Nucleus segmentation : towards automated solutions

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GLOSSARY:

2D: **Two Dimensional**: the term typically used to indicate the standard format in which images are acquired by a standard camera

3D: **Three Dimensional**: the term typically used to indicate a z-stack of 2D images referring to different optical sections

API: Application Programming Interface: a set of functions and procedures allowing the development of applications that access the features or data of an operating system, application, or other service

BBBC: **Broad Bioimage Benchmark Collection**: an open microscopy image collection for scientific purposes

CNN: **Convolutional Neural Network**: a class of deep neural networks including convolutional layers based on blocks responsible for appropriate image feature retrieval (via convolutions) and scaling (with pooling blocks)

DAPI: a widely used fluorescent stain that binds to adenine–thymine-rich regions of the DNA, thus labels the nucleus

DIC: Differential Interference Contrast: a microscopy technique that introduces contrast to images of specimen with little or no contrast upon brightfield microscopy

DNN: Deep Neural Network is an artificial neural network machine learning architecture that includes several hidden layers, and can be trained to solve more complex tasks on more complex data compared to shallow neural networks

DSB2018: **Data Science Bowl 2018**: the data science competition held in 2018 with a task to segment nuclei in microscopy images. The official, open dataset of the competition is also referred to as such, and is often used to benchmark nucleus segmentation methods

GPL: **General Public License**: a series of widely used open-source licenses that guarantee end users the freedom to run, study, share, and modify the software

GPU: **Graphics Processing Unit**: a specialized electronic circuit designed to rapidly manipulate memory to accelerate computations related primarily to graphics

H&E: **Hematoxylin and Eosin**: a combination of two histological stains: hematoxylin and eosin. Hematoxylin stains cell nuclei to purplish blue, and eosin stains the extracellular matrix and cytoplasm to pink

IF: Immunofluorescence: a staining which utilizes fluorescent-labelled antibodies to detect specific target antigens

ISBI: **International Symposium on Biomedical Imaging**: a scientific conference series dedicated to mathematical, algorithmic, and computational aspects of biological and biomedical imaging

IT: **Iterative Thresholding**: an algorithm used to define the background and foreground in an image

LoS: **line-of-sight**: the straight line between the object and the target

mAP: **mean Average Precision**: a popular metric related to measuring the accuracy of object detectors

MITK: **Medical Imaging Interaction Toolkit**: a software suite designed for medical image analysis

NEUBIAS: Network of European BioImage Analysts, a network of experts in life sciences for image data analysis.

PC: Phase Contrast: an optical microscopy technique that converts phase shifts in light passing through a transparent specimen to brightness changes in the image

RPN: **Region Proposal Network**: a fully convolutional network that simultaneously predicts object bounds and objectness scores at each position

siRNA: **short-interfering RNA**: a class of double-stranded, non-coding RNA molecules, similar to miRNA, operating within the RNA interference (RNAi) pathway

SNR: **Signal-to-Noise Ratio**: a measure used to compare the level of a desired signal relative to the level of background noise

TCGA: The Cancer Genome Atlas: a huge cancer genomic program which covers many cancer types with a patient-based, open dataset including genomic, proteomic, imaging etc. data

TIFF: **Tagged Image File Format**: one of the most common image file formats

TTA: Test-time augmentation: the aggregation of predictions across transformed versions of a test input

WSI: **Whole Slide Image**: scanned image of an entire histopathology tissue section, usually of gigapixel size, resulting in file size of gigabytes, which is difficult to handle by an image processing software

ABSTRACT

Single nucleus segmentation is a frequent challenge of microscopy image processing, since it is the first step of many quantitative data analysis pipelines. The

quality of tracking single cells, extracting features or classifying cellular phenotypes strongly depends on segmentation accuracy. Worldwide competitions have been held, aiming to improve segmentation, and recent years have definitely brought significant improvements: large annotated datasets are now freely available, several 2D segmentation strategies have been extended to 3D, and deep learning approaches have increased accuracy. However, even today, no generally accepted solution and benchmarking platform exist. We review the most recent single-cell segmentation tools, and provide an interactive method browser to select the most appropriate solution.

TOWARDS ROBUST AND AUTOMATED METHODS FOR NUCLEUS SEGMENTATION

The history [1] of detecting and segmenting single cells goes along with the first digitized microscopy images. Many research fields utilizing microscopy, such as developmental biology [2], drug discovery [3], functional genomics [4] and pathology [5] are dependent on accurate cell and nucleus segmentation as a vital part of image analysis workflows. Since image analysis has moved from a methodological research area towards data science as a result of the recent machine learning revolution, annotated datasets have become essential regarding the performance of nuclear segmentation methods. Especially, modality-independent, generalizable, and robust machine learning-based nucleus segmentation models need heterogeneous and large collections of expert-annotated images [6,7].

The level of difficulty of single-cell detection in an image, let alone precise outlining, widely varies (see **Figure 1**). In most simple cases nuclei have high contrast and are separated by proper experimental conditions (referred to as *easy* cases), hence their segmentation is not difficult (e.g. large **siRNA** - see **Glossary)** [8]. In other cases segmentation is highly *challenging*, for instance in **3D**, label-free or thick tissue sections where cells touch, overlap or have non-conventional morphology, intensity, or patterns. International competitions [6][9] have promoted the potential to overcome these issues, yet a genuinely general solution is still awaited. However, due to major advancements in this field in recent years, our community has reached an unprecedented improvement in detecting single nuclei [10]. Easy cases of segmentation, especially in 2D are not problematic anymore [11,12], while accuracy has also improved in challenging cases [6]. In addition, 3D data analysis methods have progressed with extended 2D segmentation solutions [13] or with native 3D ones [14][15]. The community has accumulated large amounts of annotated data (either by experts [6] or crowdsourcing [16]) for training machine learning segmentation models, and to evaluate the methods in public benchmarking platforms [6][17]. This review describes the specific techniques biologists can exploit for single-cell analysis. However, we emphasise that no standardized approach has been developed to date to properly compare different solutions before deciding which tool to use for a specific application.

First, the variety and extent of datasets currently available to test and train methods are presented. Next, a selection of annotation tools available for creation of training datasets for machine learning methods is introduced. Then the issues related to pre- and post-processing of images to reduce challenges inherent to complex data are

briefly discussed (see **Suppl. Mat. 1** for details on different techniques), followed by insights into 2D nuclear segmentation methods. Classical approaches that provide task-specific and general solutions for a wide variety of acquisition techniques are presented. However, most recent methods usually rely on **DNNs**, and since the target objective is related to image processing, **CNNs** are most commonly applied to segment nuclei (see in **Glossary**). As processing 3D data is one of the major challenges in single nucleus segmentation, a set of promising and successful methods appropriate to solve specific 3D segmentation tasks is discussed.

Figure 2 supports a better understanding of the definitions of detection and segmentation tasks. When identifying single cells (objects) in microscopy images automatically, i.e. using computer algorithms, the results may be either (*1*) *detections* corresponding to the localization of the objects or (*2*) *segmentations* which separate independent image regions. The former is typically represented as bounding boxes, whereas the latter may be realized by either assigning a binary label to each pixel while dividing the image into not necessarily connected regions (*semantic segmentation*) or by separating individual objects (*instance segmentation*). One may choose from a plethora of methods and software tools to perform nuclear segmentation (see section "*Segmentation methods and toolkits*"). Major challenges are discussed in the **Outstanding Questions Box**. As many of the segmentation methods considered in this review utilize *deep learning* approaches, this subset of machine learning is also introduced. Deep learning involves the training of **DNNs** for complex yet arbitrary tasks, such as detection, segmentation and classification (not strictly in our domain of cellular image analysis, but also in natural image-, video- or audio-processing). Deep learning based approaches are proven to perform

excellently on the trained domain, with the potential of extension to unseen domains [18]. Their translation is limited by the lack of publicly available datasets related to less common modalities (see section "*Annotated nucleus datasets*").

A portal[I] was developed to offer a graphical aid to select the most appropriate method for non-image analysis experts (see **Figure 2**). This portal has several advantages: (*1*) the imaging community can select the methods applicable/appropriate for their images of interest, and (*2*) developers can submit the description and best practices for their methods. Currently, the most common optical microscopy categories are considered. Notably, the web portal is declared to be maintained by the authors, yet the community is encouraged to actively contribute and eventually propose extensions to it. For benchmarking the segmentation methods, users can exploit BIAFLOWS[II] [17], a freely available web-based platform developed by **NEUBIAS**. The assessment of new proposals has been commonly performed with limited datasets and arbitrary metrics (see **Suppl. Mat. 2**); in contrast, NEUBIAS is a step forward to prevent such a biased evaluation. However, an unbiased quantitative method comparison is still impossible due to the lack of a comprehensive annotated dataset for training and testing the methods using a globally accepted benchmark platform and unified metrics (see section "*Segmentation methods and toolkits*").

ANNOTATED NUCLEUS DATASETS

Annotated datasets are used in computer science to validate the accuracy of developed algorithms. In addition, nowadays annotated datasets are also used for training machine learning models for various tasks. One of the key factors influencing the performance of segmentation models is the composition of annotated data. Ideally, a trainable model yields optimal results on a test set sampled from the same domain as training data are collected from, hence domain-specific annotated datasets serve as a valuable asset, especially when they are expert-curated. Highly specific domain datasets are usually complemented with proper metadata [19][20], such as the experimental setup, sample preparation or microscope device, and are expert-curated when it comes to annotations. However, they typically cover a narrow diversity, and are small in size. Open datasets may contain a varying number of images (see **Table 1**). An important aspect for the user to consider when either training a new model or evaluating segmentation performance on publicly available datasets is that the corresponding annotations occasionally contain such segmentations yielded by automatic methods [19] that might not be refined by an expert, thus the results might be biased. Annotated nucleus datasets displayed in **Table 1** show diversity in size (not only regarding the number of images, but also that of the objects too) and content, focusing on those widely used as benchmarks or training data. Annotations may be realized as objects (instance aware) or binary masks (semantic), are primarily 2D, and the two most common imaging modalities cover fluorescence stained cell cultures and **H&E** stained tissue sections.

