Determination Of Nanoparticle Uptake in Tumor Spheroids (DONUTS)

User Guide

Overview

This User Guide is intended for scientists working in the field of bio-nano research to assist in quantitative analysis of microscopy data of nanoparticle uptake in tumor spheroids. It uses publicly available MATLAB scripts available on GitHub (https://github.com/ElvPan/JCR-DONUTS-Analysis), developed by a multidisciplinary team. The peer-reviewed article for this work can be found under Ahmed-Cox, et al 2021, J Control Release.

Ahmed-Cox, et al, 2021

Please refer to the article above for correspondence, questions or assistance

Contents

Tables and Figures

List of Tables

List of Figures

1 Introduction to experimental and image acquisition parameters

The following subsections detail recommended experimental procedures to assist in designing experiments for the reliable and reproducible quantification of nanoparticle uptake in live tumor spheroids. This is intended as a guide and is by no means an exhaustive protocol for experimental design and acquisition parameters. The intention is that data generated can be inputted into the pipeline for the Determination of Nanoparticle Uptake in Tumor Spheroids (DONUTS) to quantify nanoparticle penetration and uptake kinetics.

1.1 Experimental setup recommendations

1.1.1 Spheroid model

Tumor cell spheroids, either with a single cell type, or with multiple cell types have been used in cancer research for several decades [1-5]. These can be prepared using an array of different methods, including low adhesion round-bottom well plates, hanging droplet, centrifugation and matrix embedding, which are comprehensively described and compared elsewhere [6-9]. For reliable imaging results of nanoparticle uptake, the initial protocol for generating spheroids must be highly reproducible. Reproducibility in our proof-of-concept study using single cell-type spheroids was determined by seeding density to a certain size (target of 500 µm), and roundness (a measure of regularity) after a set incubation time (72 hours under standard culture conditions). Size and roundness were quantified in Fiji (Is Just ImageJ), where custom packages are available and specifically designed for spheroid analysis [10]. Reproducibility will minimize downstream variation and reduce the number of samples needed for reliable and statistical comparison. It should be noted that the size of the spheroid model may influence the choice of imaging method. This is primarily due to light penetration and fluorescence detection, which is known to diminish after 150 µm, especially when using single photon microscopy [11]. In our study, we limited spheroids to 500 µm (250 µm

radius), with a red-shifted fluorescently labelled nanoparticle for increased penetration depth. Optimum spheroid size will depend on the microscopy tools available and should be addressed in early experimental design.

For the standard setup of analysis in DONUTS, it is also recommended to have a fluorescent marker of the cell membrane which helps to define the spheroid boundaries. This is used to generate a 3D mask at each timepoint, which defines the circumference and hence radii from the spheroid center when measuring particle uptake. Stable or transiently transfected cell lines with fluorescent inserts are perfectly applicable, if available. Alternatively, there are several live-compatible cell stains which are commercially available. In our study, we used Lipophilic Tracers (Molecular Probes®, Thermofisher Scientific, Australia) which come in a variety of wavelengths from 460 to 750 nm. These lipophilic tracers were added to cell suspensions prior to spheroid formation, had good retention, and direct transmission through three generations (~72 hours) of cell division. Alternatives to a membrane marker and automated spheroid boundary measurements can be found in Section 2.2.

1.1.2 Sample preparation

Imaging, particularly using a standard confocal system, is optimally conducted through glass [12]. In some cases, depending on preparation, spheroids may already be embedded in a 3D matrix in glass bottom-well plates [13,14]. In this case, sample mounting may be redundant, thus nanoparticles can be added directly to wells immediately prior to imaging (Section 1.2). In our study, spheroids were grown in low-adhesion round-bottom plastic-well plates, and therefore required a transfer to a compatible (glass-bottom) plate for imaging. It is important that spheroids are immobilized (to reduce drift during imaging) while retaining spheroid viability and 3D integrity. This is particularly pertinent if conducting time-course imaging on several spheroids simultaneously. For example, we imaged six to eight spheroids (one per well) in a glass-bottom 24-well plate (Cellvis LLC, USA). Spheroids can be effectively

embedded in sterile, molten 1% low-melt agarose (Sigma Aldrich), and then immersed in phenol red free media (500 µL DMEM + 10% FBS for a 24-well plate; Life Technologies, Australia) with nanoparticles at required concentrations. Media or PBS (500 uL for a 24 well plate) is recommended to be added to all outer wells in the plate to reduce evaporation and drift during imaging. Sample preparation for imaging should be examined using methods described in Section 1.3 on quality control.

