ANALYSIS OF LIVING ORGANISMS WITH IMAGES
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Introduction

In the twenty-first century, many inventions and discoveries related to life science had also been possible with the help of images which played a remarkable role in the field of biology. Whether it's a test of a hypothesis or the first visual presence of a cellular system, images always remain the primary evidence of its existence. Our preference for a source of information in the form of images comes from the possibility of being directly involved in visualising images. The tremendous technological developments in numerous scientific fields increased our ability to analyse and visualise life at the molecular and cellular levels. Noble prize-winning discoveries related to microscopy helped convert typical microscopes to nanoscopes, which are now more helpful for visualising images on the nano level. On the other side, only advances in microscopes were not enough. The operating system, which is dependent on computers, is also advanced. In this century, computers are not only responsible for signal transfer during image capturing, but now computers are reliable for processing the data obtained during image acquisition.

Bioimaging is a new field that started from bioinformatics and computational biology to quantify values that can be useful in research. It is present now because of Imaging and Resolution, Machine Learning Techniques and Computer Performance. This summary will mainly describe my personal experience studying stem cell differentiation processes through lamins during the Amgen Scholar Program at ETH.

What is a Bioimage

A bioimage is an image that describes a biological part of the body. It can be 2D (like X-ray) or 3D (like Computer-Aided Tomography). It helps to report the object's physical appearance without (theoretically) damaging its physical property. This technology depends on the tool that is used. Using a tool to capture images mustn't influence the outcome of the image itself. For example, during light sheet microscopy for organoids, light can have a toxic effect that can slow down the growth of organoids and influence their future behaviour.

We can divide images based on three main categories:

Maps

Representation of a quantity referred to the surface body starting from measurements in discrete points, like EEG, ECG or thermography.

Projections

Images on a projection plane starting from a centre (possibly to infinity); each point represents the integral of a physical quantity on the projection line that crosses the internal volume. Radiographs are some examples of that.

Tomography

Reconstructed images of virtual sections of the body representing various physical quantities; reconstruction is done starting from external measurements such as time of flight (ultrasound), projections from multiple angles (CT, SPECT, PET), and comparison of phase with different magnetic emitters (MRI).

How to Acquire Images

To acquire images, there are different tools

- X-ray
- Gamma-ray
- Ultrasounds
- Magnetic Fields
- Infrared light
- Atomic force microscopy (AFM)
- Light Sheet microscopy
- Positron emission tomography (PET)
- Single photon emission CT (SPECT)
- Computed Tomography
- Near Field microscopy
- Optical Coherence Tomography
- Total internal reflection fluorescence

Light Sheet Microscopy:

Light sheet microscopy is an image visualization technique. It's a fluorescence dependant image visualization technique. It utilizes a laser light sheet and only illuminates a thin slice of the sample. Signals of light due to the phototoxic effect can damage the sample. The light sheet microscopy imaging technique visualises images with the help of laser light. It illuminates the slices of the sample. It can be utilised to evoke biological specimens under a full-frame camera. In this system, the illumination beam is perpendicular to the image, creating a sheet of light through the 2D surface of the sample. It is the same as the focal plane. And during this, there is a minimal breach of light, making the object less out of focus. This setup has several benefits over other methods of imaging. A sample can be imaged quickly. Different, only one plane is excited, which means less out-of-focus light.

Pre-processing

The first step after acquiring images is to pre-process the data to reduce noise and other unwanted artifacts. The aim of pre-processing is to improve the quality of the image so that

we can analyse it in a better way. By pre-processing, we can suppress undesired distortions and enhance some features necessary for the application we are working for. Those features might vary for different applications. One of the main pillars of people in CS is "Garbage in -> Garbage out". If we give ML programs some data that may not fit reality or is too distorted, the model may not predict future results correctly.

Pre-processing techniques may depend on the tool used to acquire the image. Some tools may require more work to pre-process images. This activity is mainly done by "technicians" in the lab.

