Automated Deep Learning Models for the Analysis of Biological Microscopy Images
Haja, Asmaa

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The interest in the use of organoids in the biomedical field has increased exponentially in the past years. Organoids, or three-dimensional “mini-organs”, have the ability to proliferate and self-organize in-vitro, while displaying varying morphologies. When in culture, these structures can overlap with each other making the quantification and morphological characterization a challenging task. Quick and reliable analysis of organoid images could help in precisely modeling disease phenotypes as well as provide information on organ development. Therefore, automatization of the quantification and measurements is an important step towards an easier, faster, and less biased workflow.

In order to accomplish this, a free e-Science service (OrganelX) has been developed for localization and quantification of organoid size based on deep learning methods. The ability of the system was tested on murine liver organoids, and the data are made publicly available.

The OrganelX e-Science free service is available at
https://organelx.hpc.rug.nl/organoid/

This chapter has been previously published as:
6.1 INTRODUCTION

New advances in the biomedical field have presented us with the need for new technical and analytical approaches. The field of organoids has experienced a massive upsurge in the past 15 years. Organoids are three-dimensional mini-structures that in culture recapitulate some features and functions of the organ of origin. Ever since organoids were first described in 2009 [172], the field has continued to expand rapidly. After that, many different types of organoids, representing different organs, have been described [173]–[177]. These three-dimensional structures have become widely popular due to their ability to self-organize and proliferate while maintaining multiple biological functions [178]. Organoids can be used for multiple purposes, ranging from developmental studies to disease modeling, as well as, drug screening [178], [179].

Since it is the three-dimensional environment that allows cells to organize and behave in a more physiologically relevant manner, it is understandable how important morphology and size are [178]. It is common for researchers to track organoid morphology and size as these are the first signs of health and disease. This is often done for multiple purposes: either for developmental reasons/optimization of the culture conditions [180], [181], or in the context of different diseases [182].

Another interesting approach to studying organoid health is tracking organoids’ size in time [183]. This is done in order to determine the growth and expansion rate of the culture in time. This proliferative status of the organoids can provide relevant information regarding the developmental state of the organ, regeneration, and response to pharmacological interventions.

However, it is important to highlight that due to the way these structures are cultured, high variability in size is often observed between organoids in the same dome (Figure 6.1B and 6.1C). One of the main reasons for this is the way they are distributed in the hydrogel domes. Since these domes are three-dimensional structures, not all organoids have access to the same amount of nutrients in the medium. Often, this causes organoids around the edges of the hydrogel to have easier access to nutrients, reaching bigger sizes than those organoids contained in the middle of the dome (Figure 6.1B and 6.1C). Another complexity arising from this type of culture is the proliferative state of the cells that leads to different organoids expanding and collapsing at different rates.

Because of this high variability between organoids in the same dome, researchers need to quantify large numbers of organoids per well in order to get an accurate description of what is happening in the culture. Counting and measuring the
6.1 Introduction

Figure 6.1: Organoids display high variability in size and morphology in culture. A) Representative sketch of a hydrogel dome. Line bars represent the different Z-stack of the dome. B) Representative Sketch of the distribution of organoids in a hydrogel dome. i) Organoids accumulate in one area overlapping. ii) Organoids around the edge of the hydrogel dome develop to bigger sizes and more complex morphologies. iii) Organoids in the middle of the dome display smaller sizes and more spherical morphologies. C) Representative brightfield images exemplifying the different distributions and morphologies described in B.

Size of multiple organoids per dome is a manual process that can take several hours depending on the density of the cultures. These measurements are highly influenced by factors such as tiredness, personal interpretation, etc. This can lead to low reproducibility between measurements. Brightfield images of the cultures provide a good opportunity to get a broader impression of the health/state of the culture. These organoids can then be manually counted and measured using different softwares. To do so, scientists need to manually select the area of the organoid of interest and trace it in order to get an approximation of the size. Organoid cultures can vary in density, showing as little as a few organoids to as many as a hundred. Another difficulty of tracking an organoid’s growth in time is the need to image the cultures repeatedly (i.e. every 24 hours) and find the same organoid at every time point in order to track its growth rate. Overall, this task is highly time-consuming and has a very high person-dependent variability.