1.2 Acquisition setup recommendations

Once sample preparation has been optimised and validated, we move to imaging optimization. Initial considerations for acquisition fall under the necessary equipment for live, and ideally automated multi-position imaging. This optimally includes an incubation chamber and automated mechanical stage, with software capable of saving and switching between multiple set positions in X, Y and Z. For example, imaging to develop DONUTS was conducted on a Zeiss LSM 880 inverted laser scanning confocal microscope with incubation (37°C) and 5% CO_2 using a Plan-Apochromat 10x/0.45 M27 objective and multiblock acquisition in Zen (Zeiss, Germany). This enabled time-course acquisition of multiple spheroids in XYZ, as well as brightfield images at each time point. The interval between each time point is user defined, and will depend on acquisition speeds, pixel resolution and z-stack distance. It is recommended to prioritize z-stack acquisition to capture the spheroid hemisphere at minimum in Z, followed by a compromise between pixel resolution and time interval for imaging.

For our proof-of-concept study, single photon excitation with frame fast alignment of hardware in two channels enabled the consecutive acquisition of six spheroids with 270 µm total z-stack accumulation per spheroid within a 30-minute acquisition window. Depending on the intention of the user, this could also be extended to a greater number of spheroids for

higher throughput studies (at the expense of resolution) or preferentially increase temporal / spatial resolution for deeper, mechanistic studies of uptake.

1.3 Quality control for experimental setup

To image a dynamic process (e.g., nanoparticle uptake) in live spheroids, it is pertinent to validate spheroid viability following sample preparation and that they retain their morphological features (3D shape, size). For viability, we used a standard live/dead stain with Calcein AM and Ethidium Homodimer-1 (Molecular Probes). This confirmed continued active proliferation at the spheroid periphery, and some cell death at the core, as is typically observed in 3D spheroid tumor models (such as hypoxia, necrosis [3,15]) (Figure 1).

The retention of morphological characteristics is also critically important to prevent bias or error in nanoparticle uptake measurements. Changes in the 3D integrity of spheroid morphology can be a by-product of sample preparation, seen in Figure 2b and 2e, where spheroids were flattened by poor sample preparation (Figure 2a), compared to spheroids which were embedded in molten 1% agarose as described above (Figure 2d) and therefore retain 3D morphology at various seeding densities (Figure 2c and Figure 2f).