In recent years, more and more tools have emerged to pre-process the image. For example, during my research experience, some people used LSM980 by Airyscan where image pre-processing is almost superficial.

Image registration is another step involved in image pre-processing. In this step, images from different organisms or time points of the same organisms are allowed to compare pixel by pixel.

Some ways to pre-process the image are:

- **Resizing**

An organoid can go from under 100 um to around 700 um during development. Images that can be so much different in size may cause several problems while studying.

- **Orientation and Movement**

When organoid grows, they tend to move in different directions. At the same time, there may be the problem that the image is not centred. Not centred images can decrease the algorithm's mAP (mean average precision).

- **Grayscale**

Grayscale may depend on the number of channels you have. With light sheet microscopy, there are two channels. In any case, it's easier to study each channel separately. So, it isn't going to be a significant problem overall.

Deconvolution

It is also a well-known example of pre-processing of a bioimage. Blur signals are received during an examination of samples under the microspore. In this process, it tried to undo the blur signals to make the image clearer.

Image registration

In this process, images from a different organism or the same organism and allow to compare pixel by pixel.

Image Filtering

Another pre-processing step is to filter out the image based on the presence of a required object. Image segmentation is one of the most challenging steps among all bioimage processes.

Sometimes, pre-processing is generally confused with augmenting images. Image Augmentation is a technique that can help improve the mAP of our models. In any case, Image Augmentation doesn't always have positive results.

As a general rule, image pre-processing reduces noise and biases.

Image Segmentation

Image Segmentation divides the image into multiple elements so they can be studied effectively. In other words, it starts by enhancing the visualisation of the region of interest from the image's background. We create one or several ROIs that we can later analyse during segmentation.

When we describe Image Segmentation, we need to ask two questions:
1. **What kind of Segmentation do we want to use?**
2. **Do we want to use a Supervised or Unsupervised Tool?**

Types of Segmentations

We can define three types of segmentation:
- Semantic segmentation is a dichotomy classifier. Where each pixel of an image belongs to the background or the segmented part, that can be particularly useful when all the objects are distant from each other.
- Instance segmentation is a tool that creates multiple ROIs based on a particular group. In this case, we don't have two groups (background and ROI) but a background and multiple ROIs. It can also be helpful when there are many diverse in the same image.
- Panoptic segmentation is the union of semantic and instance segmentation. Panoptic segmentation identifies some particular classes of images and distinguishes different instances of the same type. It can be a helpful approach to label values or consider multiple ROIs independently.

Defining particular types of Segmentation can be helpful, as it may use different approaches. We can define two types of algorithms:
- **Supervised**
- **Unsupervised**

Unsupervised

Edge-based algorithms – like Otsu or multi-Otsu

The multi-Otsu threshold is a thresholding algorithm used to separate the pixels of an input image into several different classes, each obtained according to the intensity of the grey levels within the image. Multi-Otsu calculates several thresholds determined by the number of desired classes. The default number of classes is: The algorithm returns two threshold values for obtaining three classes. A red line represents them in the histogram below.

Image partitioning

Bioimage usually requires an image portioning step to analyse specific objects. The objects of interest are separated from the background image in this process. Image partition can comprise identifying a bounding box across objects or recognising the set of pixels composing each object. This process is called segmentation. Images with fewer objects can be distinguished by hand, but on the other side the datasets are more readily available than an automation system is required. Deep learning methods from computer vision have increased object detection, segmentation speed, and accuracy in biological images.

Deep learning methods be further divided into several categories:
1) Troubleshooting approaches

It's a division of bioimage pixels under different groups. Reimage pixels determine the threshold values. Bioimages intensity lower than threshold level categorised under one group and images intensity higher than threshold level placed under other groups.
2) Region growing approaches

These approaches use data regarding intensity or bioimage edge data to extract a pre-defined image region.

Classifiers

Classifiers are also an essential type of segmentation in this process; bioimage is converted from image space into feature space trained classifiers to differentiate under which pixel class image fall.