Table 1. Open datasets of annotated nucleus for single-cell analysis purposes

Name	Individual objects (O) or binary masks (BM)	2D/3D	Microscopy	Staining	Sample	# images	# objects	Ref.
BBBC032	O	3D	confocal	fluo	mouse embryo blastocyst cells	1 (172*)	1220	[21]
BBBC033	O	3D	confocal	fluo	mouse trophoblast stem cells	1 (32*)	585	[21]
BBBC034	O	3D	brightfield/fluorescent	x/3 fluo**	hiPSC 3D	1 (52*)	790	[19]
Scaffold-A549 Dataset	O	3D	fluorescent	Hoechst +DiL	lung cancer tissue	21	800 (+10000 w/o labels)	[22]
BBBC039	O	2D	fluorescent	Hoechst	U2OS cells	200	23.615	[10]
CoNSeP	O	2D	brightfield	H&E	colon tissue	41	24.319	[23]
CryoNuSeg (TCGA[IV])	O	2D	brightfield	H&E	various tissues	30	7.596	[24]
DSB2018	O	2D	various	various	various tissues and cells	841	37.530	[6]
Janowczyk et al.[V]	BM	2D	brightfield	H&E	breast tissue	141	~12.000	[25]
LIVECell	O	2D	phase-contrast	label-free	cell cultures	5 239	1.686.352	[26]

Lizard	O	2D	brightfield	H&E	colon tissue	291	495.179	[27]
MoNuSeg2018	O	2D	brightfield	H&E	various tissues	44	28.846	[28]
NuCLS	O***	2D	brightfield	H&E	breast tissue	N/A	222.396	[16]
NucMM	O	3D	electron microscopy/ micro-CT	label-free	brain tissue	2	~170.000 + ~7.000	[29]
PanNuke	O	2D	brightfield	H&E	various tissues	481	205.343	[30]
S-BSST265	O	2D	fluorescent/ confocal	IF/ DAPI	various tissues and cells	79	7.813	[20]
TCGA[IV] images processed by Irshad et at.	N/A	2D	brightfield	H&E	kidney clear cell renal carcinoma tissue	63	N/A	[31]
TCGA[IV] images processed by Kumar et at.	O	2D	brightfield	H&E	various tissues	30	21.623	[28]
TNBC	O	2D	brightfield	H&E	breast tissue	50	4.022	[32]
Wienert et al.	O	2D	brightfield	H&E	various tissues	36	7.931	[33]
TissueNet Version v1.0	O	2D	fluorescent	various stainings	various tissues	6990	~1.200.000	[7]

International challenges, such as the annual **ISBI** or competitions hosted by e.g. Kaggle with industry partners, inspire those in the field of research and development to propose new technologies and methods or combine existing ones for a new purpose. One of the most successful and widely used segmentation methods, *U-Net [34]* (see in **Supplementary Material 4**) arose from the 2015 ISBI Cell Tracking Challenge [35], and has been the basis for several novel CNN architectures ever since (see section "*Segmentation Methods and Toolkits***"**). Similar competitions contribute to the development of this field with invaluable collections of microscopy images, on which developers may benchmark their novel approaches according to standard evaluation metrics (typically **mAP**) in a fairly comparable way. In recent years the **DSB2018** [6] dataset has been applied as such, since its image set comprises various types of microscopy modalities, magnifications, labels, sources etc. This might also provide insight into the expected model performance. Generally, datasets originating from challenges are carefully validated by field-expert annotators [6][15] (usually biologists and pathologists), promoting their further applicability to train new models. Notably, annotations of the training set are usually released instantly, while test set annotations may remain private even after the challenge is concluded [35]. Dataset size strongly depends on the task, e.g. a competition in 2D instance segmentation (like DSB2018) generally has a larger number of annotated images than a tracking [35] or a 3D segmentation task [19][21].

Conclusively, a key contribution of the bioimage analysis community to this field is the release of open datasets of annotated images, in as many varying imaging modalities as possible. Data sharing is highly encouraged, especially in case of intrinsically challenging microscopy types, such as label-free imaging (notably, LIVECell [26] is a promising step in this direction) or generally in 3D. Provided in an open way, these annotated datasets could inspire method developers to increase their focus on less frequent modalities, and release pre-trained models for those as well. Also, they enable users to benchmark (evaluate the performance of) available methods on this data. Additionally, experiment-specific unlabelled image sets (e.g. TCGA[IV]) may also promote progress in case an annotated subset is shared later independently [24,28][31]. Finally, as annotated datasets require an appropriate software tool that the experts (or generally, annotators) can use to create the labels, various annotation software solutions are collected in the following section.

TOOLS FOR ANNOTATION

Countless software tools are available to create annotations for single-cell segmentation training or validation, with a widely varying spectrum of functionality. These tools are designed either for specialists, such as biologists and pathologists, or for method developers. Options for annotation typically include freehand drawing, point, ellipse or polygon labelling, all of which may be exported to formats suitable for different applications. The finer the representation (annotation) of the object is, the more information it provides for a model when used as training data. While object location marked simply by a centre point or bounding box coordinates is sufficient for

detection or even classification training, contours (boundaries) labelled either semantically (binary) or in an instance-aware way are usually used to train segmentation. Equivalently, the same types of annotated data may be utilized to assess the accuracy of different methods.

Even though labelling several images tends to be time-consuming for a single expert, even students [16] can learn how to create accurate annotations when curated by experts, yielding large annotated datasets via joint and shared efforts. Semi-automatic annotation achieved by initial segmentation methods offers a convenient solution to speed up the annotation process for experts, and is often preferred by the community. Such annotation methods also help to increase [36][37] the agreement between experts, which is a common problem source in annotation. Alternatively, a consensus of multiple annotators may be used [31][6] at the object- or pixel level; crowdsourced annotations [16] are easier to combine this way. Commercial solutions and free-to-use software, including but not limited to those applied in cell biology, are described in detail in **Suppl. Mat. 3** and **Suppl. Table 2**.

Plugins or extensions to existing open-source software, such as *ImageJ/Fiji [38,39]* or *MITK [40]* are popular choices preferred by bioimage analysts already experienced with the given software. The *Fiji* plugins *Trainable Weka Segmentation* [41] and *LabKit[III]* use machine learning to train pixel classification similarly to *ilastik* [42] (see **Suppl. Mat. 3-4**), while *AnnotatorJ* [36] applies a *U-Net* to assist contour annotation. Assistance in the *MITK* plugin *3D-Cell-Annotator* [43] exploits active surfaces with shape descriptors in 3D, while *NuClick* [18] uses its own CNN for histopathology images.

Larger image analysis projects not primarily intended for annotation, but for a rather more comprehensive evaluation of the sample images (*Cytomine* [44][45], *ilastik [42]*, *DeepCell* [46], *QuPath* [47]), including e.g. the segmentation or classification of cells, may also provide convenient solutions for annotation. Still, each has its target application: e.g. *QuPath* is a desktop tool suitable for **WSI** analysis, while *Cytomine* processes WSIs online in a collaborative way, and DeepCell improves its segmentation DNN with annotation collaboration.

Standalone software packages (*Diffgram*, *LabelImg, Segmentor* [37]) offer a lightweight, specific solution for annotation: *Segmentor* [37]is intended for 3D annotation, *Make Sense* and *Diffgram* have additional online interfaces, and the latter also supports deep learning. Online tools (*VGG Image Annotator*, *Kaibu*, *supervise.ly, Piximi annotator*) require no installation and have no specific hardware requirements. However, it is worth noting that online service-based platforms (*Lionbridge.AI* or *Hive*) require that raw data are sent out of the laboratory, which might be undesirable in case of sensitive (e.g. patient-related) images.

Nonetheless, genuinely general-purpose image editing applications, such as *GIMP* (**GPL** licence, free) or *Photoshop* (Adobe, commercial) may also be used to create annotations at the expense of more cumbersome export, e.g. in the case of instance annotation labels.

Conclusively, several options are available, depending on the specific requirements of a project or experiment. Tools that provide multiple implementations (e.g. both local and online) might be ideal for more users.

SEGMENTATION METHODS AND TOOLKITS

Single nucleus segmentation methods may work with raw images, but in more challenging cases (e.g. **Figure 1. j-v**) the quality of the analysis (and specifically that of single nucleus segmentation) benefits from additional pre- and post-processing steps (e.g. illumination correction [48][49] or denoising [50] prior to the analysis, mask refinement or test time augmentation (**TTA**) [51] applied as post-processing). Application of these methods depends on the task and the desired quality of the result; some of the most commonly used processing steps are described in **Suppl. Mat. 1**.

Nucleus segmentation is traditionally performed using a data-specific workflow that contains various filtering and thresholding methods, followed by morphological operations and processing steps (*ImageJ/Fiji* [38,39], *QuPath* [47], *CellProfiler* [52]). Segmentation using pixel classification, based on classical machine learning methods has been used for challenging data for a decade, with early versions of tools including e.g. *DeepMIB [53]* and *ilastik* [42]. The fundamental difference between classical image processing-based nucleus segmentation and that with classical machine learning is the input required from the user: in the former case, manual parameter setting and fine-tuning is expected in different processing

modules in the pipeline, which is still capable of yielding very high accuracy at the expense of time-consuming re-parameterization for each new experiment. The latter enables users to rely on automated feature extraction and learning by still providing examples manually, which most likely also need to be repeated in experiments. Notably, appropriate pre-processing of input images (e.g. intensity scaling) can help to unify the range of optimal parameters in both cases. The nuclear segmentation task has moved towards robust and automated approaches with *U-Net [34]* (see in **Suppl. Mat. 4**), which was a breakthrough for deep learning-based nucleus segmentation (and in the field of deep learning-based segmentation in general). In contrast to image processing and classical machine learning, deep learning-based methods require fewer input parameters from the user, and are generally more straightforward to apply between experiments than in the case of classical approaches. Nonetheless, pre-processing also increases the accuracy of CNNs in most cases. *U-Net* still serves as a baseline for semantic segmentation tasks, and is (*1*) used as part of recent general nucleus/cell segmentation pipelines, such as *Cellpose* [12] and *StarDist* [54], and (*2*) utilized or further developed in *nnU-Net* [55] and *UNet++* [56]. Even though *U-Net* is a semantic segmentation framework, it can be extended to instance segmentation with post-processing. One typical solution is to classify pixels into three classes where one class represents nuclear edges, and as such, it can aid instance segmentation [10]. Computationally *U-Net* is relatively simple, thus it is possible to train a basic *U-Net* on workstations or even laptops with a **GPU**.

Another breakthrough in deep learning-based instance segmentation was *Mask R-CNN* [57]. This network was designed for the segmentation of natural images,

however, it has been adapted for nucleus segmentation in methods such as *nucleAIzer [11]*. *Mask R-CNN* is built over a *CNN* feature extraction backbone and **RPN** *[58]* to suggest possible object regions. These proposals are classified and used for binary mask prediction. *Mask R-CNN* outputs a list of masks allowing overlaps, whereas the output of *U-Net* is an image with no overlaps. However, two recent extensions to *U-Net*-based *StarDist, MultiStar [59]* and *SplineDist* [60], enable segmentation of overlapping objects. *NuSeT* [61] combines RPN, *U-Net* and watershed post-processing to optimize segmentation of crowded cells. *Mask R-CNN* requires more computational resources than *U-Net*, still it can be trained on a modern workstation or laptop.

Even though many segmentation methods are not deep learning-based (*MINS [62,63]*, *XPIWIT [64]* etc.), the field has recently tended to shift towards approaches based on deep learning (e.g. *ilastik [42]* now offers **DNNs**). This includes bundles of specific deep learning methods for segmentation and pre-processing which could be used on Google Colab (*ZeroCostDL4Mic* [65], *Segmentation of stochastic optical reconstruction microscopy (STORM) images* [66]), or other client-server architecture (*ImJoy* [67], *DeepCell Kiosk [46][68]*, *HistomicsML2* [69]) with provided separate pre-trained models (*CDeep3M* [70], *nucleAIzer* [11], *Cellpose* [12]). *ImageJ* users can also utilize deep-learning based segmentation with plugins and pre-trained models (*DeepImageJ* [71]). The majority of the methods discussed here are deep learning-based (see **Table 2**), which require hardware resources due to the parallelizable and heavy computational costs of DNNs, hence **GPU** acceleration is advised, especially for training. Cloud-based solutions often meet this requirement.