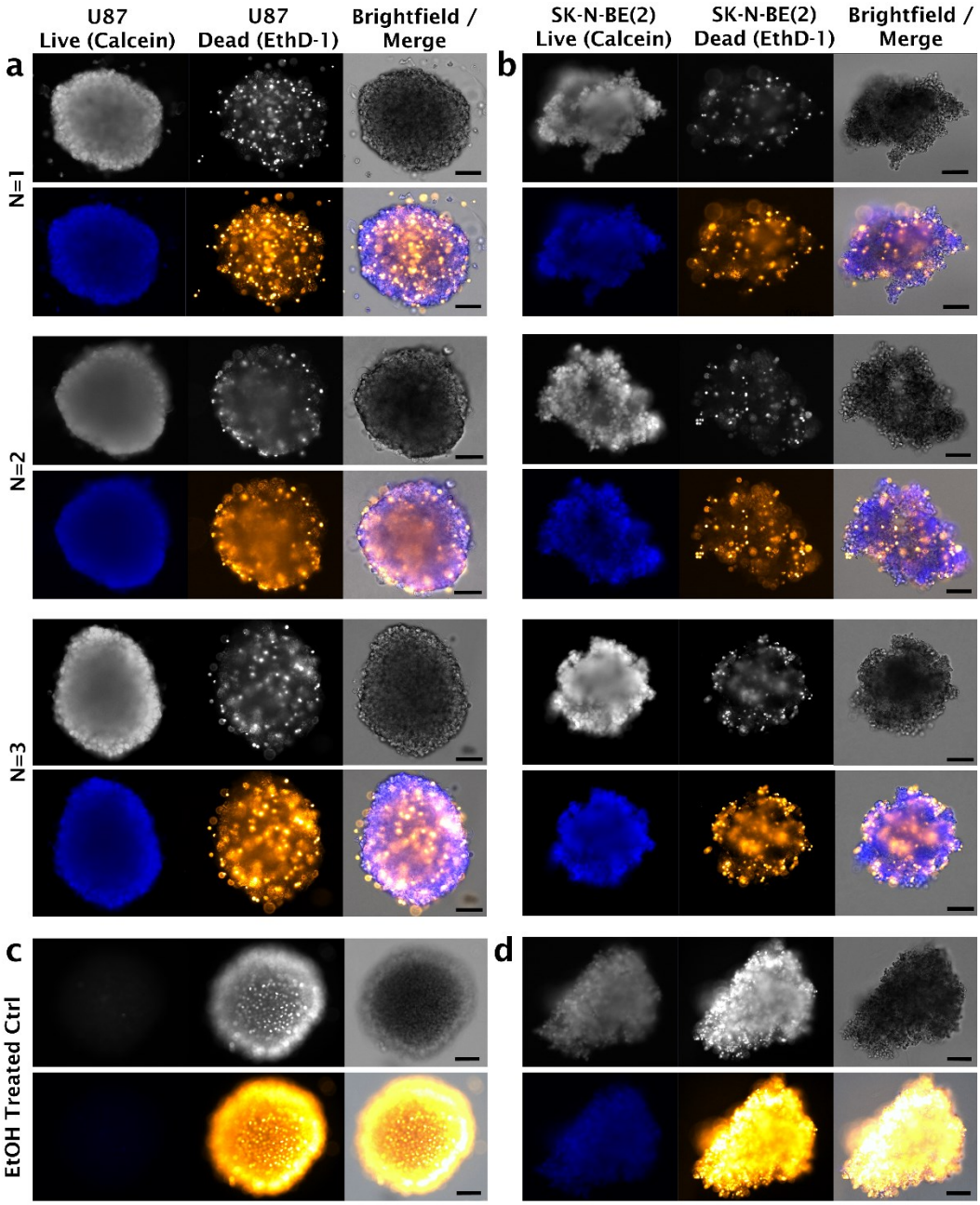

Figure 1: Spheroid viability and characteristics visualized through a live/dead assay using Calcein-AM and ethidium homodimer-1 (EthD-1). Representative brightfield and live (blue)/dead (orange-yellow) images of (a) U87 and (b) SK-N-BE(2) cell spheroids, which were grown for 3 days in low adherent round-bottom well plates with an initial seeding density of 2×10^3 cells. Viability is contrasted against ethanol (EtOH) treated controls for (c) U87 and (d) SK-N-BE(2) respectively. Fluorescence intensity per channel is consistent across all panels to ensure visualisation without saturation. Scale bar, 100 µm. From Supplementary Data (Ahmed-Cox, *et al* 2021, J Control Release).