OrgaQuant [184] is an open-source implementation that addresses this issue. However, this tool does not perform a pixel-wise segmentation of organoids, instead, it uses a bounding box to detect an organoid. Thus, the area of an
towards automatization of organoids analysis: a deep learning approach to localize and quantify organoids images

organoid cannot be computed accurately. Additionally, OrgaQuant does not allow for user interventions and does not relate organoids from different stack images of a z-stack image. Another group [185] has also published annotated organoid images, in which each organoid is surrounded by a bounding box created by experts. However, these data can only be accessed upon contacting the authors. Due to the use of bounding boxes, the correct area of each organoid cannot be computed. This is important since the growth characteristics are crucial for organoid research. Even though they trained a deep neural network to detect and track organoids, this model is not publicly available. In order to overcome these problems, we have extended the OrganelX e-Science service [71] to segment and analyze organoid cultures. This system allows researchers to upload their brightfield images in order to quantify the number of organoids present in the image as well as to measure their area. The system allows researchers to calculate the average area of multiple organoids of a culture or only of those of interest. It also offers the possibility to select multiple organoids of interest in different Z-stacks of the dome (Figure 6.1A). Moreover, the system indicates the organoids that are found in different stacks. Another application would be to track organoids in time using images acquired at different time points.

The contributions of this work are presented as follows: (1) Design of a deep-learning algorithm and processing pipeline dedicated to organoid-image analysis; (2) Development of an e-Science service that detects, segments, and analyses organoids images; (3) Testing the system on a use case to evaluate the area and growth analysis; and (4) Providing public data for the use case.

6.2 Method and Implementation

6.2.1 Murine Liver Progenitor Organoids Culture

Ductal fragments from male C57BL/6J mice were isolated following the published protocol by Broutier et al [186]. Mice between 3 to 5 weeks of age (Jackson Laboratory, Bar Harbor, ME, USA). Ethical approval was obtained from the Central Authority for Scientific Procedures on Animals (CCD) of the Netherlands and from the University of Groningen Ethical Committee for Animal Experiments (Animal Use Protocol Number: 171504-01-001/3). These fragments were kept in expansion medium consisting of Advanced DMEM/F12 supplemented with 10mM HEPES, 1% (v/v) GlutaMax, 1% (v/v) Penicillin-Streptomycin (all Gibco), 1% B-27 Supplement (Invitrogen) 1% N-2 Supplement (Invitrogen), 10mM Nicotinamide (Sigma Aldrich), 1.25mM N-Acetylcysteine (Sigma Aldrich), 10% RSpondin Conditioned Medium (provided by Calvin J. Kuo), 30% Wnt3a CM (provided by Hans Clevers), 100 ng/ml Noggin (Petrotech) 50 ng/ml HGF
(Peprotech), 100 ng/ml FGF-10 (Peprotech), 50 ng/ml EGF (Peprotech), 10nM Leu-gastrin. Three days after the isolation Noggin and Wnt3a CM were removed from the medium. The medium was changed every 2-3 days. Liver progenitor organoids were passaged every 6-7 days. Insult-exposed organoids were grown in the same medium but in the absence of amino acids.

6.2.2 Organoid Imaging

Organoid cultures were followed over time with an AxioObserver Z1 compound microscope (Carl Zeiss), 2.5x and 5x objectives, and an AxioCam MRm3 CCD camera (Carl Zeiss) in brightfield. Raw images were processed using the ZEN 3.1 blue edition software. The whole well was imaged by selecting 9 tiles that were later on stitched together. On average 20 different Z-stacks were imaged in order to ensure all organoids in the Matrigel dome were detected. The number and size of organoids were assessed in the whole well. For the first case study, a set of 95 organoids were manually counted. For the second case study, and tracking in time of the organoids, a set of 25 organoids were randomly selected and measured. Images, of the same cultures, were acquired every 24 hours and the same 25 organoids were measured at every time point.

6.2.3 The OrganelX e-Science Service

For the purpose of studying organoids in culture, the free e-Science web page\footnote{https://organelx.hpc.rug.nl/organoid/} offers different functionalities. The service offered by the website begins after uploading a CZI file by the user. A CZI file, which is suitable for microscope image data and captured by a Carl Zeiss microscope, contains tile images for each stack as well as image stacks. It is to be mentioned that the system will be further developed to include different types of images files. In addition to the CZI file, the user needs to provide a short experiment ID and sample ID. This is beneficial for future comparisons between experiments and samples in the experiment. The user will be redirected to a page that tracks the request’s progress and presents the final results in an interactive way.