Several methods mentioned above could be used for 3D datasets (see **Table 2**). Segmentation of 3D nuclear images with deep learning is not straightforward. The major limitation is that the annotated data in the field are less abundant compared to the planar case. There are several deep learning-based methods developed by the medical image analysis community facing a similar challenge. However, in the case of medical images, usually only one or a few objects need to be segmented. This task is different from and less difficult than nucleus segmentation, where hundreds of instances should be segmented even when they touch. For example, segmenting a medical image by combining the segmentations of 2D images may provide acceptable accuracy. In contrast, nucleus segmentation is an instance segmentation task where this approach alone is less likely to work in crowded parts of the image, but the connected components of the stacked 2D segmentations can be used as a seed image for the watershed transform to compute the final 3D instance segmentation [72]. Besides, 3D segmentation is more demanding in terms of computational resources (especially GPU memory and file sizes) when a dense 2D method is extended directly to process 3D images. Introduction of a further dimension may lead to substantially growing complexity (for example in case of differential geometry-based approaches) and more complex spatial dependencies in case of CNNs, however, this phenomenon termed 'the curse of dimensionality' is especially problematic, thus more training data and more computational resources are required. Still, several tools are specifically developed for the 3D segmentation task [73], and some deep learning based methods developed for 2D segmentation are also extended to 3D. The *IT3DImageJSuite* is an *ImageJ* (*Fiji*) [38,39] plugin that involves several algorithms (including iterative thresholding and watershed). *LoS* [74] approximates the convex decomposition of the objects with spectral clustering.

OpenSegSPIM [75] is a *MATLAB* application which performs instance segmentation by applying a pipeline of filters in a semi-automatic manner. *RACE* [76] and Ruszczycki *et al.* [15] first compute the 2D segmentation on the z-slices, and then combine them to 3D objects. Similarly to *BioImageXD* [77], *Fiji* [38,39] and *Icy* [78], *Vaa3D* [79] uses a pipeline consisting of Gaussian filtering, adaptive thresholding, distance transformation and 3D watershed [80], while the MITK plugin *3D-Cell-Annotator* [43] uses active contours for semi-automatic 3D segmentation. In contrast, most recent methods apply deep learning techniques to segment nuclei. These include *QCANet [81]*, developed to analyze mouse embryos in 3D, *3DeeCellTracker* [82], intended for tracking after the segmentation of nucleus instances, and the algorithm proposed in Lapierre-Landry *et al.* [83] which performs watershed segmentation on the probability map, and supervoxel clustering to achieve the final instance segmentation.

Self-supervised and unsupervised learning approaches decrease or even eliminate the need of annotated training data. A few of such methods for nuclear segmentation have appeared recently [84,85][86]. These methods show competitive results, though their accuracy does not exceed that of the supervised state-of-the-art methods. Self-supervised segmentation for histopathology images [85] uses *ResUnet-101* and requires a minimum of annotated data for fine-tuning. Another approach [84] uses an attention mechanism, and does not require annotated data. *AD-GAN* [86]uses a sophisticated training approach based on GAN, does not require annotated data and also works for both 2D and 3D.

Table 2 and **Suppl. Table 3** report the list of tools mentioned above, whilst **Suppl. Mat. 4** includes their short descriptions.

Table 2. Relevant tools for nucleus segmentation

Algorithm: A complete method to segment nuclei. An algorithm can be shared as a source code for developers in e.g. a GitHub repository or can be implemented as a user-accessible method in a platform. *Pipeline*: A workflow of image processing algorithms to segment nuclei, allowing the user to set parameters for each step of the workflow or even change the included algorithms to optimize segmentation tailored to the specific data. *Platform*: A software package that includes multiple algorithms or pipelines for nucleus segmentation, and often has a defined API to include additional methods as well.

2D/3D/Both	Tool name	Pipeline/algorithm/platform	Code availability	Year	Reference	GUI/Tutorial/Biaflows/GPU/Cloud
2D	U-Net	Algorithm	Yes	2015	Ronneberger et al.[34]	N/N/Y/Y/N
2D	SegNet	Algorithm	Yes	2015	Badrinarayanan et al. [87]	N/N/?/Y/N
2D	Mask R-CNN	Algorithm	Yes	2017	He et al.[57]	N/Y/Y/Y/N
2D	QuPath	Platform	Yes	2017	Bankhead et al.[47]	Y/Y/N/Y/N
2D	UNet++	Algorithm	Yes	2018	Zhou et al.[56]	N/N/N/Y/N
2D	Segmentation of Nuclei in Histopathology Images by deep regression of the distance map	Algorithm	Yes	2018	Naylor et al.[88]	N/Y/N/Y/N

2D	Multi-scale Cell Instance Segmentation with Keypoint Graph based Bounding Boxes	Algorithm	Yes	2019	Yi et al. [89]	N/N/?/Y/N
2D	HoVer-Net	Algorithm	Yes	2019	Graham et al.[23]	N/Y/N/Y/N
2D	CIA-Net	Algorithm	No	2019	Zhou et al.[90]	N/N/N/Y/N
2D	Bend-Net	Algorithm	No	2020	Wang et al.[91]	N/N/N/Y/N
2D	nucleAIzer	Algorithm, Pipeline	Yes	2020	Hollandi et al.[11]	Y/Y/N/Y/Y
2D	MultiStar	Algorithm	Yes	2020	Walter et al.[59]	N/N/N/Y/N
2D	Instance-Aware Self-supervised Learning for Nuclei Segmentation	Algorithm	No	2020	Xie et al. [85]	N/N/N/Y/N
2D	Self-supervised Nuclei Segmentation in Histopathological Images Using Attention	Algorithm	Yes	2020	Sahasrabudhe et al. [84,85]	N/N/N/Y/N
2D	Triple U-Net	Algorithm	Yes	2020	Zhao et al.[92]	N/N/N/Y/N

2D	"High-resolution deep transferred ASPPU-Net for nuclei segmentation of histopathology images"	Algorithm	No	2021	Chanchal et al.[93]	N/N/N/Y/N
2D	NucleiSegNet	Algorithm	Yes	2021	Lal et al.[94]	N/Y/N/Y/N
2D	SplineDist	Algorithm	Yes	2021	Mandal et al.[60]	N/N/N/Y/N
2D	Contour Proposal Network	Algorithm	Yes	2021	Upschulte et al.[95]	N/N/N/Y/N
2D	HistomicsML2	Pipeline, Platform	Yes	2021	Lee et al.[69]	Y/Y/N/Y/Y
2D	STORM	Pipeline	Yes	2021	Mela et al.[66]	N/N/N/Y/Y
2D	MSRF-Net	Algorithm	Yes	2021	Srivastava et al. [96]	N/N/N/Y/N
3D	"3D cell nuclei segmentation based on gradient flow tracking"	Algorithm	No	2007	Li et al.[97]	N/N/N/N/N
3D	Vaa3D	Platform	Yes	2010	Peng et al.[79]	Y/Y/Y/Y/N
3D	IT3DImageJSuite	Platform	Yes	2013	Ollion et al.[98]	Y/Y/N/N/N
3D	LoS	Algorithm	Yes	2013	Asafi et al.[74]	N/Y/N/N/N

3D	"Automated cell segmentation with 3D fluorescence microscopy images"	Algorithm	No	2015	Kong et al.[99]	N/N/N/N/N
3D	OpenSegSPIM	Platform	Yes	2016	Gole et al.[75]	Y/Y/N/N/N
3D	RACE	Platform	Yes	2016	Stegmaier et al.[76]	Y/Y/N/Y/N
3D	U-Net (3D)	Algorithm	Yes	2016	Cicek et al.[100]	N/N/N/Y/N
3D	"Segmentation of fluorescence microscopy images using three dimensional active contours with inhomogeneity correction"	Algorithm	No	2017	Lee et al.[14]	N/N/N/N/N
3D	DeepSynth	Algorithm	No	2019	Dunn et al.[101]	N/N/N/Y/N
3D	"Three-Dimensional Segmentation and Reconstruction of Neuronal Nuclei in Confocal Microscopic Images"	Algorithm	Yes	2019	Ruszczycki et al.[15]	N/N/N/N/N

3D	"Semi supervised segmentation and graph-based tracking of 3D nuclei in time-lapse microscopy"	Algorithm	Yes	2020	Shailja et al.[102]	N/N/N/Y/N
3D	"A deep learning pipeline for nucleus segmentation"	Pipeline	No	2020	Zaki et al.[103]	N/N/N/Y/N
3D	"Combined detection and segmentation of cell nuclei in microscopy images using deep learning"	Algorithm	No	2020	Ram et al.[104]	N/N/N/Y/N
3D	QCANet	Algorithm	Yes	2020	Tokuoka et al.[81]	N/Y/N/Y/N
3D	Allen Cell and Structure Segmenter	Platform	Yes	2020	Chen et al.[105]	Y/Y/N/Y/N
3D	3D-Cell-Annotator	Platform	Yes	2020	Tasnadi et al.[43]	Y/Y/N/Y/N
3D	"Nuclei detection for 3D microscopy with a fully convolutional regression network"	Algorithm	No	2021	Lapierre-Landry et al.[83]	N/N/N/Y/N
3D	3DeeCellTracker	Platform	Yes	2021	Wen et al.[82]	N/Y/N/Y/N

Both	MINS	Platform	Yes	2014	Lou et al.[62,63]	Y/Y/N/N/N
Both	XPIWIT	Algorithm	Yes	2016	Bartschat et al.[64]	Y/Y/N/Y/N
Both	ilastik	Platform	Yes	2018	Berg et al.[42]	Y/Y/Y/Y/N
Both	DeepImageJ	Platform	Yes	2019	Gómez-de-Mariscal et al.[71]	Y/Y/N/Y/N
Both	ImJoy	Platform	Yes	2019	Ouyang et al.[67]	Y/Y/N/Y/Y
Both	"A coarse-to-fine data generation method for 2D and 3D cell nucleus segmentation"	Algorithm	No	2020	Zhao et al.[106]	N/N/N/Y/N
Both	Cellpose	Algorithm	Yes	2020	Stringer et al.[12]	Y/Y/Y/Y/Y
Both	CDeep3M	Platform	Yes	2020	Haberl et al.[70]	Y/Y/N/Y/Y
Both	StarDist	Algorithm	Yes	2020	Shmidt et al.[13] ; Weigert et al.[54]	N/Y/Y/Y/N
Both	NuSeT	Platform	Yes	2020	Yang et al.[61]	Y/Y/N/Y/N
Both	nnU-Net	Platform	Yes	2021	Isensee et al.[55]	N/Y/N/Y/N
Both	DeepMIB	Platform	Yes	2021	Belevich et al.[53]	Y/Y/N/Y/N
Both	InstantDL	Pipeline, Platform	Yes	2021	Waibel et al.[107]	N/Y/N/Y/N

Both	ZeroCostDL4Mic	Pipeline, Platform	Yes	2021	von Chamier et al.[65]	Y/Y/N/Y/Y
Both	DeepCell Kiosk	Pipeline, Platform	Yes	2021; 2016	Bannon et al. [46,68]]; Van Valen et al.[46,68]	Y/Y/Y/Y/Y
Both	AD-GAN	Algorithm	No	2021	Yao et al. [86]	N/N/N/Y/N
Both	Embedding-based Instance Segmentation in Microscopy	Algorithm	Yes	2021	Lalit et al. [108]	N/Y/?/Y/N

