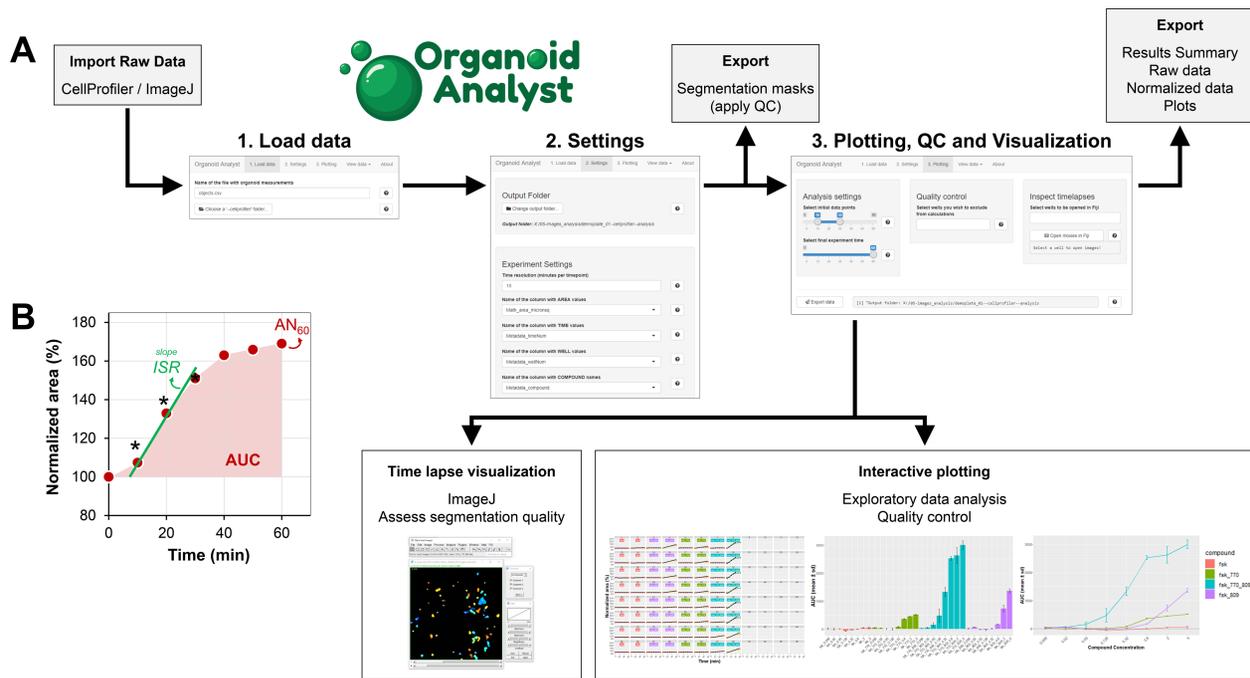


Fig. 3. Common image analysis challenges in the FIS assay. (A) Uneven background fluorescence in widefield microscopy images can be corrected with the pseudo-flat field background correction implemented in ImageJ. (B) During organoid swelling fluorescence intensity of the calcein dye is proportionally reduced due to dilution ('raw'). Thresholding these images often produces ring-shaped objects which underestimate the cross-sectional area ('thresholding', arrows). Applying the classical 'fill holes' algorithm results in overestimation of cross-sectional area if there are voids between organoids ('thresholding + fill holes', arrowheads). The 'conditional fill holes' algorithm implemented in ImageJ solves this problem ('thresholding + conditional fill holes'). (C) Example of the per-object quality control implemented in CellProfiler and ImageJ, where form factor ( $4 \times \pi \times \text{Area} / \text{Perimeter}^2$ ) was used to exclude dead organoids (form factor  $< 0.3$ , arrowhead). Scale bars: 250  $\mu\text{m}$

quality control information and file locations. Remapping of file locations and per organoid quality control is also supported;