Organoids in each image stack are automatically detected and segmented using Mask-RCNN\[^{167}\]. Mask-RCNN is a state-of-the-art instance segmentation framework based on a convolutional neural network that outputs a mask channel consisting of a pixel-wise prediction of organoids’ location. As training this framework from scratch is a non-trivial task, we employ and adjust a pre-trained model\[^{187}\]. It is relevant to mention that the computation time for each uploaded CZI file varies and depends on the number of stacks, images sizes, and the number
of organoids found in each image stack. With this in mind, an optional step is offered for the user to provide an email address so that a notification will be sent to the email address when the computation is completed. Each of the segmented organoids will get a unique number (ID).

Upon completing this step, an automated analysis step begins for grouping and assigning identical organoids of different stacks. Organoids of different stacks are called identical when their mask center-point is within a certain distance and the Intersection of Union value of their bounding boxes is within a certain range.

Once all internal computations are finished, the results will be presented on the page the user was redirected to (or notified if an email address where provided). The number of the successfully detected and segmented organoids for all stack images will be given. The user can interactively perform the following operations:

- Select a stack image and preview the organoids that are detected by MASK-RCNN for that specific stack image. The corresponding number of detected organoids for this stack will be specified.

- Select multiple organoids of interest. As a result, the area of each selected organoid will be shown. Additionally, the minimum, maximum, average, and total areas will be marked. The area of each organoid depends on the unit scale given by the CZI file. A radio button is provided to determine whether to select identical organoids of all stacks or to only consider organoids of the chosen stack.

- Extract and download a summary zip file containing:
  (i) PNG images of all stacks including the selected and unselected organoids.
  (ii) CSV file of the identical organoids represented by unique id and corresponding name.
  (iii) Excel file, in which each sheet includes information of the selected organoids (ID, bounding box coordinates, mask border, and the area) for each stack image.

It should be noted that multiple compute requests can be simultaneously handled. The size of the CZI file should be smaller than 1.5GB. The width and height of the stack images can be of any size. However, the bigger the image size is, the more computational time would be needed. For each request separately, all above-mentioned computations will be computed and represented in the redirected page within a maximum of 12 hours after uploading the CZI file. Currently, the results will be deleted after a week of the submitted request.
6.3 VALIDATION AND RESULTS

To validate the accuracy of OrganelX along with its interactive tool we defined and compared two scenarios.

6.3.1 Segmentation and Area Calculation for One-Stack Images

For a randomly chosen stack image, we manually annotated 95 organoids and compared their cross-sectional area against the computed area by OrganelX. It is to be mentioned that the segmentation of one stack image using OrganelX was completed in a few minutes while the time for the manual segmentation of the same stack was around 1 hour. Furthermore, while manual counting allowed us to segment 95 organoids, OrganelX was able to count twice as many organoids. Figure 6.2A shows the chosen stack image in addition to the manually segmented organoids in red. The black and blue points in Figure 6.2B denote the cross-sectional area of those manually and OrganelX segmented organoids, respectively. The results demonstrate that the overall computed areas are nearly identical with a minor difference for some organoids. To demonstrate the accuracy of this result in a number, the average of the absolute differences between manual and OrganelX segmented areas was calculated and divided by the total manual segmented areas. An inaccuracy of less than 0.01% was obtained.

6.3.2 Segmentation and Growth Area Over Time

Once the ability of the system to replicate manually annotated data was assessed, its ability to track organoids in time was evaluated.

In this case study, liver progenitor organoids have been grown in either complete medium (referred to as control) or in medium lacking all amino acids (referred to as starvation), which are essential for growth. Organoids in starvation medium, although coming from the same initial culture as the control ones, have impaired growth and smaller size due to the insult introduced at time point 0H (hours). To illustrate the results, these organoids have been tracked over time by imaging them every 24 hours. In order to study the growth rate, 25 organoids for each medium were randomly selected and their cross-sectional areas were measured every 24 hours using brightfield images. Figure 6.2C demonstrates the growth at time point 0 and after 96 hours for organoids in the control and starvation medium.