Most of the listed tools require some effort from the user to install, prepare the environment, do the pre-processing of the input if needed, and finally to run it. The amount of time and effort primarily depends on the computational background of the user, and on the tool itself. Cloud-based tools (usually supplied with web GUI) could be the primary starter choices for life scientists. However, there is a trade-off: cloud-based versions of tools have limited customizability, while local versions are more flexible, and the user does not need to share the data with third-party services. In the latter case the quality of the documentation also matters to assure proper setup. In **Table 2** we provide information on whether the tool is documented properly (only official documentation was taken into account). The algorithms quite often lack detailed official documentation, though provide the most flexibility (usually are parts of the complex pipelines), and for the most popular ones unofficial documentation or

tutorials and third-party implementations exist too. The potential performance of a tool is obviously an important concern for the user, and it might be challenging to decide on choosing the appropriate tool. The user may decide based on the community's preferences. Alternatively, a reliable comparison of the performance of the different tools can support decision-making. However, apart from BIAFLOWS[II] and automatic challenge submission systems (e.g. Kaggle or ISBI[VI]), the microscopy image analyst community lacks (*1*) an evaluation platform for the objective comparison of nucleus segmentation methods, using (*2*) a standardized evaluation metric in a transparent way. Thus, a consensus on utilizing a single, standardized platform is eagerly awaited. Since challenge portals only provide this functionality for the datasets of given challenges, a more inclusive platform, such as BIAFLOWS is suggested. Even though the relevance of newly published methods is usually supported by some quantitative segmentation results, it has several shortcomings from the user's point of view as follows. (*1*) The test dataset might not suggest relevant performance when the dataset size is too small, or covers a single imaging modality only. On the other hand, approaches developed for specific microscopy images (such as H&E or fluorescence confocal images) or segmentation scenarios (e.g. crowded cell culture) are intended to work in their given domain of images, and should not be expected to perform just as well on more extensive or general datasets. (*2*) When comparison to prior methods is performed and reported, the number of tested methods is usually low, and (*3*) additional model- or data-specific modifications might have been applied to the compared methods (or the test images as pre-processing), thus merely literature-based comparisons of accuracy scores may confuse the user (see **Suppl. Mat. 2**).

CONCLUDING REMARKS

Recent years have brought significant improvements in nucleus segmentation, including large annotated datasets, new high-accuracy 2D/3D strategies, deep learning approaches, and segmentation benchmarking platforms, however, establishing a genuinely general solution for nucleus segmentation is still an unmet need. In this review and the accompanying web-based portal[l] we aimed to cover the missing link between recent advancements and users' needs by providing a detailed overview on the available means for nucleus segmentation. The concluding remarks below are focused on crucial limitations and future goals.

The ***first crucial point*** is to cover more modalities of microscopy data for both 2D, and especially 3D, with open datasets of annotated images. Current methods are expected to work when trained on additional microscopy data modalities [18,109]. Most datasets include H&E stained tissues or fluorescently labelled cell cultures (see **Table 1**) which are two of the most widely used modalities in practice. However, further microscopy types (e.g. DIC, light-sheet or phase contrast) lack such publicly available annotations, except a recently published, large, label-free dataset [26]. Even though researchers can train existing deep learning methods on their own nowadays, these models remain private (unless released on e.g. GitHub, zenodo, Kaggle or in a Napari [110] plugin; on the first three platforms datasets may also be deposited [32]) and the initial datasets are small, resulting in suboptimal model generalization. For a given modality of interest, generalization is also a crucial point for medical applications: the data should be as diverse as possible to promote robust models. Diversity from a computer vision point of view would include various regions

of tissue with the distinct visual appearance of both the target objects and the surroundings, as well as covering several phenotypes of cells, different batches or slightly different experimental setups. An extensive annotated dataset including most (if not all) modalities occuring in single-cell analysis experiments with respect to the type of microscopy, sample and label could definitely improve existing trainable methods. Besides, it would offer the possibility of releasing genuinely general pre-trained models, and would also serve as a standard dataset, similarly to the widely used COCO dataset [111] in computer vision.

The **second crucial point** relates to solving common microscopy challenges for both 2D and 3D data, such as: touching, overlapping and irregularly shaped nuclei [54][59][60][61]. Either dataset design or model architecture can be beneficial for a solution. Current methods achieve various levels of success in overcoming these issues, thus further developments are needed.

The **third crucial point** is the lack of (*1*) a globally accepted benchmark platform for comparison, and (*2*) a unified metric for tool evaluation. BIAFLOWS and Kaggle are available solutions to overcome these issues. However, still most publications presenting novel methods or tools typically provide limited comparisons (either in terms of the data used in evaluation or the number of methods compared) and use not standardized metrics. Accordingly, the results published by different authors are often difficult to compare.

The **ultimate goal** is to develop an algorithm, and train it so that the resulting single model would be able to accurately segment nuclei in a variety of microscopy

modalities. Some of the available algorithms and models are aimed to meet this requirement [11,12], and the field is moving towards a generally applicable solution. While a quantitative comparison of the methods available for each modality is beyond our intention, it is worth mentioning that deep learning tends to provide fine accuracy in segmenting nuclei in images obtained with different microscopy techniques, as shown at the DSB 2018 challenge [6][112].

ACKNOWLEDGEMENTS

RESOURCES

    I.   https://biomag-lab.github.io/microscopy-tree/

    II.   https://biaflows.neubias.org

    III.   imagej.net/plugins/labkit

    IV.   http://cancergenome.nih.gov/

    V.   http://www.andrewjanowczyk.com/deep-learning/

    VI.   https://biomedicalimaging.org/

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FIGURE CAPTIONS

Figure 1. Diversity of optical microscopy images representing nuclei. The inner circle shows standard examples of each type (e.g. widefield, confocal, light-sheet, DIC, PC images), while the outer circle presents more difficult cases. Finally, common challenging cases (e.g. multinucleated cells, irregular morphology, elongated shape, heterogeneous samples) regarding nucleus segmentation are reported in the corners. **c**,**d**, **l**, **f**, **g**, **o, t** are images from our laboratories/collaborators; **a**, **s, j** are from BBBC collection; **e**, **n** are from the TCGA collection; **r** is from the LIVECell dataset; the remaining ones are from the internet (see **Supplementary Table 1** for the sources).

Figure 2. A sample-driven guide to select an appropriate method for single nucleus segmentation. Firstly, based on the images of the given experiment the user can determine the category (e.g. widefield, confocal, light-sheet, DIC, PC images) and select the corresponding node in the interactive online tool *unbias^{I}* according to the sample, label and microscopy type. Then, a list of segmentation methods is shown in the table on the right, including the method description and implementation if available alongside pre-trained models. The list may be filtered with the buttons above the table by dimension (2D/3D) and challenging segmentation issues (e.g. elongated nucleus in smooth muscle tissue). Finally, the goal of the experiment (e.g. object-aware segmentation or additional phenotyping i.e. classification) guides the user to select the appropriate segmentation method.

TITLE:

Nucleus segmentation: towards automated solutions

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OUTSTANDING QUESTIONS BOX:

- Can existing tools reliably segment touching/overlapping/cluttered or morphologically challenging nuclei? Do training datasets include such examples of nuclei to enable learning of the visual representation?

- How to make general-purpose models that work accurately for many different imaging modalities?

- How to define a standardized approach to quantitatively compare the existing solutions, and properly assess new methods to overcome current comparison challenges?

- What are the future steps developers should take towards a general solution for nucleus segmentation?

Supplemental Information

Nucleus segmentation: towards automated solutions

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SUPPLEMENTARY MATERIAL 1

Optional pre-processing steps:

1) Each segmentation method requires its own input format (e.g. file types, annotations, image size, etc.). To match these requirements, a basic, but practical suggestion is to try analysing sample datasets (if provided) and perform ***input pre-processing*** prior to the usage of the method. Besides the images themselves, the annotation formats required by different methods are also not uniform: different file formats (TIFF or PNG, in most cases), all objects in the same file or one annotation file per object, the pixel values. These should also be considered prior to using the given method.

2) Uneven illumination affects every image acquired by a microscope. It is often overlooked, but it can introduce considerable bias to image measurements. ***Illumination correction*** methods are applied to solve this problem. One of the most common approaches is known as the "rolling ball" algorithm [1] which is implemented in many image processing platforms. More advanced methods (*BaSiC* [2] and *CIDRE* [3]) aim to model the illumination pattern for correction.

3) ***Dataset augmentation*** is used to increase both the number and diversity of the training examples to train more robust models. Conventionally, modified

(rotated, noised, cropped, rescaled) versions of training images are added to the dataset. Another approach to dataset augmentation is to use **image style transfer** models (for instance CycleGAN-based framework *Pix2Pix* [4]) which allows generating additional images in two ways: (*1*) the input image is style-transferred to simulate either a different tissue, cell line or microscopy modality, and (*2*) another approach is to firstly simulate annotations creating representative labels, then use these labels as the input to the style transfer model to get reconstructed examples of nuclei in the desired image modality [5]. The disadvantage of this is that separate training is required making it more time-consuming and less user-friendly.

4) In certain circumstances or experimental setups, all or several images might be acquired with undesirable noise. In this case, **denoising** approaches can be applied as a preprocessing step to improve the image **SNR**. For this purpose, classical image processing methods such as median filter [6] or gaussian filter [6][7] are widely used. Also, recently, deep learning-based methods appeared for this task. *Content-Aware Image Restoration* (*CARE*) [8] is a deep learning-based method to improve the SNR. The models are specific to experimental setups and trained with pairs of low and high SNR, thus it requires ground truth data and is not suitable to use for imaging of novel biological structures. *Noise2Void* [9], uses only noisy images and is, therefore, more suitable in case of the absence of denoised data. For a more complete review of denoising approaches, we point the reader to the following recent review [10].

5) The fluorescent labelling process is expensive, time-consuming and may have undesirable effects on the samples, thus fluorescent stains cannot be used in all experiments. However, segmentation of nuclei from transmitted light images

is more challenging than from fluorescent microscopy images. One possible solution to this is **in-silico labelling** [11][12][13]: prediction of fluorescent stains (such as Hoechst for nuclear staining) from transmitted light images.

6) To handle very high-resolution large size microscopy images, one possible solution is to split (**tile or crop**) the image into smaller ones and use these for training or prediction. After prediction of the tiles they can be merged back to one mosaic at the original image size [14].

Optional post-processing steps:

1) Prediction **mask refinement** either with classical methods (watershed, thresholding) or with a deep learning model specifically trained to refine segmentation masks provided by other methods in the pipeline.

2) **Test-time augmentation (TTA),** to make predictions using the transformed input images (i.e. rotated, flipped) and then merge the predictions of all versions of the input image with consensus voting. In case of *U-Net* simple averaging and thresholding are generally used, while in the case of *Mask R-CNN* majority voting is typically approached to infer the object and averaged masks for the final objects. The solution was tested [15] on the DSB2018 dataset and showed minor improvement of the prediction scores, but another application of test-time augmentation is to observe and analyze uncertainty in predictions.