3. **Plotting:** This feature enables exploratory data analysis with interactive plotting tools, including per-well normalized swelling kinetics ( $AN_{w,t}$ ) and per-treatment summaries (mean  $\pm$  standard deviation) of normalized swelling data. Three organoid swelling reporters are calculated: the area under the

curve (AUC) at an arbitrary experimental endpoint, the percentage of area increase ( $AN_t$ ) and the initial swelling rate (ISR) (Fig. 4B and Section 3). *Organoid Analyst* interacts with Fiji/ImageJ to display time lapse data overlaid with segmentation masks. This feature enables assessing segmentation performance. Quality control of wells with unreliable data is supported;



**Fig. 4.** *Organoid Analyst* data analysis workflow. (A) *Organoid Analyst* is a web application which runs in the web browser. It is served from a local server provided by the R shiny package. A tab bar at the top of the screen guides the user through data loading, defining settings, plotting and results export. Screenshots show each of these steps. (B) Graphical representation of the three swelling reporters computed within *Organoid Analyst*: area under the curve (AUC), initial swelling rate (ISR) and percentage of area increase (AN<sub>t</sub>). Both AUC and AN<sub>t</sub> refer to the period ranging from time 0 to the endpoint defined within *Organoid Analyst*. All reporters are derived from the normalized swelling kinetics plot

4. **Data export:** Raw and normalized numerical data in tabular format (CSV or XLSX), exploratory plots (PNG) and quality-controlled segmentation masks (PNG) can be exported. Raw and normalized data tables can also be visualized online.

## 3 Materials and methods

### 3.1 FIS reference and demonstration datasets

To validate our novel FIS analysis workflow part of a previously published well-characterized dataset of five genotypes consisting of organoids harboring homozygous F508del or F508del compound heterozygous A455E, R117H, S1251N or class I (dele2.3 and Y1092X) mutations (Dekkers *et al.*, 2016) were re-analyzed with the ZEISS Zen Blue software. All FIS assays were performed in biological duplicates and technical triplicates, except for one F508del/A455E organoid culture (n=2). We have included a demonstration dataset (Supplementary File S1) of an F508del/F508del organoid culture (mock-) treated with (a combination of) VX-770 and/or VX-809 for training purposes.

## 4 Algorithms

### 4.1 File renaming

The *htmrenamer* R package enables renaming files exported by ZEISS (TIF), Leica Microsystems Navigator (TIF) and MatrixScreener (OME-TIFF) software and placing them into a standardized hierarchical folder structure (Table 1). Image acquisition metadata generated by LAS X MatrixScreener (XML and LRP) is read by the Leica renamer and used for file handling. The ZEISS renamer requires user input regarding the multiwell plate type. Leica Navigator files are processed without the need of additional experimental metadata. Images are renamed according to well descriptors provided in a plain text tabular TXT file (microscope infile) which contains 7 columns separated by a double dash ('--'). Two columns correspond to the compound and its concentration (or any other two relevant items). The remaining columns contain well

coordinates. Each row describes one well and its contents. Renamed files are given a prefix identical to the infile file name to identify the experiment.

### 4.2 Conditional fill holes

The swelling of fluorescently labeled organoids produces a decrease of the average fluorescence intensity due to dye dilution, which is mainly noticeable in the lumen (central volume) of the organoid (Fig. 3B, 'Raw'). A global threshold of such images may generate ring-shaped objects whenever fluorescence intensity in the luminal region is near background levels (Fig. 3B, 'Thresholding'). If organoids are densely seeded, swelling may produce organoid-free voids that are misclassified as objects whenever the fill holes operation is applied (Fig. 3B, 'thresholding + fill holes'). The conditional fill holes algorithm (Fig. 3B, 'thresholding + cond. fill holes') removes these artifacts by assuming that organoid radial expansion (i.e. swelling) is much larger than lateral displacement during the time lapse. The conditional fill holes algorithm is the following (Supplementary Fig. S3):

- The fluorescence image (A) is thresholded with holes unfilled (B).
- Pixels located in holes in the thresholded image are identified (C).
- The intersection of 'hole pixels' with the final thresholded image in the previous time point is calculated (D). All pixels have a zero value at the first time point. Since organoids expand radially, the resulting image will highlight the pixels where fluorescence was diluted below the threshold value compared to the previous frame (true holes in organoids). Background pixels are not part of this image because they will be below the threshold value.
- The final thresholded image is the union of images B and D (E).