Figure 6.2D demonstrates the area of those selected organoids over the experi-

2 https://organelx.hpc.rug.nl/organoid/test_example
Figure 6.2: Evaluation results. (A) Representative bright-field image of a liver progenitor organoid culture at day 3 after passaging with medium to small structures. 95 organoids have been selected and manually measured (indicated by red contour). Scale bar, 1000 µm (B) Size of 95 individual organoids measured manually compared to the same organoids measured by OrganelX. (C) Representative bright-field images of liver progenitor organoids imaged at time points 0H and 96H. The upper row shows control organoids in complete condition medium and the bottom row shows organoids in a medium depleted of amino acids (Starvation). The scale bars present 1000 µm (D) Cross-sectional area of 25 random organoids tracked in time, measured every 24 hours. The Black and solid line represents control conditions measured manually. The Grey and the dotted line represents control conditions measured by OrganelX. The red and solid line represents starvation conditions measured manually. The light red and the dotted line represents starvation conditions measured by OrganelX. Error bars indicate the SEM.

As it can be seen, the area of the organoids measured by OrganelX is very similar to those manually segmented especially for those in starvation medium. Only small differences of the areas are reported for those in the control medium after 48 hours. This is due to the fact that organoids are growing, their sizes increase over time, and OrganelX fails to quantify the size of such large organoids. This dissimilarity highlights the importance of continuously training OrganelX in order to optimize its response to bigger organoids.
6.3.3 Publicly Available Data

The e-Science website offers the tested data to be publicly available\(^3\). Table 6.1 demonstrates the number of stacks in each file, the total number of detected organoids in the dome, as well as the average of all areas of the detected organoids in the dome.

Table 6.1: The number of stacks in each CZI file (S), the total number of detected organoids in the dome (O), the average of the total detected organoids area in the dome in \(\mu m\) (A).

<table>
<thead>
<tr>
<th>Time</th>
<th>Control</th>
<th>Starvation</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 H</td>
<td>16 (S), 295 (O), 20951.03 (A)</td>
<td>16 (S), 219 (O), 19353.19 (A)</td>
</tr>
<tr>
<td>24 H</td>
<td>24 (S), 292 (O), 21617.07 (A)</td>
<td>24 (S), 222 (O), 20319.44 (A)</td>
</tr>
<tr>
<td>48 H</td>
<td>27 (S), 302 (O), 24782.65 (A)</td>
<td>27 (S), 221 (O), 22521.13 (A)</td>
</tr>
<tr>
<td>72 H</td>
<td>20 (S), 298 (O), 25058.80 (A)</td>
<td>20 (S), 226 (O), 20824.84 (A)</td>
</tr>
<tr>
<td>96 H</td>
<td>26 (S), 295 (O), 24240.08 (A)</td>
<td>26 (S), 171 (O), 18044.25 (A)</td>
</tr>
</tbody>
</table>

6.4 DISCUSSION AND CONCLUSION

This chapter introduces an extension to the OrganelX e-Science website that automatically detects and segments organoids upon uploading a CZI file containing different stack images. It also offers functionalities such as selecting organoids of interest, computing their area, and counting the number of organoids in each stack image. The performance of the website was evaluated using two scenarios, in which OrganelX has shown the ability to accurately segment organoids. Regardless of the small variability in individual sizes, OrganelX is able to track down in time the size of multiple organoids with a similar outcome as manual measurements. Moreover, OrganelX offers the possibility to easily increase the sample size (e.g. from 25 organoids up to hundreds) in order to produce a more descriptive result. Segmented CZI files with more than 30,000 detected structures were made publicly available. It is to be noticed that for this quantification, each individual stack image has been regarded as an independent image. OrganelX has only been tested on murine liver organoids, which present a common cystic morphology. However, it is possible to also measure any other types of organoids. As long as these organoids have an approximately spherical morphology they should be detected. The method has not yet been tested on non-cystic morphologies such as murine intestinal organoids. For the future, OrganelX will be trained to work with different morphologies.

\(^3\) [https://organelx.hpc.rug.nl/organoid/public_data](https://organelx.hpc.rug.nl/organoid/public_data)
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