SUPPLEMENTARY MATERIAL 2

The diversity among the different nucleus segmentation tools and the lack of an uniform strategy of evaluation make the performance determination and the quantitative assessment of new methods very challenging. Most of the papers describing a new method also propose some limited assessments, but the inconsistent use of segmentation accuracy metrics makes it nearly impossible to merge the results of different papers and define *a priori* which method is the best for a specific dataset of images. For example, in the *Plantseg* original paper [16] the variation of information metric is used, for the original *U-Net* plus watershed paper [17] the aggregated Jaccard metric, whereas *Cellpose [18]* uses an average precision metric. Also, besides the conventional Jaccard Index, the Aggregated Jaccard Index metric (AJI) is used in papers like [19] and [20], in which the AJI not only estimates the overlap of the true positives but also accounts for false positives and false negatives present in the segmentations. *StarDist [21]* reported scores on 10% of a custom fluorescent subset of the DSB2018 dataset [22], trained on the remaining part of this subset. *EmbedSeg [23]* used the same subset for their evaluation. Thus, comparing both their mean average precision (mAP) scores to other methods evaluated on the entire DSB test set (also comprising of histopathology images) would be irrelevant. Accordingly, these two specific methods however, can be fairly compared. Additionally, the authors [21][23] presented accuracy within the IoU thresholds 0.5 to 0.9 in contrast to the DSB2018 competition [22] where 0.5 to 0.95 was used. Notably, mAP reported on the DSB2018 dataset includes the number of false negative objects, while traditionally precision does not. In [24] listed 20 deep learning approaches for histopathological image segmentation reporting for each of them the metrics used in the original paper to assess the proposed method. The first game-changing attempt to propose a

systematic comparison of nucleus segmentation architectures on complex stained images was proposed in [22]. This work reported the results of the 2018 Data Science Bowl, which challenged participants to segment nuclei in a variety of stained 2D light microscopy images without the need for any manual interaction or adjustment. The goal was to investigate whether any modern solutions, such as large capacity deep-learning models, could provide a single unifying solution without requiring manual configuration. The evaluation strategy was on the basis of identifying object-level errors. This was accomplished by matching target object masks with predicted objects submitted by participants and then computing true positives and false positives. In order to match target masks and predicted objects, the Intersection-over-Union (IoU) score was computed for all pairs of objects using IoU. A minimum IoU threshold was selected to identify correctly segmented objects and any other predicted segmentation mask below the threshold was considered an error. With all true positives, false positives, true negatives and false negatives, they created a confusion matrix and computed precision, recall and F1 scores using a fixed IoU threshold. Finally, the official competition score S was defined using multiple IoU thresholds. However, this work considered just 2D images, while in [25] quantitatively compared the segmentation performance of 9 segmentation tools using two publicly available 3D nuclear stained datasets related to a cancer spheroid and a mouse embryo, imaged with a light-sheet fluorescence microscope (LSFM) and a confocal microscope, respectively. The tools tested were randomly assigned to 5 expert computer scientists working with microscopy images on a daily basis who set different parameters of the tools to achieve the best segmentation they could. Performance was evaluated simply using the Jaccard Index value obtained between the segmentation masks and the ground truth. A milestone was achieved in the same year in the paper [26] that

introduced *BIAFLOWS*, a community-driven, open-source web tool enabling to reproducibly deploy and benchmark bioimage analysis workflows on annotated multidimensional microscopy data. It is worth noting that *BIAFLOWS* extends *Cytomine [27]*, a web platform originally developed for the collaborative annotation of high-resolution bright-field bioimages and also shows qualitative (visualized) comparison of segmentation results on example datasets (e.g. the heterogeneous DSB2018 dataset) achieved by some of the most commonly used methods, hence users can better understand the differences between method results including segmentation errors and challenging cases. Today, 6 methods have been compared using *BIAFLOWS* on the DSB2018 dataset, but since training a new model is not available within *BIAFLOWS*, pre-trained models with different characteristics provided by the authors of original methods have been compared. Notably, these models are not trained on a standardized dataset, therefore performance comparison on a public dataset might yield unexpected results. Recently, [28] evaluated and compared the segmentation effectiveness of 5 deep learning architectures (*U-Net*, *U-Net ResNet*, *Cellpose*, *Mask R-CNN*, *KG instance segmentation*) and two conventional algorithms (*Iterative h-min based watershed*, *Attributed relational graphs*) on complex fluorescence nuclear images of various types. Using a publicly available dataset of images from specimens of different diagnosis, they showed that instance-aware segmentation architectures and Cellpose outperform the U-Net architectures and conventional methods on complex images in terms of F1 scores, while the U-Net architectures achieve overall higher mean Dice scores. In addition, they provided quantitative results demonstrating that images annotated by under-graduates (the so called "silver images") are sufficient for training instance-aware segmentation architectures to efficiently segment complex fluorescence nuclear images. Finally, [29]

implemented and quantitatively compared 5 representative deep learning pipelines, alongside a highly efficient non-deep learning method named *MARS [30]*. The deep learning methods were trained on a common dataset of 3D cellular confocal microscopy images. An extensive, publicly available annotated confocal image dataset and a new segmentation quality evaluation which isolates segmentation errors due to under/over segmentation, were adopted. First, they analyzed the quality of segmentation on overall stacks, including all cell layers. Next, the confocal stack was split into different cellular layers and the segmentation quality of the 5 pipelines for each layer was studied. Finally, they evaluated the segmentation quality on synthetic images with commonly occuring aberrations. For quantitative assessment, a Volume averaged Jaccard Index (VJI) metric measuring the degree of overlap averaged over the cell volume was used to estimate overlap between the predicted segmentations and ground-truths, and two additional metrics that identify the rates of over and under segmentation were applied. The rate of over-segmentation is the percentage of cells in the ground truth associated with multiple regions in the predicted segmentation. Conversely the rate of under-segmentation is the percentage of cases where several regions in the groundtruth are associated with a single cell in the predicted segmentation. Concluding, despite today comparing heterogeneous image analysis methods is still tedious and error prone, the different approaches recently described and *BIAFLOWS* help the developers of new methods fairly and quantitatively compare their approach with the state-of-the-art ones without the need of proposing new assessment and comparison strategies.

SUPPLEMENTARY MATERIAL 3

This supplementary material contains short descriptions of the annotation tools.

1. ***AnnotatorJ*** [31]: The plugin has various annotation and export options, integrated deep learning (*U-Net*) as well as classical algorithm (e.g. region growing) assistance, classification option and z-stack annotation support. It allows contour edit, instance and semantic object annotation, bounding boxes and custom Keras model import. It is open-source and cross-platform (as *ImageJ/Fiji* [32,33]).

2. ***Trainable Weka Segmentation*** [34]: Applies machine learning to train pixel classification from drawn labels (similarly to *ilastik*), using the Weka framework. It does not support deep learning or further annotation types.

3. ***NuClick*** [35]: Uses its own CNN to assist in annotation of histopathology H&E stained images (also showed the ability to work on IHC and Pap Smear modalities). The user only needs to click on the nuclei to annotate them.

4. ***LabKit***: Suggested as an annotation tool by the authors of *StarDist [21]*, *Labkit* allows labelling and pixel classifier training similar to *Trainable Weka Segmentation* and *ilastik*, alongside 3D object annotation.

5. ***3D-Cell-Annotator*** [36]: It is a tool integrated into the segmentation plugin of the *Medical Imaging Interaction Toolkit* (*MITK*) version 2018.04. It is based on 3D active contours with shape descriptors. It works in a semiautomatic fashion requiring an initial seed point for each object to start contour evolution. Finally, a label is associated with each object.

6. ***Cytomine*** [27][37]: A bioimage analysis software with a wide range of general image processing functionality that also offers several annotation options, has an online version and offers deep learning support. It can process WSIs online, operates in a collaborative way of iterating the annotations: Cytomine puts emphasis on the expert curation (e.g. multiple pathologists can collaborate on the same WSI).

7. ***ilastik*** [38]: A software using user-defined labels to train pixel classification (with machine learning, similarly to the *Trainable Weka Segmentation* plugin). See also the "*General frameworks for 2D images*" section.

8. ***Segmentor*** [39]: An open-source tool for 3D annotation of light-sheet microscopy images, developed by Borland *et al.* The tool is cross-platform (e.g. MacOS, Windows, Linux). Segmentor allows loading annotations from other tools and has built-in intensity thresholding but does not have built-in deep learning capabilities.

9. ***Diffgram***: Aids annotation with deep learning models (Tensorflow-based) and image processing algorithms (e.g. *GrabCut*). It has an online and local (*Python*) version, as well as a paid plan.

10. ***LabelImg***: It is a lightweight object annotator in *Python* via bounding boxes with export to popular data formats (*Pascal VOC*, *YOLO*).

11. ***Make Sense***: Offers annotation assistance via pre-trained deep learning models (*SSD* on *COCO*, and *PoseNet*), has an online and a local (TypeScript) version.

12. ***VGG Image Annotator***: A lightweight, online tool with multiple annotation options.

13. ***Kaibu***: An online application also realized as an *ImJoy* [40] plugin and invokable in a Jupyter notebook [41], with multiple drawing options, labelling and export to a json file.

14. ***Supervise.ly***: A web-based annotation tool with deep learning model support (e.g. *YOLO*, *U-Net*, *Mask R-CNN*) including training, has multiple annotation options and is free for research.

15. ***Lionbridge.AI*** and ***Hive***: Two service-based tools (project management) with wide functionality and paid plans, outsourcing the annotation task, intended for general purpose object annotation (such as computer vision applications) on natural photos. They are available at https://lionbridge.ai/services/image-annotation/ and https://thehive.ai/, respectively.

16. **DeepCell Label**: Part of the *DeepCell [42]*, it allows both to create annotations from scratch or to correct the predictions of the segmentation model. When used in collaboration (or crowdsourcing), annotations serve to improve the segmentation DNN of DeepCell. The application is web-based, it can be deployed either locally or on the cloud service.

17. **QuPath** [43]: A local open software with workflow-based processing options intended for whole slide image (WSI) analysis. Annotation is possible in terms of drawing ROIs. See also the "*General frameworks for 2D images*" section.

SUPPLEMENTARY MATERIAL 4

In this section, we present short descriptions of the tools/methods to segment single nuclei.

Not deep learning-based tools working on 2D images:

1. **MINS** [44,45]: *MINS* works on fluorescent 2D and 3D images. It was designed for segmenting single cells in embryos, but it can be easily used for other multicellular models as well. It is implemented into three subsequent modules: detection, segmentation, and cell position classification. Tutorials and an easy-to-use GUI leads the user through the modules. *MINS* currently only works on local machines.

2. **XPIWIT** [46]: It is an XML-based wrapper application for the *Insight Toolkit* (*ITK*) containing several segmentation algorithms. One of the most promising algorithms for nucleus segmentation included in *XPIWIT* is the *Threshold of Weighted intensity And seed-Normal Gradient* dot product image (*TWANG*) segmentation [47]. The authors provided a GUI and tutorials, but programming experience is required to effectively use *XPIWIT*. Currently, *XPIWIT* does not work in the cloud.

Deep learning-based tools working on 2D images:

1. **Cellpose** [18]: It is a generalist segmentation method based on *U-Net* for predicting gradients (horizontal and vertical separately) that form a vector field used to define individual objects. It is mainly designed for cells but includes a pre-trained nucleus segmentation model as well. It does not work with RGB data such as H&E stained images (automates or needs information of which channel is used for segmentation). It also includes an annotation tool. It has a GUI or can be run from the command line. Predictions can also be performed in the cloud. An extensive user guide is provided.

2. **EmbedSeg** [23]: It is an embedding-based instance segmentation method designed for microscopy images. The claimed key features are the ability to accurately segment different shapes and low GPU-memory footprint, compared

to other methods (U-Net, Mask R-CNN, Cellpose). Code examples and detailed introduction on the GitHub page are provided. It does not have its own GUI.

