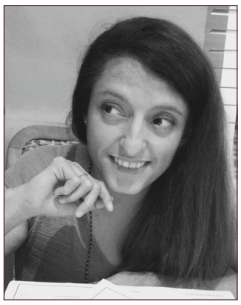
INSIDIA: the Imagej macro for *in-vitro* spheroid invasion assay analysis



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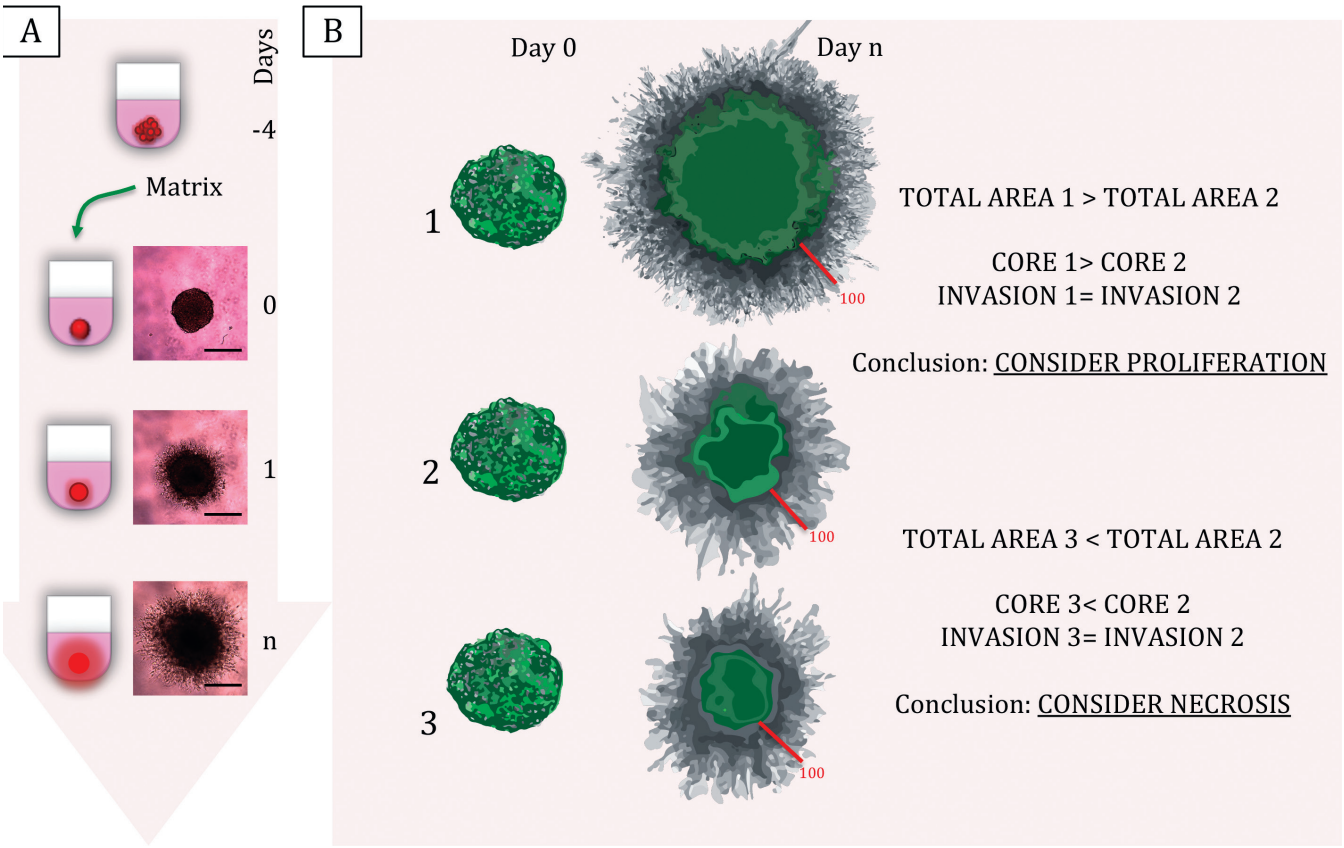
While *in vitro* assays are generally recognised as, to some extent, not fully representative of the *in vivo* situation, they do allow potential for more mechanistic investigations and high throughput evaluation. Indeed, *in vitro* assays able to characterise cancer aggressiveness, in particular invasion, are becoming more complex and compelling [1]. This complexity recognises nuisances in phenotypical and molecular processes distinguishing different patterns of tumour invasion and the impact of cancer cell microenvironment in terms of, for example, cell-, matrix- and soluble factor-interactions. The various patterns of tumour invasion [2] are relevant to understanding the dysfunctional biology, role of molecular pathways and response to experimental treatments. Furthermore, *in vitro* assays, especially when combined with microfluidics platforms, represent the high-throughput screening choice for cancer therapeutics, not possible in live animal models [3].