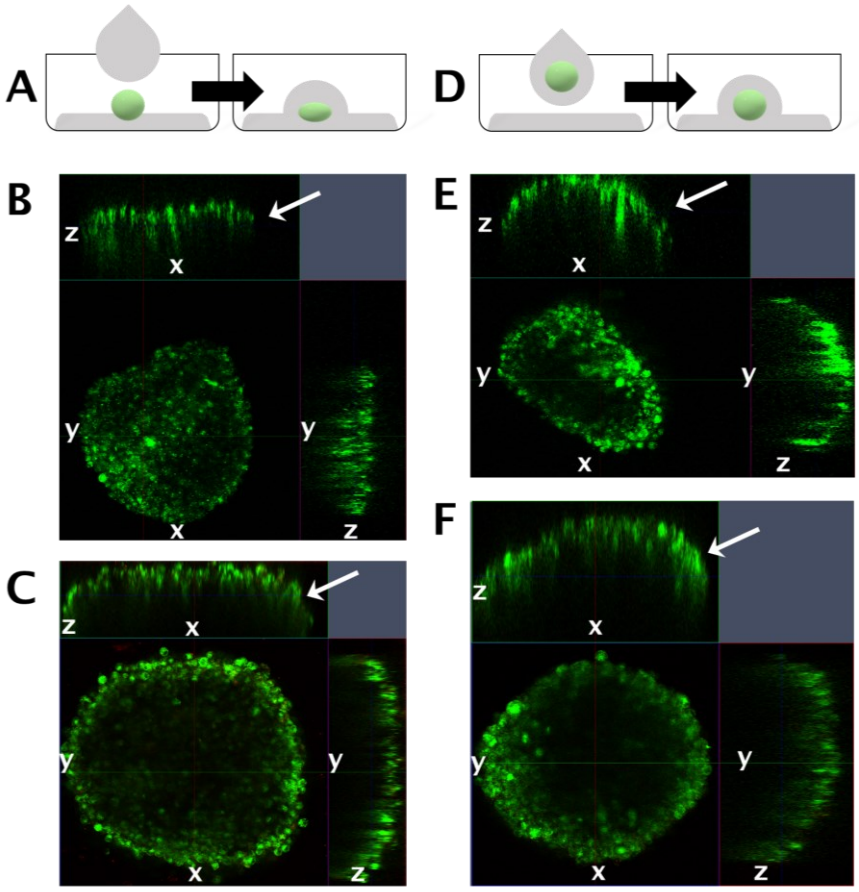

Figure 2: Determination of optimal sample preparation during spheroid imaging to preserve 3D isotropy. (a) Preliminary sample preparation where spheroids (green) were placed between two layers of 1% low melt agarose (grey). **(b)** U87 spheroid seeded at 1 x 10^3 and **(c)** 2 x 10^3 cells with membrane dye (DiI) for 72 hours before sample preparation by method in (A). **(d)** Refined sample preparation where spheroids (green) were immersed in molten 1% low melt agarose and gently "dropped" onto a second layer of agarose in the well. **(e)** U87 spheroid seeded at 1 x 10^3 and **(f)** 2 x 10^3 cells with membrane dye (DiI) for 72 hours before sample preparation by method in (D). Images are the mid-plane and orthogonal projections (X, Y and Z) of single photon confocal z-stacks of the spheroid hemispheres. White arrows highlight the shape of the spheroid in XZ, with the curvature indicating the impact of sample preparation.

2 Analysis for the Determination Of Nanoparticle Uptake in Tumor Spheroids (DONUTS)

2.1 Overview and summary

The DONUTS platform covers several analysis scripts to measure nanoparticle uptake in tumor spheroids which draw upon previous work [16], and were henceforth designed and validated in Ahmed-Cox, *et al* 2021, J Control Release. The primary script, titled DONUTS_3D_T, measures nanoparticle uptake in 3D tumor spheroids and is designed to handle four-dimensional (4D) data files, corresponding to 3D data across time (*t*) (Figure 3a and Figure 3c, detailed Section 2.3). An accessory script, titled DONUTS_2D_T, builds upon previous work presents the early 2D iteration of our analysis platform, and quantifies nanoparticle uptake in a user defined z-slice (2D plane) across time (*t*) (Section 2.4).

There are also several scripts to extend and support the DONUTS package (detailed in subsequent sections) that include: 1) kinetic analysis scripts (Figure 3e and Figure 3g, detailed in Section 2.5 and Section 2.6), 2) a suite to simulate nanoparticles in spheroids for validation and modelling (Figure 3b, Figure 3d and Figure 3f , detailed in Section 2.7.1), and 3) the script for quantifying nearest neighbor distances of nuclei in spheroids at a single timepoint (*t*) (Figure 3h, detailed in Section 2.7.2). For validation, there are also four test datasets which have been uploaded to Figshare and can be accessed in the relevant sections below, and in Table 1.