3. ***Mask R-CNN*** [48]: It is an instance segmentation method originally for natural images, nevertheless, it can also be trained to segment nuclei. Besides segmentation, it also provides a classification of the segmented objects. As it is a general segmentation method, there is no "official" guide for nucleus segmentation, but guides can be found elsewhere. Code examples are provided. It does not have its own GUI.

4. ***MSRF-Net*** [49]: This method is designed for medical segmentation tasks, including segmentation of microscopy images. In the described tests this method significantly outperforms baseline methods U-Net and U-Net++ on the DSB2018 dataset. The instructions on the GitHUb page are not well detailed. The method does not have its own GUI.

5. ***Multi-scale Cell Instance Segmentation with Keypoint Graph based Bounding Boxes*** [50]: This method is primarily designed for nuclei/cell segmentation. This method firstly makes detections (bounding-boxes) by detecting and then grouping of keypoints. Segmentation is then performed inside detected bounding boxes. In the described tests, this method performed significantly better than the baseline *Mask R-CNN*. Code examples are provided on the GitHub page, though there is no detailed tutorial. The method does not have its own GUI.

6. ***nnU-Net*** [51]: It is mainly based on *U-Net*, the key feature of this pipeline is an automated adaptation strategy for different datasets based on heuristics rules and the method learns other parameters directly from the dataset. No GUI or cloud execution is provided. Has an extensive user-guide.

7. ***nucleAIzer*** [5]: A pipeline mainly based on *Mask R-CNN*. As a first step, the pipeline performs resizing of the images to make the mean nucleus size uniform. To adapt the model to different types of cells and stainings, style transfer is used for dataset augmentation. After a *Mask R-CNN*-based segmentation *U-Net* is used to refine the output masks. The pre-trained model works on all kinds of images from fluorescence microscopy to H&E stained images. Predictions can be performed in the cloud, a user tutorial is provided on the project's site.

8. **SegNet** [52]: This segmentation is based on encoder-decoder architecture, the encoder part is based on VGG16 [53]. Can be used as a baseline for semantic segmentation tasks. This is a bare neural network architecture, so no GUI or cloud execution is provided out of the box. Guides can be found online.

9. **StarDist** [21]: The name *StarDist* is related to the core idea of the approach: star-convex polygons. The implementation is based on a modified *U-Net* with two output layers, one for object probability for each pixel and a polygon distance output layer. Includes pre-trained models for fluorescence nuclei and brightfield H&E staining. The method does not have a built-in GUI, but code examples are provided in the form of Jupyter Notebooks.

10. **U-Net** [54]: It is based on the encoder-decoder architecture, and is the winner of the ISBI 2015 cell tracking challenge [55]. Provides good baselines for semantic segmentation tasks. This is a bare neural network architecture, so no GUI or cloud execution is provided out of the box. Guides can be found online.

11. **UNet++** [56]: It is a *U-Net* with redesigned skip connections to exploit multiscale features in image segmentation and a pruning scheme to speed up inference (with a minor negative impact on accuracy). This is a neural network architecture, no GUI or cloud execution is provided out of the box. Guides can be found online.

12. **Contour Proposal Networks for Biomedical Instance Segmentation** [57]: It is a method based on the idea of fixed-sized representation based on Fourier Descriptors and uses object detection networks as a backbone. This is a neural network architecture, no GUI or cloud execution is provided out of the box. No user guide is provided either.

13. **MultiStar: Instance Segmentation Of Overlapping Objects With Star-convex Polygons** [58]: In essence, it is very similar to *StarDist*, for better handling of overlapping objects an additional output branch was added for overlap predictions. This is a neural network architecture, no GUI or cloud execution is provided out of the box. Example code is provided, but there is no user guide.

General frameworks for 2D images:

1. **CDeep3M** [59]: It is a semantic segmentation tool based on a convolutional neural network (CNN) architecture for image segmentation. It works as an online platform offering a place for web tools. The GUI is provided, predictions can also be done in the cloud. Extensive user guides with video tutorials are provided.

2. **DeepCell Kiosk** [42],[60]: It is a cloud-native implementation of the *DeepCell* ecosystem. It is designed to allow researchers to easily deploy and scale a deep learning platform for biological image analysis. Once launched, users can drag-and-drop images to be processed in parallel using publicly available or custom-built TensorFlow models. This method is also used for the whole-cell segmentation of tissue images [61]. The web-based GUI can be used out of the box. An extensive user guide on installation and usage is provided.

3. **DeepImageJ** [62]: It is an *ImageJ* framework and a deep learning model repository. It provides trained models for different tasks and it contains *U-Net* 2D/3D, *Mask R-CNN*, *StarDist*. The GUI is provided out of the box. The site of the project has extensive text and video tutorials.

4. **DeepMIB** [63]: It is a user-friendly (has a GUI and extensive tutorials) software package written in *MATLAB*, suitable for different types of microscopy images (2D and 3D electron and multicolor light microscopy). Deep learning architectures are available (and pretrained): 2D&3D *U-Net*, 3D *U-Net* for anisotropic datasets and 2D *SegNet [52]*.

5. **Ilastik** [38]: It is a user-friendly (has a GUI and extensive tutorials) interactive tool for a variety of microscopy image analysis tasks, including segmentation. The underlying segmentation method is similar to the *Weka Segmentation [34]*.

6. **ImJoy** [40]: It is a serverless web application that can be used locally, either a local machine or remote servers can be used for computation. The segmentation plugin of ImJoy is based on *U-Net*. The GUI is web-based. The user guides are provided.

7. **InstantDL** [64]: It is a framework that solves a wide range of tasks, 2D and 3D semantic, 2D instance segmentation and 2D&3D in-silico staining. It was designed to be user-friendly, the user only needs to specify the inputs (more parameters and modifications are available for advanced users). Semantic segmentation and in-silico staining are performed by *U-Net*, instance

segmentation is performed by *Mask R-CNN*. It does not have a GUI, though the documentation is extensive and supported by video tutorials.

8. **ZeroCostDL4Mic** [65]: A framework designed to allow the usage of segmentation (based on *U-Net* and *StarDist*) without any coding or even hardware for deep learning, as it was developed mainly for Google Colab (cloud execution) and does not have a separate GUI. Besides 2D&3D segmentation, the framework provides object detection (based on *YOLOv2* [66]), denoising for 2D&3D data (based on *CARE* and *Noise2Void*). The framework is well-documented.

9. **QuPath** *[43]*: Designed primarily for whole slide image (WSI) analysis, it provides options for initial intensity scaling based on staining channels even in H&E with colour deconvolution, automatic cell segmentation based on classical image processing algorithms to find nuclei, classification with machine learning methods and various tests for statistical analysis. Its open source implementation is written in Java and utilizes ImageJ's image processing capabilities, has a user-friendly GUI and documentation. It is possible to utilize a *StarDist* segmentation extension in *QuPath* with possible GPU acceleration.

Purpose-specific frameworks for 2D images:

1. **HistomicsML2** [67]: It runs as a containerized server application that provides web-based user interfaces for analysing whole-slide imaging data. It can be deployed on GPU servers or cloud platforms. The project wiki provides the detailed documentation.

2. **NuSeT** [68]: A deep learning tool for reliably separating and analyzing crowded cells. Combines *U-Net*, *Region Proposal Network [69]*, and watershed to segment nuclei. Focuses especially on touching or overlapping nuclei. This framework can handle 2D/3D data and pre-trained models are available. The tool has its own GUI, the documentation is on the project's GitHub page.

3. **Segmentation of stochastic optical reconstruction microscopy (STORM) images** [70]: Authors compared *U-Net*, *Mask R-CNN* and *ANCIS* [71] (Attentive neural cell instance segmentation) architectures for super-resolution

microscopy and provided Google Colab (Jupyter [41]) notebooks (to run in cloud) and for local machines.

4. ***HoVer-Net*** [72]: performs classification and segmentation of 2D H&E stained histology images. Pretrained models for different histology datasets are provided on the project GitHub page, together with the instructions to run it.

5. ***Segmentation of Nuclei in Histopathology Images by deep regression of the distance map*** [73]: an algorithm for nuclei segmentation of 2D H&E stained histology images. The segmentation task is formulated as a regression of the intra-nuclear distance maps. This is done to overcome the problem of touching nuclei. The method does not have a GUI but has instructions for running on a GitHub page.

6. ***Triple U-Net*** [74]: an algorithm for nucleus segmentation of 2D H&E stained histology images. It consists of three U-Net-like branches: RGB branch, a Hematoxylin branch and a Segmentation branch; the features extracted from these branches are then aggregated by novel methods proposed in the paper. The method does not have a GUI or well detailed instructions for running on a GitHub page.

7. ***NucleiSegNet*** *[75]*: an algorithm for nucleus segmentation of 2D H&E stained histology images. It consists of three parts: a robust residual block (for feature extraction), a bottleneck block, and an attention decoder block (object localization). The method does not have a GUI but has instructions for running on a GitHub page.

8. ***Self-supervised Nuclei Segmentation in Histopathological Images Using Attention*** [76]: the method for nuclear segmentation of 2D H&E stained images. The method is self-supervised, meaning that it is trained with an auxiliary task (identification of magnification level) and does not require ground-truth annotations for segmentation (only for validation) and then post-processing steps applied. Tested on three H&E datasets and provides competitive performance. The method does not have a GUI; it has an instruction on a GitHub page.

Methods for 2D images but without a freely available implementation:

1. ***A Coarse-to-Fine Data Generation Method for 2D and 3D Cell Nucleus Segmentation*** [77]: A two-step deep learning approach for segmenting 2D and 3D data. The method combines detection with a sparse annotation strategy for training a 3D deep learning model, without requiring extensive full voxel annotation. Such models typically face a critical challenge: the insufficient availability of training data due to various difficulties related to annotation. A full voxel annotation strategy incurs high workloads and costs because only experts can annotate biomedical images properly, and currently, no direct annotation technique is available for 3D biomedical images. GUI is not mentioned in the paper.

2. ***CIA-Net: Robust Nuclei Instance Segmentation with Contour-aware Information Aggregation*** [78]: This method is based on the idea of multilevel information aggregation between two task-specific decoders: nucleus decoder and contour decoder for hierarchical refinement of the details of nuclei and the contour. GUI is not mentioned in the paper.

3. ***CPP-Net: Context-aware Polygon Proposal Network for Nucleus Segmentation*** [79]: It is a method based on *StarDist.* Compared to *StarDist,* there is an additional distance map. It includes additional refinement steps (context enhancement) for final segmentation. GUI is not mentioned in the paper.

4. ***FANet A Feedback Attention Network for Improved Biomedical Image Segmentation*** [80]: The key idea is a feedback mechanism which allows the use of information from the previous training epoch in the current one. It was tested on different datasets, including DSB2018. GUI is not mentioned in the paper.

5. ***Bend-Net*** [81]: An encoder-decoder type of network with the special bending loss, designed specifically for histopathology images. This loss function penalizes large curvature (which are probably touching nuclei), and gives small penalties to points with small curvature (typical for nuclei in histopathological images). According to the paper, it can outperform *Hover-Net* for several histopathology datasets. GUI is not mentioned in the paper.