### 4.3 Data normalization

Normalization of high-content data within *Organoid Analyst* is performed as described below. Let us represent:

- Well numbers (i.e. identity) by  $w$ ;
- The number of minutes since the beginning of the kinetics by  $t_i$ , where  $i = 0, 1, \dots, m$  is the sequential time frame identifier;
- The number of objects segmented in each image by  $n$ ;
- The cross-sectional area of each object by  $A_j$ , where  $j = 1, 2, \dots, n$ .

Each image is summarized by the sum of the areas of all of its objects:

$$A_{w,t_i} = \sum_{j=1}^n A_{j,w,t_i}$$

Then, a normalized swelling kinetics is computed ( $AN_{w,t_i}$ ) by setting the initial time point to 100%:

$$AN_{w,t_i} = \frac{A_{w,t_i}}{A_{w,0}} \times 100\%$$

The normalized swelling kinetics is offset ( $ANO(w, t_i)$ ) in order to place the baseline at 0%:

$$ANO_{w,t_i} = AN_{w,t_i} - 100\%$$

The swelling kinetics is reported as the area under the curve (AUC) measured at  $t = 60$  min. The AUC for each well and time point ( $AUC(w, t_i)$ ) is the discrete trapezoidal integration of the normalized and offset kinetics curve. The discrete integration is performed iteratively as follows:

$$AUC(w, t_i) = \begin{cases} 0 & \Leftarrow i = 0 \\ AUC(w, t_{i-1}) + \frac{ANO_{w,t_i} + ANO_{w,t_{i-1}}}{2} \times (t_i - t_{i-1}) & \Leftarrow i > 0 \end{cases}$$

The initial swelling rate (ISR), which is the slope of a line fitted to the region of maximal swelling in normalized kinetics curves, is also calculated, together with a simplified reporter, the percentage of area increase,  $AN(t)$ . Replicate wells are summarized by their mean, standard deviation (SD), standard error of the mean (SEM) and counts.

## 5 Results

To validate the open-source FIS analysis workflow we used part of a published reference dataset (Dekkers *et al.*, 2016). This dataset contains confocal fluorescence microscopy images of FIS assays performed with intestinal organoids harboring five different CFTR genotypes (2 donors per genotype with 2–3 experimental replicates per donor). In each experiment, organoids were treated with eight forskolin concentrations (0.008–5  $\mu$ M) in the presence or absence of CFTR potentiator VX-770 and/or CFTR corrector VX-809. The dataset was analyzed with ZEN Blue (ZEISS) or the open-source FIS analysis workflow with image analysis being performed with Fiji/ImageJ (Fig. 5A) or CellProfiler (Fig. 5B).

Comparison of the results obtained by the open-source FIS analysis workflow with the results obtained by using proprietary tools demonstrated high data similarity among (i) the proprietary and open-source approaches regardless of the image analysis step being performed with ImageJ or CellProfiler and the (ii) ImageJ *versus* CellProfiler image analysis pipelines in the open-source workflow (Fig. 5). Bland-Altman analysis revealed agreement between the proprietary tools and ImageJ (Supplementary Fig. S1) or CellProfiler (Supplementary Fig. S2). Specifically, ~95% and ~94% of AUC pairs fell within the limit of agreement regions of the Bland-Altman plots when image analysis was performed using CellProfiler or ImageJ, respectively. Measurement biases were small compared to the data dynamic range. Additionally, interpretation of AUC dose-response curves to determine responsiveness to modulators and effective compound concentrations (Fig. 5) resulted in the same

conclusions when using the proprietary and open-source analysis approaches regardless of the image analysis being performed by CellProfiler or ImageJ.

In conclusion, the re-analysis of a published dataset with our open-source FIS analysis workflow yielded equivalent quantitative information and biological conclusions, validating the approach and showing it can replace proprietary and custom workflows currently in use.