Watershed

It's a process in which different objects present in an image are separated. It's an algorithm that is used for segmentation. In this algorithm, values generated by pixels, and built-in settings by the user, used a local topography. The algorithm floods basins from the markers until basins attributed to different markers meet on watershed lines. In many cases, markers are chosen as local minima of the image, from which basins are flooded.

In the example narrated below, overlapped circles are separated. For this purpose, one subtracts an image far from the background. The distance between these maxima is selected as markers, and the basins flooded by the algorithm from such markers differentiate the circles along a watershed line.

Besides that, there is another tool known as Random walker segmentation. This algorithm method is similar to the watershed. The difference between watershed and random walker is that this method uses a Random approach. The idea is that the user labels a small number of pixels and other pixels are classified based on the closest labelled point.

Supervised

Supervised approaches have the initial need of having some data for training and test to before using the software.

Pixelwise classifier of Ilastik

The Pixel Classification workflow assigns labels to pixels based on pixel features and user annotations. Once the features are selected, interactively train a Random Forest classifier from user annotations. Random forests or random decision forests are an ensemble learning method for classification, regression and other tasks that operates by constructing many decision trees at training time.

CellPose and Omnipose

CellPose and Omnipose are generalist algorithms for cellular segmentation with human-in-the-loop capabilities. That can allow segmenting new images using previous models or creating your own.

If you need to create your model, using CellPose and Omnipose would require annotations. Annotation is the activity of letting know to the algorithm what it should consider during training and validation. After that, there is the possibility of training the algorithm and saving that for future use. Cellulose and Omnipose were the same platforms in 2021. After that year, the developer decided to divide and create new platforms. The main difference between Cellpose and Omnipose is in the size of the cells. Omnipose requires a longer training time even though it's more precise with long cells.

Mask-RCNN

It is defined as a variant of a Convolutional Neural Network. This network stands for Region-based convolutional neural network. This variant is the futuristic network for image and instance segmentation. This network is a region-based neural network and developed on top of Faster R-CNN. This machine learning model is computer-based. It is used to detect objects and vision tasks carried out by computers. The basics of this image portray the basis of region-based CNN. This system uses the boxes around the regions and then assesses convolutional networks autonomously on all the Regions of interest to classify regions of the image into a selected class. CNN can be defined as a type of artificial neural network. It depends on a mathematical operational system known as convolution. Basically, these neural networks are used to process pixel data.

Further, it is used to process and recognise images. It's a feed-forward neural network in this process; signals progress in the network without making any cycles or loops. In recent years convolutional neural networks have gained more interest in bioimage analysis due to their high accuracy and no need for the direct involvement of an expert. This network consists of pooling, activation of functions, and convolutional and connected layers. A high level of reasoning is accomplished in connected layers in which all the neurons relate to previous layers. The last layer in this network of connections is known as the loss layer. This layer estimates the error amount between desired and actual output. These convolutional layers perform a function to extract the desired features of the bioimage. During this process, several arrays are applied throughout the input tensor. A product should be determined among the input tensor and element kernel to access the output amount in the tensor of output. The layers decrease the number of parameters by downsampling the in-plane dimensionality. Nonlinearities are known as activation functions in which a single number is counted and performs further operations. ReLU (rectified linear unit), Tanh and Sigmoid are the most used activation functions. There are five popular deep CNNs for the classification and extraction of features.

- AlexNet.
- GoogLeNet.
- U-Net.
- Visual geometry group network (VGGNet).
- Residual network (ResNet).