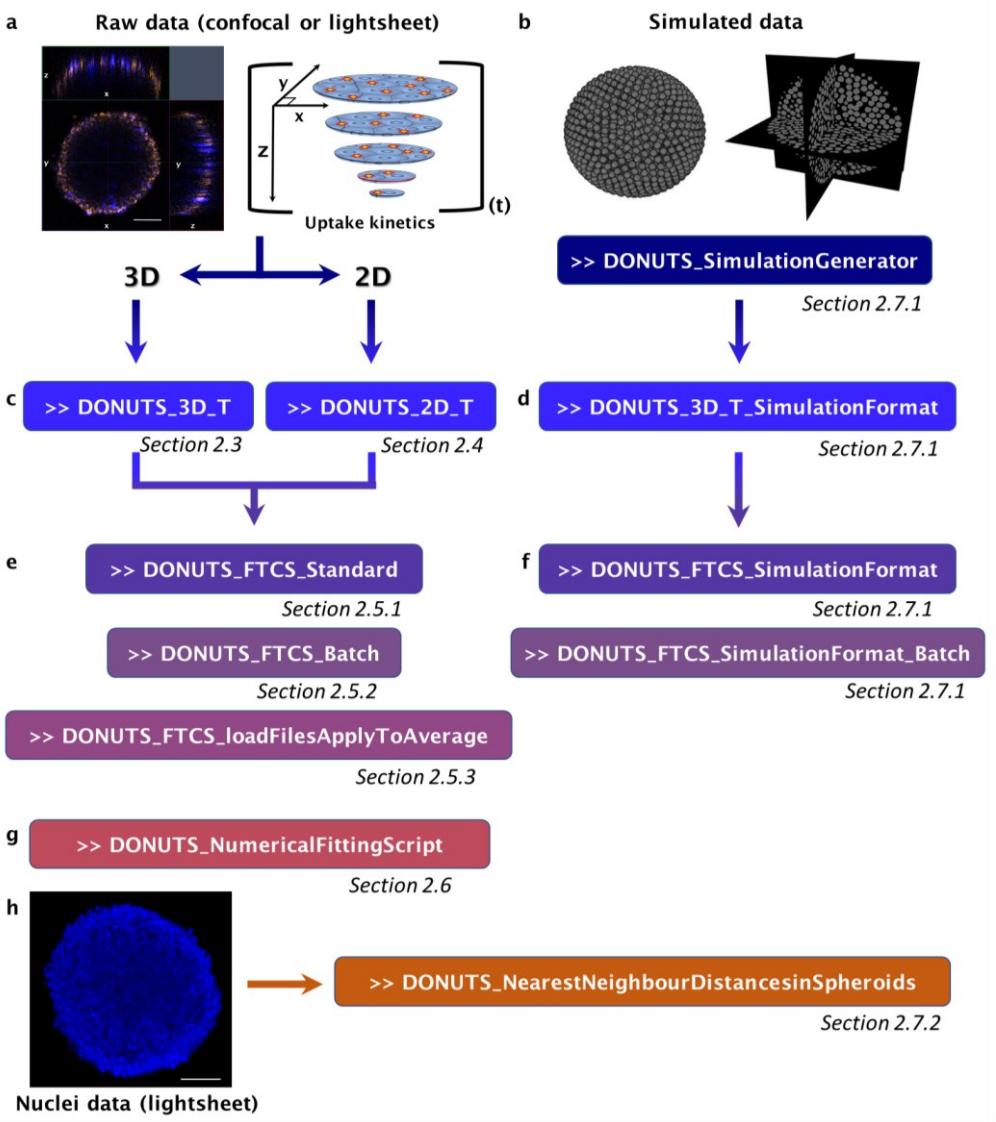

Figure 3 Workflow for the Determination of Nanoparticle Uptake in Tumor Spheroids (DONUTS) scripts and associated analyses. DONUTS requires either **(a)** raw imaging data of nanoparticle uptake in 2D z-slices of a spheroid, or across 3D data and time or **(b)** simulated data using the DONUTS_SimulationGenerator script. For analysis of nanoparticle uptake over distance and time using **(c)** biological imaging data, users are directed to either DONUTS_2D_T, or DONUTS_3D_T, or for **(d)** simulation data, DONUTS_3D_T_SimulationFormat. **(e)** For downstream kinetics, users have the option of several iterations of DONUTS_FTCS scripts, the Standard, Batch and ApplytoAverage versions, or **(f)** the simulated counterparts, in Standard or Batch. **(g)** For biological data, users may instead elect to run a computational model of nanoparticle uptake kinetics using DONUTS_NumericalFittingScript. **(h)** Accessory analysis can be conducted by computationally segmenting spheroids to measure nearest neighbor distances between adjacent nuclei using DONUTS_NearestNeighborDistancesinSpheroids.

Table 1 Figshare links to relevant test datasets for validation of DONUTS scripts

Dataset	Test for Section	Figshare Link
A	2.3 – DONUTS_3D_T	https://figshare.com/s/b39a40af214fcf691f71
B1, B2	2.4 – DONUTS_2D_T	https://figshare.com/s/d01a278923d9a8165786
C	2.7.1 – Simulation Package	https://figshare.com/s/56bd1cc2f3e5efec0b33
D	2.7.2 – DONUTS_NND	https://figshare.com/s/fc3a63136bad16ac17f5