6. ***High-resolution deep transferred ASPPU-Net for nuclei segmentation of histopathology images*** [82]: a method for nucleus segmentation in H&E stained histopathology images. It uses deep and wide encoder-decoder with

atrous spatial pyramid pooling architecture to handle a widely varied spectrum with a large number of artifacts. According to the paper, the method could reach better results for three datasets vs *DIST*, *SegNet* and *U-Net* with attention. GUI is not mentioned in the paper.

7. **Instance-Aware Self-supervised Learning for Nuclei Segmentation** [83]: a self-supervised method for nuclear segmentation of 2D H&E stained images. The training consists of two self-supervised auxiliary tasks: scale-wise triplet learning (in this task the scale of the sample is the class label) and nuclei count ranking. The network has encoder-decoder architecture (with ResNet-101[ref] as a backbone). Encoder is trained with the auxiliary tasks and then the network is fine-tuned for the segmentation task. The method claims to be state-of-the-art (as for October 2021) on the MoNuSeg dataset (AJI metric). GUI is not mentioned in the paper.

8. *AD-GAN* [84]: an end-to-end unsupervised approach based on generative adversarial network. This method can handle both 2D and 3D data, and claims to provide competitive performance versus supervised methods. GUI is not mentioned in the paper.

Not deep learning-based tools specific for 3D images:

1. *IT3DImageJSuite* [85]: It is an ImageJ Suite that contains several algorithms for 3D segmentation. In particular, the *3D Iterative Thresholding* (*IT*) is a tool designed to test all the single threshold levels and collects the objects yielded at different values, fulfilling some a priori criteria defined by the user. No programming experience is required to use *IT3DImageJSuite*, tutorials and GUI help the users. Currently *IT3DImageJSuite* only works on local machines.

2. *LoS* [86],[87]: It is a fully automated local intensity-based thresholding method to segment 3D fluorescence images. This method is based on the intrinsic definition of *line-of-sight* (*LoS*), used to cluster the surface points and separate the borders of the nuclei. *LoS* works on local machines but its implementation in Mathematica and limited documentation make its usage not easy.

3. *OpenSegSPIM* [88]: It is designed to easily segment nuclei or cells and compute several features without requiring human interaction, except for setting a few initial parameters: an approximate nucleus diameter measurement and

intensity adjustment of the image contrast. Its documentation and GUI make using the tool easy. Currently it does not work in the cloud.

4. **RACE** [89]: It is an open-source analysis framework designed for large-scale 3D images taking advantage of state-of-the-art multi-core processors and graphics cards for processing terabyte-sized datasets within a reasonable time (e.g. 1–2 days). It requires single-channel 3D TIFF image stacks of fluorescently labelled cell membranes or nuclei and seeding points. *RACE* works on local machines. The authors provided a manual and implemented a GUI to improve usability.

5. **Vaa3D** [90]: It is designed for large-scale bioimage visualization, analysis and management. *Vaa3D* has a rich set of functions and plugins, including the cell segmentation toolkit. It is a fully automatic gradient vector flow-based segmentation tool. *Vaa3D* comes with documentation, user manuals, testing data and video tutorials. It does not work in the cloud and it is easy to use.

6. **3D-Cell-Annotator** [36]: It allows to segment single cells and nuclei in 3D using 3D active contours with shape descriptors as prior information for true single-cell annotation in a semiautomatic fashion using initial seeding points. It comes with a user manual, video tutorial, documentation and GUI. Currently it does not work in the cloud.

7. **Three-Dimensional Segmentation and Reconstruction of Neuronal Nuclei in Confocal Microscopic Images** [91]: A 3D segmentation algorithm for crowded, overlapping objects in the 3D space. The algorithm assumes that the objects have regular shapes therefore the surface of each object can be reconstructed by processing multiple XY-planes. Currently no implementation and documentation is provided.

Deep learning-based tools specific for 3D images:

1. **Allen Cell Structure Segmenter** [92]: It is a toolkit developed for the 3D segmentation of intracellular structures in fluorescence microscope images. This toolkit brings classic image segmentation and iterative deep learning workflows together to generate high-quality segmentations. The software originally used *Jupyter* [41] notebooks to construct the segmentation workflows,

but it also has a *Napari* interface developed recently. To train the segmentation models some technical knowledge may be needed. It has extensive documentation with video tutorials.

2. **QCANet** [93]: It is a 3D instance segmentation method designed to analyze the development of fluorescently labelled mouse embryos. The algorithm consists of a *Nuclear Detection Network* that detects the object centers and a *Nuclear Segmentation Network* that separates the background from the object. Then, in a post-processing step, 3D watershed is applied to label the segmented image using the output of the detection network. Once the source code is downloaded, the user has to execute it from the command line. The readme file shows basic examples and the details of the configuration.

3. ***Semi-supervised segmentation and graph-based tracking of 3D nuclei in time-lapse microscopy*** [94]: It describes a simple 3D CNN used to extract the object probability map for the input image. Then, a watershed and a supervoxel algorithm (SLIC) are applied simultaneously to obtain a roughly segmented image and an over-segmented one. The two images are then merged and processed using a novel boundary correction algorithm to obtain more accurate object boundaries. The software can be executed in the command line once the source code is downloaded. The readme file shows basic examples.

4. ***3DeeCellTracker*** [95]: It is a 3D instance segmentation pipeline aimed to track cells in 3D such as cells of the brain of moving worms or tumor spheroids. The pipeline consists of two major parts: segmentation and tracking. The segmentation part uses a 3D *U-Net* and a 3D watershed is applied to construct the final instance segmentation. *Jupyter [41]* notebooks are provided for running the software. Video tutorials are available that show the installation of the software, tracking functionality, manual annotation with the *ITK-snap* and correcting the labels.

Methods for 3D images but without a freely available implementation:

1) ***A Deep Learning Pipeline for Nucleus Segmentation*** [96]: An end-to-end computational pipeline to train and evaluate the performance of machine learning-based nuclear segmentation algorithms. This pipeline was first used to

generate preliminary nuclear labels of several different cell types, then manually corrected in an interactive fashion and used to train and evaluate the performance of different *CNN*-based architectures for nuclear segmentation. The pipeline was based on the *Snakemake* language, a generic workflow management software where individual computational tasks are chained together to form a pipeline. Each task is configured by a set of input files and parameters, by a call to a bash script or a *Python* script that accepts the input files and processes them, and by a set of output files generated by the task. The pipeline was developed as a reproducible workflow capable of evaluating any number of different nuclear instance segmentation models. As a first step in the pipeline, a pretrained CNN model or a traditional nucleus segmentation algorithm is used to generate preliminary sets of labels for the nuclei. These preliminary labels generally contain gross segmentation mistakes, such as false positives, false negatives, splits and merges, which can be manually corrected using an interactive, web-based interface. The implemented pipeline runs either on clusters or on a local server equipped with NVIDIA™ GPUs. The Snakemake pipeline for models training and testing is deposited in a dedicated Github repository: *https://github.com/CBIIT/nci-hitif*

2) ***Combined detection and segmentation of cell nuclei in microscopy images using deep learning*** [97]: A 3D convolutional neural network to simultaneously segment cell nuclei and detect their centroids in confocal microscopy images. Mirroring the co-dependency of these tasks, the proposed model consists of two serial components: the first part computes a segmentation of cell bodies. In particular, they adopt the *U-Net* architecture as the basis of the segmentation network. The motivation behind this architecture is that the contraction and expansion paths of the architecture capture the context around the objects in order to provide a better representation of the object. The second module identifies the centers of these cells. This serial architecture is motivated by the observation that the detection task is rather straightforward given a segmentation map, yet annotations are easier to obtain for the detection task.

3) ***DeepSynth*** [98]: This method uses synthetic data to train a modified 3D *U-Net*. In this version of the *U-Net*, the network consists of leaky *ReLU* activations and the authors employ a combination of the dice and binary cross-entropy loss to

achieve better accuracy. As a post-processing step, a 2D watershed is used on each orthogonal plane to separate the overlapping nucleus instances. The code is available on request.

4) ***Nuclei detection for 3D microscopy with a fully convolutional regression network*** [99]: Lapierre-Landry *et al.* recently presented *VRegNet*, a two-step encoder-decoder segmentation network based on the *V-Net* architecture, a popular 3D semantic segmentation architecture similar to 3D *U-Net* but using the Dice loss to prevent class imbalance, combined with a new regression-based fully convolutional network. Precisely, in *VRegNet* nuclei are first segmented from the background using *V-Net*, then the regression network is used to directly identify centroids. *VRegNet* is designed to segment nuclei in large 3D fluorescence datasets.

5) ***3D Cell Nuclei Segmentation Based on Gradient Flow Tracking*** [100]: A physical model is proposed to segment each nucleus instance. The authors argue that computing only the gradient of the image and tracking the points to reach the intensity maxima to detect cells could lead to suboptimal accuracy because of the noisy areas present in the center of the objects and the cluttered gradients present in images containing irregularly shaped objects. Therefore a gradient vector diffusion algorithm is proposed that diffuses the gradient vectors from the high magnitude areas to the low magnitude parts. The proposed equation also produces a smooth vector field even in noisy areas. After the diffused gradient vector field is constructed, the points are tracked back to the sinks that correspond to the nucleus instances. The tracking can be implemented efficiently by not tracking the pixels that are tracked back during the processing of a different pixel previously.

6) ***Segmentation of fluorescence microscopy images using three dimensional active contours with inhomogeneity correction*** [101]: In another interesting paper, a deep mathematical model is developed to segment nucleus instances in fluorescent images. The proposed model is a 3D active contour model with a region-based data term that considers the image inhomogeneities during the surface evolution. The input image is modelled as the pointwise multiplication of the homogeneous Image and the inhomogeneous field added to a zero mean gaussian. The proposed energy

functional is then minimized by solving the corresponding partial differential equation.

7) ***Automated Cell Segmentation with 3D Fluorescence Microscopy Images [101,102]***: A pipeline with several steps to segment cell instances in 3D fluorescent images. First, the image is interpolated using cubic interpolation, then a Gaussian filter is applied. Then, the gradient vector field is computed for the pre-processed image and an adaptive threshold is applied to the image to separate the foreground and background pixels. As a post-processing step, the object boundaries are smoothed using morphological operation.