The first CNN was the AlexNet regarding good performance in detecting and classifying the object. AlexNet and VGGNet work on a similar network, but VGGNet owns extra layers of convolutional. VGGNet contains several constituting layers, which include pooling rectification, three fully connected layers and thirteen convolutional layers. But compared to VGGNet in AlexNEt all layers are piled in one place. The inception module was firstly introduced in GoogLeNet. In this inception module, a sparse optical structure estimates in CNN to achieve more efficient computation by dimensionality reduction. When GoogLENet was introduced, it consisted of twenty-two layers having layers related to rectified linear operations. Two fully connected, three conventional and pooling layers. AlexNet possesses more parameters as compared to GoogLeNet. U-Net has a U-shaped architecture it contains two paths, i.e., contracting and expansive paths. These paths give these CNNs a U shape. It is designed especially for biomedical image segmentation. CNN contains two 3x3 unpadded convolutions. It has 23 layers of convolution depending on unpadded convolutions, followed by a 2x2 pooling operation and a ReLU. Lastly, I will talk about RestNet most convenient and easiest process in the bioimage dataset. It displayed acceptable performance of

classification. In this CNN, layers learn the functions when they receive the input instead of learning from already available functions. This process can decrease the time of training.

Labelling

Once you have the segmented values, you need to demultiplex them. Demultiplexing comes from Electronics, where the idea is to start with the input and divide that into different outputs. In our case, we had to divide it into three other proteins (tubulins, lamins and cell membranes). Once a mask is present, there is also the possibility of labelling different values based on some particular features.

For example, in the biological world, there is the possibility of defining some proteins that may differ in roundness, density (given by light intensity), surface area and much more. Several tools (such as region properties on skimage) can be useful. That could lead to finding some 'standard' values that we already know their form. Indeed, we would be especially curious to study their location in this case. That could cause information loss with some particular shapes of the same category. For example, during the project with lamins, there were some lamins incredibly bright. Others were hard to see, and some lamins were smaller than others. At the same time, other models are becoming more and more used. For example, morphometrics is a Napari's plugin that gives valuable information on different dimensions to label values effectively. It operates a Random Forest Classification where many parameters are considered to find a particular value. Napari is one of the most used image viewers for Python. Many applications are based in Napari.

Before, we talked about RCNN. In this case, segmentation and labelling are done in just one image. Even though this approach is speedy and efficient, it may not be the best when there are complex images. Many parts of the images could not be considered, leading to a loss of potentially helpful information.

Transfer Learning and Model Zoo

In machine learning (ML), there is a research problem known as transfer learning. It is explained as knowledge gained, used for solving a problem, and can be easily used for different issues that can be related to the first problem. To understand this phenomenon, we can say that knowledge gained during recognising cars can also be used to identify tracks. There is a need for many datasets while examining multiple cells. The pre-defined models can be used for different cells. This way, the researcher can easily annotate their data in less time and have high precision. Besides that, it can be more helpful than examining all hyperplane points to locate the global minimum. But there is a problem with using this approach. The overfitting values mean data used for training and validation can be unique from what we have found in real issues.

Overfitting and Augmentation

To increase the Mean Average Precision of a Model, there is also the possibility of Augment Data. Data Augmentation happens when the AI model uses the input images and some copies that can be rotated, flipped, and changed in colour and brightness. Data Augmentation is generally positive. At the same time, Overfitting values can also happen in this situation. In our story, increasing brightness and flipping the image caused a decrease in the mAP of the segmentation model.

From Offline to Online

Since AI models are becoming increasingly complex (and they may require too much data and space), there are several possibilities to train the model. One example is ZeroCostDL4Mic, a collection of self-explanatory Jupyter Notebooks for Google Colab that features an easy-to-use graphical user interface.

When quantifying images, there are some crucial parameters to consider:
- Number of objects
- Size of the object
- Morphology of objects
- Spatial distribution
- Fluorescent intensity
- Object tracking

Image Quantification

Once bioimages are detected further step is quantification. It can be counted and categorised on type shape or its dynamics. It can be done on an object, or it can be looking at the specific aspect of the object. Manual categorisation of an object is time-consuming, and even if experts can do it, it shows the reflection of biasness. Yang and colleagues devised a deep learning model independent of observer or expert to evaluate microscopy image focus quality, providing an absolute quantitative measure of image focus. It can be performed at all times and on every scale of length.