## 6 Discussion

The FIS assay is a well-established assay to assess the pre-clinical efficacy of CFTR-modulating compounds. Until now no standardized workflow for the analysis of FIS data existed. We have presented a complete workflow for the analysis of FIS data from raw microscopy files into publication ready datasets. The workflow provides intuitive and almost entirely graphical interfaces for quantifying FIS data on personal computers. All tools in this workflow are free and open-source (R, ImageJ and CellProfiler). The main advantages of our workflow are that it is specifically tailored to the FIS assay—thereby increasing analysis reproducibility and standardization—and can be used in laboratories without skilled bioimage analysts and/or data scientists.

The workflow was designed to process confocal and widefield data regardless of the microscope system being used when performing the assay (currently only ZEISS and Leica systems are supported). *htmrenamer* accomplishes manufacturer agnostic capabilities without the need for database administrators by not matching the standardizing file access functionality provided by an OMERO database (Allan *et al.*, 2012). Future updates of *htmrenamer* may extend support of additional manufacturers.

We developed two alternative analysis pipelines using Fiji/ImageJ and CellProfiler. CellProfiler is recommended for most situations and can easily be adapted to analyze confocal three-dimensional datasets (volume *versus* time) and different imaging modalities (e.g. brightfield). Object tracking is supported in both CellProfiler and Fiji/ImageJ. The Fiji/ImageJ analysis provides the ‘conditional fill holes’ algorithm, which improves the segmentation of organoids that swell significantly, e.g. organoids having high CFTR residual function or which express wild-type CFTR. Regardless of the image analysis platform, we recommend testing the image analysis parameters on several images/wells representative of the whole dataset before initiating batch analysis as thresholding or quality control parameters may not perform equally well on all images. Some common image analysis challenges and suggestions on how to address them when using ImageJ or CellProfiler are included in the corresponding GitHub repositories.

The statistical analysis tool—*Organoid Analyst*—is a novel and essential resource for the CF research community designed to enable integrated analysis of FIS data. *Organoid Analyst* provides an interface between microscopy images and quantitative data thereby being a user-friendly tool for data analysis and assessment of the overall quality of data obtained from FIS experiments. Image visualization—where raw images are overlaid with segmentation masks—allows for validation of the image analysis steps and (if necessary) informs on how to improve it.

*Organoid Analyst* features for the first time the initial swelling rate (ISR) as a reporter of organoid swelling. Even though biological validation is beyond the scope of this publication, we believe the ISR may be an informative FIS parameter, as it circumvents relevant shortcomings of the AUC: (i) organoid swelling is a three-dimensional process which may not be well represented in two dimensions, (ii) organoids do not swell indefinitely and (iii) fluorescence dilution may complicate organoid segmentation. Correlation between AUC and ISR measurements from the FIS reference and demonstration datasets (Supplementary Dataset S1) was very high ( $r \geq 0.97$ ,  $P < 10^{-15}$ ; data not shown).

Several image analysis tools for organoid morphometric characterization have been published but these tools are not specifically tailored to FIS analyses as they (i) only perform segmentation and feature extraction (Bulin *et al.*, 2017; Harna