NND, Nearest neighbor distance

2.2 Initial MATLAB setup

The DONUTS package of scripts run in MATLAB and require standard installation (with standard toolbox including Elmat, Uitools, Datafun, Ops, Polyfun, Datatype (tabular), Iofun packages) and added to it Curve fitting, Optimization, and Image Processing Toolboxes. The core of the scripts uses OME Bioformats to read in imaging files [17] (https://docs.openmicroscopy.org/bio-formats/5.7.1/developers/MATLAB-dev.html) and have been modified for input compatibility with all major imaging systems (Zeiss, Leica, Olympus, Nikon). Default output of analysis is typically as a Microsoft Excel spreadsheet (for downstream analysis), a raw MATLAB workspace (.mat file) for downstream scripts (particularly in Section 2.7.1) and occasionally some visual accompaniment (either as an image or video). Once installation is complete, users may proceed to mapping paths to DONUTS scripts and commencing analysis. Moving forward, important steps during setup and analysis will be marked by an arrow in text, as below.

- **User Input Required:** Users must first Set Path, to map the folders and subfolders that contain necessary scripts.

For users unfamiliar with MATLAB, please refer to Figure 4 which provides a visual guide to assist initial setup which requires mapping of the relevant folder with subfolders of analysis scripts. Under the HOME Tab, click on the Set Path icon (in orange) and then select Add with Subfolders (in blue) to search the local drive for location of analysis scripts. The subfolder should contain the DONUTS scripts, and any accompanying scripts, including those detailed in Acknowledgements (Section 4). Once users are satisfied with the path, select the folder (in purple) and Save and Close (in pink) to proceed to initiating the analysis.