When there is the possibility of creating a model, there are three different possibilities.
- **Individual Object Simulation**

Object simulations focus on understanding how an object behaves under certain conditions. One of the most used libraries for Object Simulation is Simpy. In general, defining particular features and seeing how they would evolve over time can be helpful. When there is just one variable (like surface, volume or surface length), there is the possibility of describing and using Ordinary Differential Equations. Ordinary differential equations have an order higher than one. The way to solve them computationally is by going to the first order with additional variables (x, y, z). Thanks to our dataset, we are interested in defining the coefficients of these variables.
- **Stochastic Differential Equations**

A stochastic differential equation (SDE) is a differential equation in which one or more of the terms is a stochastic process, resulting in a solution which is also a stochastic process. SDEs various model phenomena, such as stock prices or physical systems subject to thermal fluctuations. In this case, there may be some particular discontinuities, such as jump processes. Random differential equations are conjugate to stochastic differential equations. Random differential equations are like having partial or ordinary differential equations with noise.
- **Network theory**

The network theory starts by defining any element of the network as a node, trying to find correlations between all of them (using edges). That can make real complex computational problems to solve far more accessible than expected. Given a problem, nodes are the main elements of the problem, while edges are the relationships of the problem. Network theory can explain several issues, from airport and plane distributions to protein and bond interactions. Graph Neural Networks are becoming increasingly important when describing

network theory. It starts from the idea that representing any phenomenon through a matrix is possible. Graph Neural Networks are related to Convolutional networks as a node is related to a pixel.

Indeed, biological images can be seen as graphs, and there is the possibility of editing, manipulating and modifying values based on that. While working with Graph Neural Network, there is a need to understand what's a node and what's an edge. A node is the basic component of our analysis; in general, it could be a plane in an airport, a person in a group or a musical note in a song.

An edge is a link between nodes. For example, a plane can go more frequently from Rome to Zurich, London, Madrid and New York. At the same time, a plane can go more frequently to Zurich than to New York. That's why edges can be weighted. A weighted edge is when you give a higher value to an edge than another. In the field of machine learning, it's called "Graph Attention Networks". Attention-based models are helpful when there is a need to give priorities. For example, in the sentence: "Snow White eat a red poisonous apple", our attention is focused on the poisonous apple, not on its colour.

During my experience, we modelled lamins'position as nodes of the graph and their distance as edges. There is the possibility of defining a maximum space to study neighbourhood analysis. Defining standard features of the neighbourhood (such as the number of nodes, average distance, and geometric distribution) can give us insightful information on how future communities can evolve.

Graph Neural Networks are becoming increasingly important because they help simplify many complex tasks, giving a computation advantage in solving the problem faster and with higher precision.

Future Road Map

Bioimage analysis was the first step taken a century ago, but it's still a long journey where biologist instinctively trusts the findings. A significant technical advancement in the capacity of image visualisation systems to produce big data worries the interaction between image analysis and acquisition. In bioimaging experiments, it's a thumb rule to fetch as much as image data can be taken further and let the software do the job. Microscopists, computer scientists and biologists need to develop intelligent and precise imaging systems that help analyse larger image datasets. There is also a need for innovative instrumentation in which a smart mechanism can help maximise data transparency and increase the content of information regarding bioimages. This could be beneficial because we can first acquire bioimage at low resolution and then identify the required area by using several detection methods. After that, only obtain the bioimages of that area in high resolution.

In future, we expect the system of bioimage analysis will be automated. The capacity of the current bioimaging analysis system is already a step ahead. It's already involved in automation, and a vast analysis can be performed by biologists on already available information. In biology, images are not the only trace of lead. Other omics such as transcriptomics, metabolomics and proteomics provide general data and information on biological processes. A combination of all these processes with bioimage can complete the whole process compared to the fields described individually.