3D cancer cell or tumour sample spheroids represent an evolving model accommodating a number of *in vivo* characteristics such as formation of a compact "tumour" core and the formation of pH, nutrient, and oxygen gradients [3]. One assay incorporating the ability to study *in-vitro* invasion in a 3D manner with potential to examine the above interactions is the 3D spheroid-sprouting assay of Vinci et al. [4] which, since its publication in 2012, has become increasingly popular. The assay (Figure 1A) basically consists of suspending a compact sphere of cancer cells within an extracellular matrix and following over time the growth of the sphere and the cell migration from the sphere into the 3D matrix environment. The extracellular matrix of course can be modified with different molecular and cellular components. The models can be subjected to a range of cytotoxicity and cell growth analyses and progression of the cancer spheroid monitored over time by image capture.

Images recorded using a simple inverted laboratory microscope will capture over time the extent and pattern of neoplastic cell invasion from the spheroid mass. However, the quantitation of such behaviour has, to date, been varied and generally limited in scope. The basic analysis has involved the measurement of the overall area occupied by the expanding cellular mass but such simple geometrical information under-powers the potential of the assay to reveal biological differences. For example a cancer cell spheroid may be divided in two distinct zones: a 'Core' (the original spheroid mass that has undergone varying extents of proliferation) and an 'Invasive Front' of cells detaching from the Core and invading through the surrounding extracellular matrix. Figure 1B shows two cell lines invading in the same way may undergo different modifications of their core; if cells in the core keep proliferating (Figure 1B.1) the last will increase and consequently also the total area occupied by the cellular components, while if cells are not proliferating (Figure 1B.2) or possibly undergoing apoptosis, autophagy or necrosis (Figure 1B.3) the resulting total area will be smaller. Both cases have to be considered, especially for tumours like Glioblastoma multiforme (GBM), in which necrosis in the centre of the tumour is a defining histological feature. Notably, GBM cells have long been recognised as having the propensity to invade deep into the surrounding normal brain parenchyma before they recommence cell division and form distant foci of the tumour. Conversely, invading medulloblastoma cells tend to move short distances between their divisions as they follow the histoarchitecture of the cerebellum.

With the methodological advances in undertaking more complex 3D spheroid invasion assays and the large amount of data images that can be captured there is a real need for image analysis support approaches that will allow for multi-parametric analysis of the spheroid models. While some such software is available it is most often designed for specific microscope platforms, is not customisable and not available as an open-source utility.

To this end we have developed a macro-code implemented as free software on the ImageJ platform which enables high-content screening of spheroid images.



The open-source macro, INSIDIA (INvasion SpheroID Imagej Analysis), automatically detects spheroids from background and derives several quantitative parameters from the images [8]. Some of the features of INSIDIA include:

- Batch processing of a large amount of images (estimated rate of 3s per image on an i5 core and 8GB RAM PC),
- Possibility for the operator to check different steps of the image preparation for an optimal subsequent analysis,
- Ability to detect and separate the cellular mass from the image background on grey scale images through implementation of Frangi filter [5],
- Geometrical description of the spheroids with parameters like area, perimeter, convex polygon and circularity,
- Detection of the core and invasive edges based on radial profiles of images and on maps of grey intensity gradients inside the spheroid [5, 6],
- Ability for the operator to get customisable parameters.

The utility of the 3D-spheroid-sprouting assay is high with applications in investigation of aberrant cancer biology and drug screening in the context of varying micro-environments. Our work is particularly focused on malignant gliomas which invade characteristically through white matter tracts, along perivascular channels or along ependymal and pial linings [7]. As such we are exploring variations in invasion as a function of matrix components such as hyaluronic acid and tenascin C (white matter tracts) or laminin and collagen (major components of the perivascular niche). Correspondingly, the impact of different cell types co-cultured in the spheroid or in the matrix is possible within the model.

Here we have highlighted our open-source macro, INSIDIA [8], that will expand the quantitative information that can be derived for the growth and invasion of cancer cells in a 3D-spheroid-, or even organoid invasion assay.

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