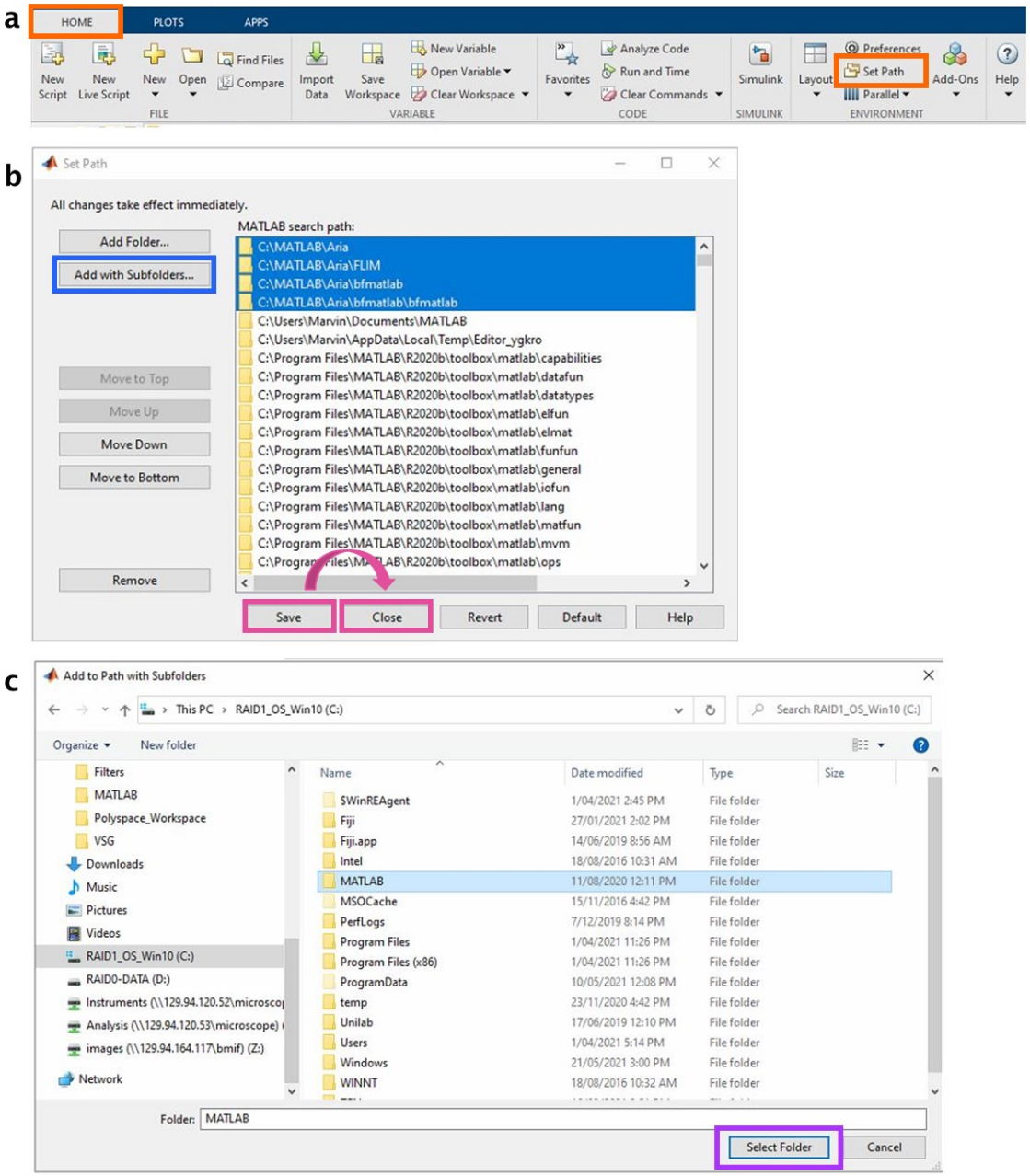

Figure 4 Familiarization with the MATLAB user interface and setting a working path. **(a)** Under the HOME Tab (in orange), click on Set Path to open a dialog box in **(b)** and then select Add with Subfolders (in blue). This will open a second dialog box in **(c)** which enables users to search for the folders containing analysis scripts, select those folders (in purple) and then return to (b) to save changes and close the dialog boxes (in pink).

- **User Input Required**: Select Run and choose the file you would like to analyse.

After double clicking the analysis script file on the left-hand side of the window, MATLAB will automatically open the EDITOR Tab. Select Run (in orange, Figure 5) to initiate the script that is opened in the Editor window and proceed with analysis.