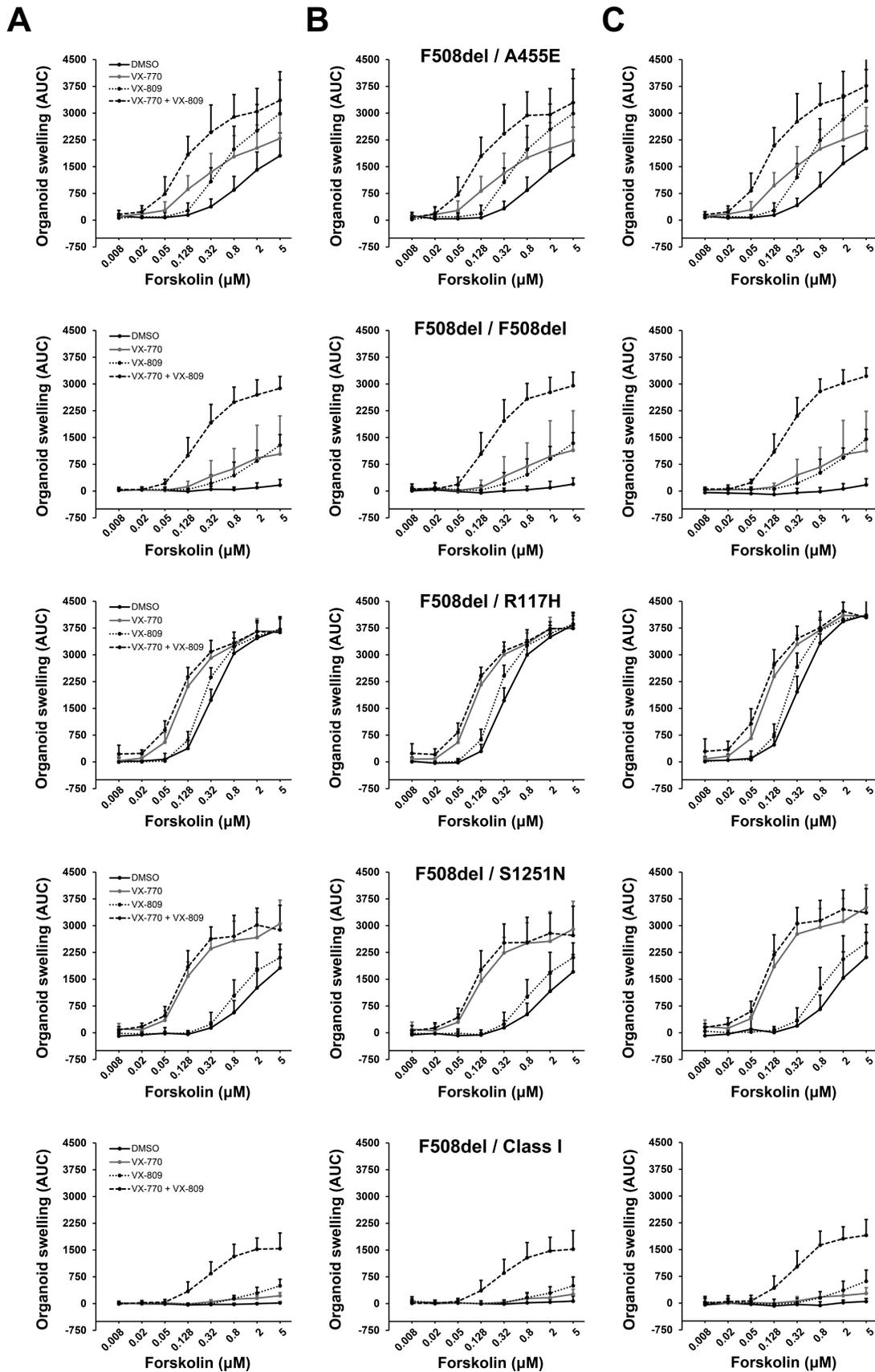


Fig. 5. FIS analysis workflow performance. Fluorescence microscopy images of organoids with five different CF genotypes, originally described in (Dekkers *et al.*, 2016), were analyzed with the open-source FIS analysis workflow to obtain corresponding AUC values. The image analysis step was performed with either (A) ImageJ or (B) CellProfiler. Original results obtained from proprietary tools (ZEN Blue) are shown in (C)

*et al.*, 2014), (ii) analyze brightfield rather than fluorescence images (Borten *et al.*, 2018; Kassis *et al.*, 2019), (iii) do not address metadata handling, (iv) have closed source codes (Akerfelt *et al.*, 2017) or (v) are developed for non-intestinal organoids (Booij *et al.*, 2017).

To the best of our knowledge, this publication presents the first open-source workflow for FIS assay analysis, i.e. an end-to-end, auditable and reproducible protocol to extract high-content statistical data from raw FIS time-lapse images. The workflow is cross-platform, based on commonly available software tools and extensively documented for users with minimal image analysis and data science skills. This workflow's relevance is further demonstrated by its use in recent publications (e.g. Silva *et al.*, 2020).

Overall, we anticipate that the open-source FIS analysis workflow presented here will improve inter-laboratory replication of FIS data and allow establishing the assay in more laboratories. The workflow is expected to contribute to improved prediction of CFTR modulator efficacy in individual CF patients by generating high-quality FIS data. We propose that this workflow becomes part of standard operational procedures (SOP) for FIS analysis.

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