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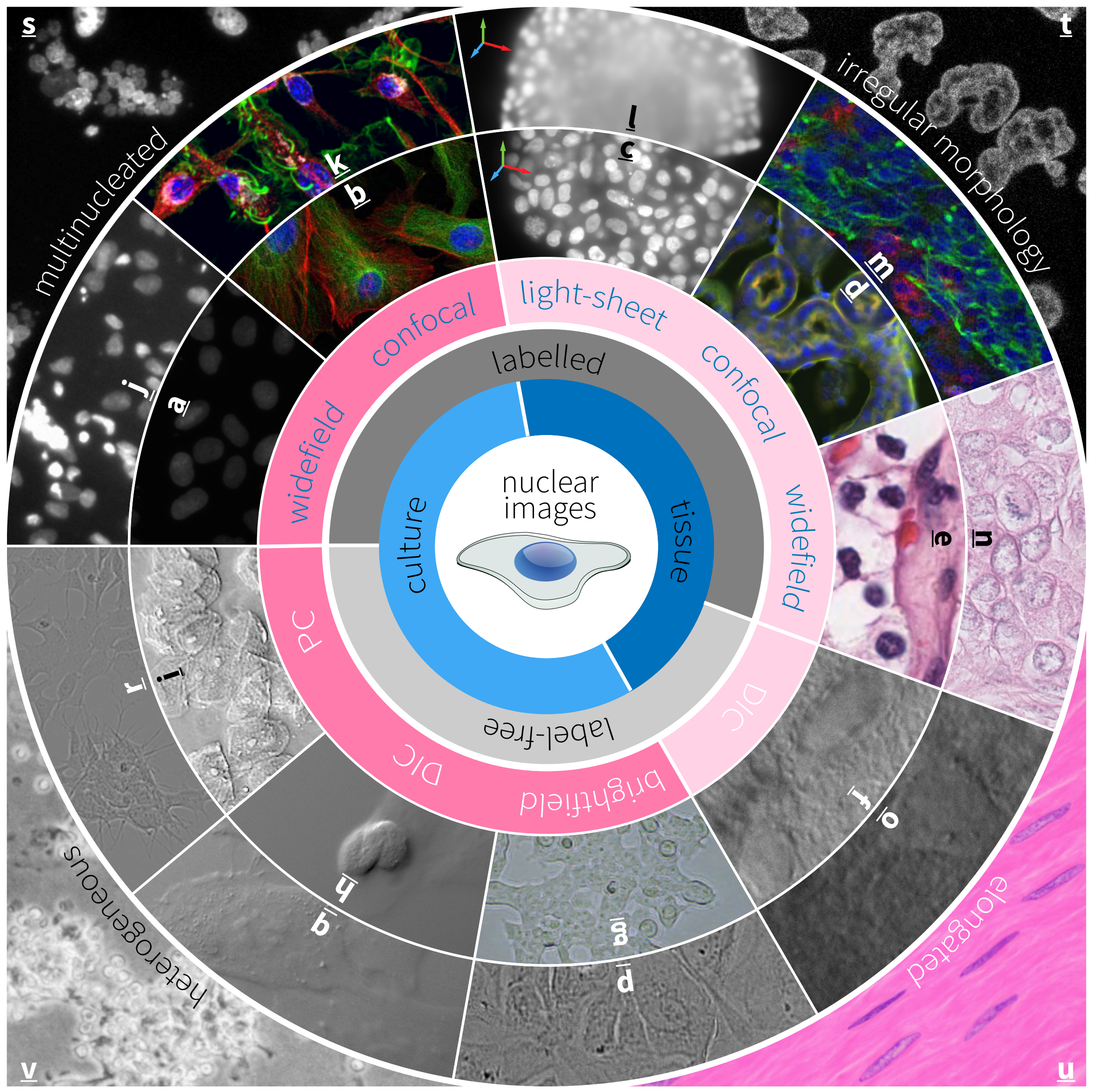21 Schmidt, U. *et al.* (2018) Cell detection with star-convex polygons. In *Medical*

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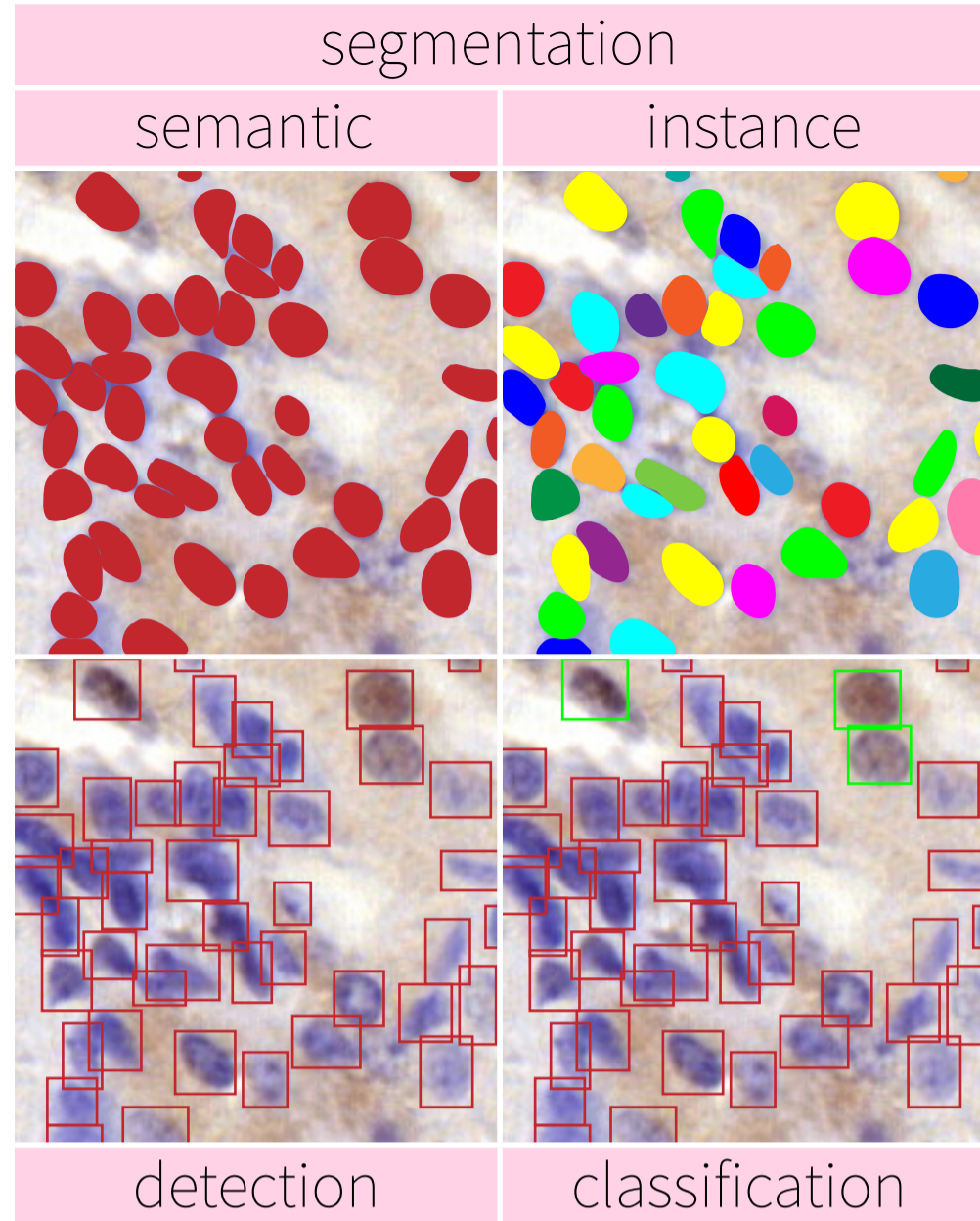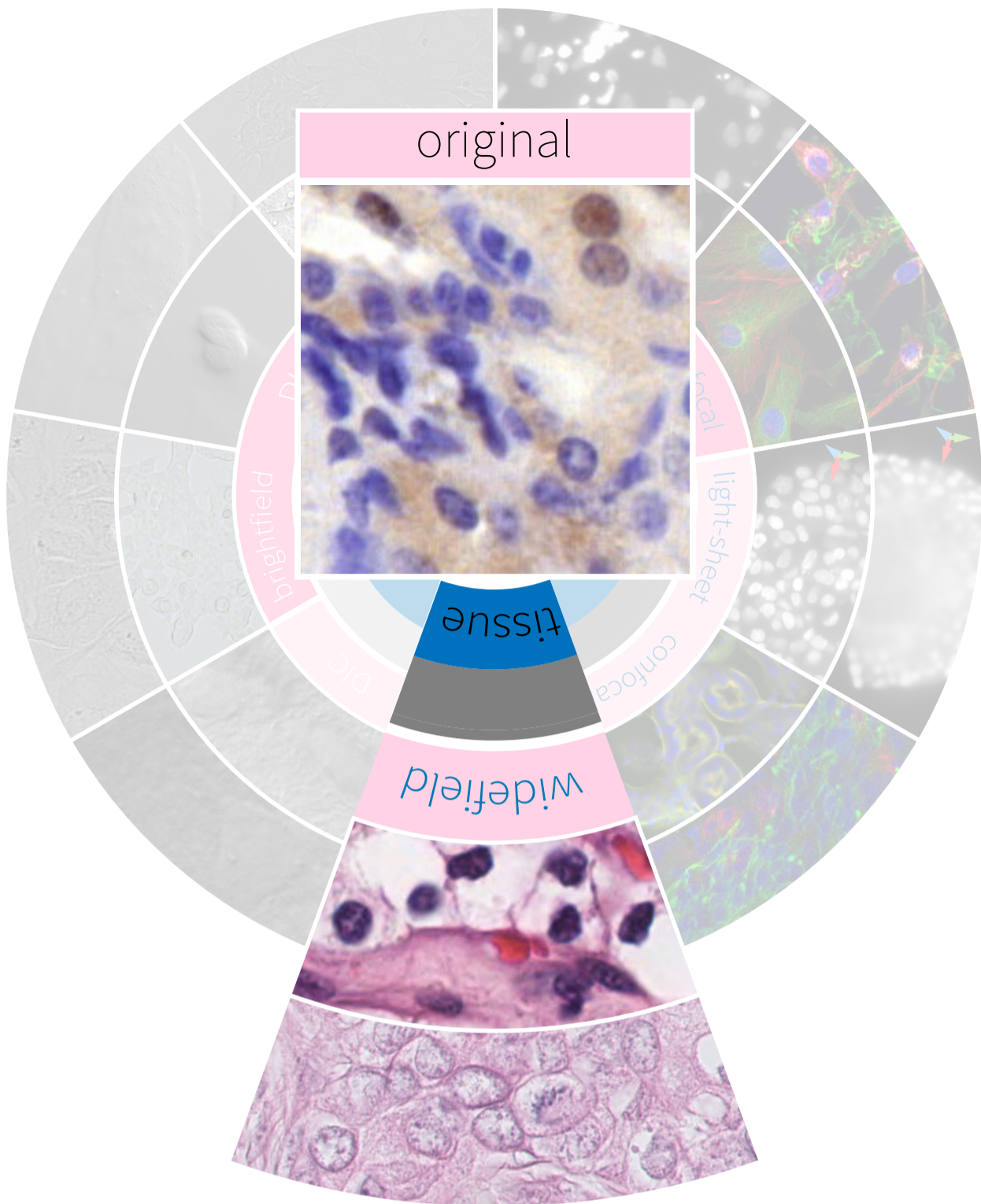
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irregular morphology

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confocal

widefield

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DIC

label-free

DIC

brightfield

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r

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o

h

g

q

g

d

heterogeneous

elongated

v

u

what is my DATA like?

what do I want to DO?

original

tissue

widefield

segmentation

semantic

instance

detection

classification

Microscopy image type selection tree

Culture

Do you think your method works on this kind of data? E-mail us at email@address.c
Get help here

Methods:

2D

3D

Challenges:

elongated   concave   deviating   wobbling   heterogen

fragmented

Name	Author	Year	Journal	Links	Github ★	Forks	2D	3D	Challenges
nucleAlzer	Hollandi et al.	2020	CellSyst	code paper site	34	12	✓	x	elongated concave deviating wobbling heterogen fragmented
U-Net	Ronneberger et al.	2015	MICCAI 2015	code paper	3569	1750	✓	x	elongated concave deviating wobbling heterogen fragmented

Fluo

Labelled

Confocal

BF

Microscopy

Culture

Label-free

BF

DIC

PC

sample   label   microscopy   methods