Experts in the field
- Arne Seitz
- Sage Daniel
- Christian Tischer
- Anne Carpenter
- Kevin Eliceiri
- Jason Swedlow
- Ernst Stelzer

Common Tools to Use
1. **Denoising**
2. **Noise2Noise**
3. **Noise2Void**
4. **Segmentation**
5. **Stardist**
6. **Cellpose**
7. **Detection**
8. **Yolo**

Definitions

Intersection over Union — IoU

IoU computes intersection over the union of the two bounding boxes, the bounding box for the ground truth and the bounding box for the predicted box by an algorithm.

Training – Validation – Test Sets

Training data: set using a supervised learning method, for example, using optimisation methods such as gradient descent.

The validation data set provides an unbiased evaluation of a model fit on the training data set while tuning the model's hyperparameters.

Validation datasets can be used for regularisation. It can happen by early stopping (stopping training when the error on the validation data set increases, as this is a sign of over-fitting to the training data set). In this case, there are also local minima to consider

The test data set is a data set used to provide an unbiased evaluation of a *final* model fit on the training data set if data in the test data set has never been used in training (for example in cross-validation).

Unsupervised learning

Unsupervised learning is a type of artificial intelligence (AI) that allows software applications to become more accurate at predicting outcomes without having a particular label.

Machine Learning is divided into three main categories:
- Supervised Learning
- Classification
- Regression

It is defined by its use of labelled datasets to train algorithms that classify data or predict outcomes accurately.

- Unsupervised Learning
- Dimensionality Reduction
- Clustering
- Reinforcement Learning

Unsupervised learning is a type of algorithm that learns patterns from untagged data.

Reinforcement learning (RL)

is an area of machine learning concerned with how intelligent agents ought to take actions in an environment to maximise the notion of cumulative reward.

ROIS

Regions-of-interest (ROI) for detected objects.

MASKS

Masks for the detected objects

Class

Class integers for the detected objects. For example, after classification is possible to define different categories inside a npy array. That can be possible by creating different groups. Every class has a particular number (1, 2,3..)

Scores

Confidence Probability for each predicted class.

Convolutional layers

Convolutional layers can be described as a type of layer which looks like a filter which processes the image. These values can be learned during the training process. In the convolutional layer, each neuron is connected to a small group of adjoining neurons found in previous layers.

Data augmentation

Data augmentation is an approach to increasing the quality and size of the training sets. It includes conventional training such as geometrical operations like flips, translations, rotations, random cropping, high and low levels of contrast and intensity, random copping of the bioimage and transformations of non-rigid images like elastic deformations.

Dense Layer

It is a layer in which all the neurons present in the previous layers are associated with the presentation layer.

Ground Truth

It can be defined as when a known output is corrected with respect to the input provided.

Image Patch

An image patch is a small and rectangular piece of a larger image. It helps in reducing computational costs during the training.

Input Data
This data is known as data provided to a Machine learning model.

Loss
It can be defined as when a function evaluates a model's prediction and how closely it matches.

Layers
These can be defined as A set of different artificial neurons interconnected in a neuron network.

Output
It's a date which comes out from a machine learning model.

Pooling
It's a process combining associated neurons having or guided by an average minimum or maximum operator.

Signal-to-noise ratio
It is a measurement of the quality of the image. It's the ratio between digital image mean intensity value to standard deviation of these values.

Style Transfer
In this method, a reference is needed. An image is used as a reference from that specific style learned based on that image.

Training
It is a process in which the parameters of a machine learning model are optimised. In this, the best map inputs are transferred into preferred outputs.

Testing sets
It is a collection of already known input and output pairs. These sets are used during training to evaluate the machine learning model's performance on unknown data.

Transfer learning
The transfer learning method is a model developed for a specific task, and that machine learning model can be further used for a different task.

Weights
These are Neural network parameters, and during the training process, these parameters are adjusted.

Voxel
It's a 3-D which is equal to a pixel.

Acknowledgement

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