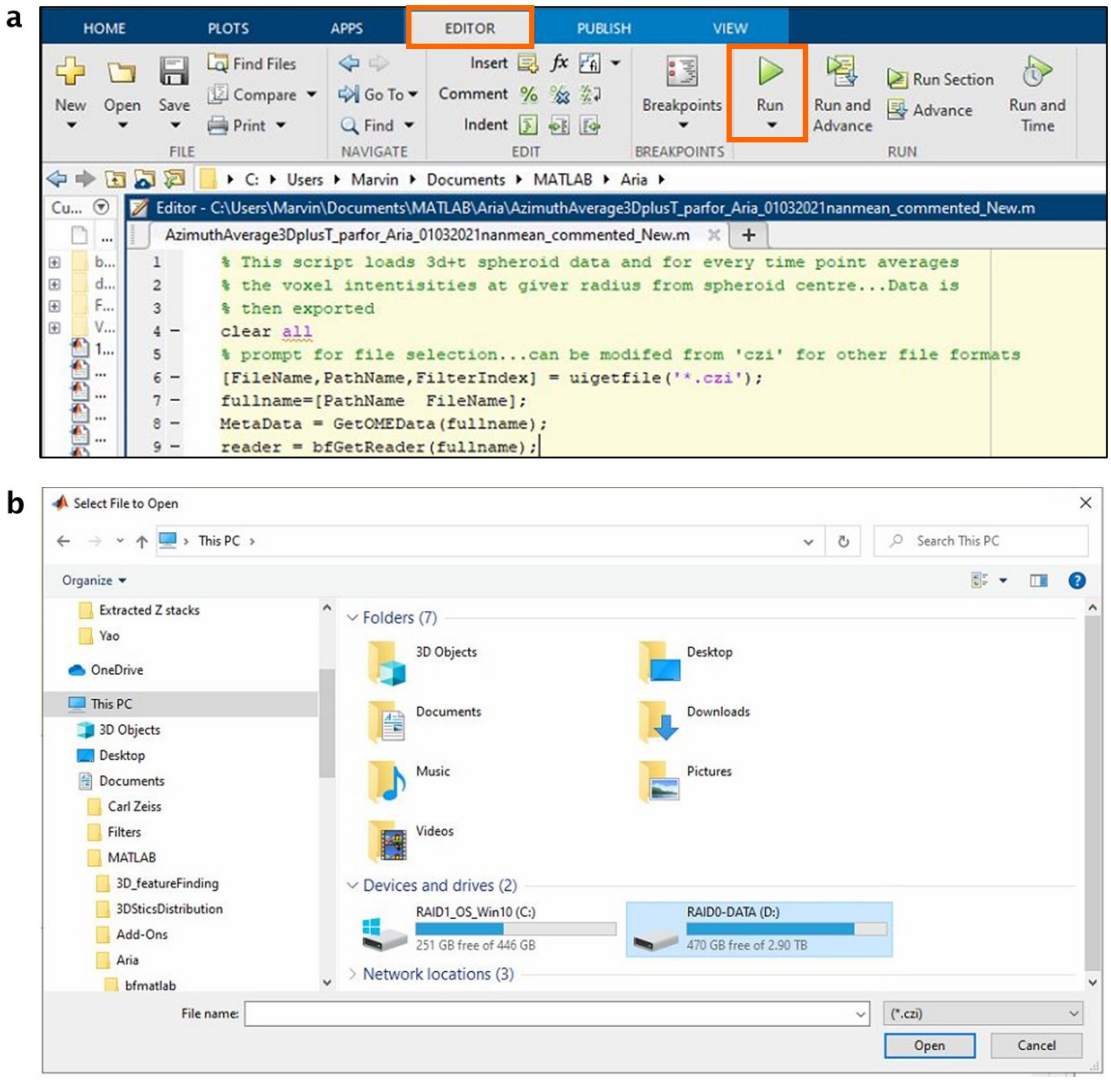

Figure 5 DONUTS Initiation. **(a)** Under the EDITOR Tab, select Run to initiate analysis script open in the Editor window. A dialog box will open **(b)** to select the file to analyse. File selection prompts MATLAB to load the data and proceed with analysis.

2.3 DONUTS_3D_T: Step-by-step walkthrough with comments

The following is a step-by-step guide to assist users for the DONUTS_3D_T analysis script, and follows installation of MATLAB, and relevant toolboxes described above.

For DONUTS_3D_T, it is recommended to run the script on the raw data files and follow user friendly prompts as the script progresses through analysis. For raw data files, MATLAB will automatically read in metadata (such as pixel resolution in XY and Z). The file size, number of time points and computer specifications used for data analysis will impact how long each file takes to analyse. For reference, files of 25 – 35 Gb (total of 24 time points per file) analyzed on a computer with dual 2.60 GHz processors, 128 Gb RAM and a 64-bit operating system will take 15 – 90 minutes to complete analysis on each file. The output data is in the form of an easy-to-manage Microsoft Excel spreadsheet which can be used to visualize trends in nanoparticle uptake data across distance (r) and time (t) and enable further analysis for nanoparticle kinetics (described in Section 2.5 and 2.6).

- **User input required**: Select Run and chose the file you would like to analyse.

MATLAB will first prompt for file selection (recommended to be on a local drive) and will automatically load data from the first time point as well as any relevant metadata (Figure 5).

A test dataset (Test Dataset A) has been provided for validation of DONUTS_3D_T and downstream analyses scripts. This has been reduced to a file with six timepoints covering 2 – 12 hours of nanoparticle (30 nm size) uptake in a tumor spheroid which takes approximately 15 minutes to analyse.

- **User input required**: Define boundaries and 3D center of spheroid.

As established in Section 1.2, data acquisition of nanoparticle uptake into tumor spheroids over time was established with a membrane marker in channel 1, and nanoparticle

fluorescence in channel 2. Initially, the script will use data from channel 1 (membrane channel) of the first timepoint (t_1) to define the boundaries and center of the spheroid. For some experiments, or by user choice, fluorescence signal from the nanoparticle channel can also be used to define the spheroid boundaries. In this case, it is recommended to be a static boundary defined at a set timepoint (i.e., the final timepoint tested, t_n), which can then be converted to a binary 3D mask and retrospectively, applied to the full time-course of nanoparticle uptake. Cautions arise from possible shifts or growth of the spheroid over time, which means that the 3D mask of the spheroid at t_n is likely to be over-estimated and may influence results. There is also the option for users who may be inclined to manually define the spheroid boundaries at certain intervals over time. In both instances, users are welcome to contact the authors for assistance in implementing a manual mask compatible with the DONUTS pipeline.

Assuming data is available from channel 1, user input will be required to select the boundary of data containing the spheroid which is done using the pop-up box “Please select range containing spheroid” (Figure 6a) with a simple click and drag selection tool. Users will then be prompted to select the spheroid center in XY (Figure 6b) and YZ (Figure 6c) using a crossline target. Users will note that in YZ, sample data has only been captured to the equatorial (mid-plane) of the spheroid, thus showing a spheroid hemisphere in Figure 6c.

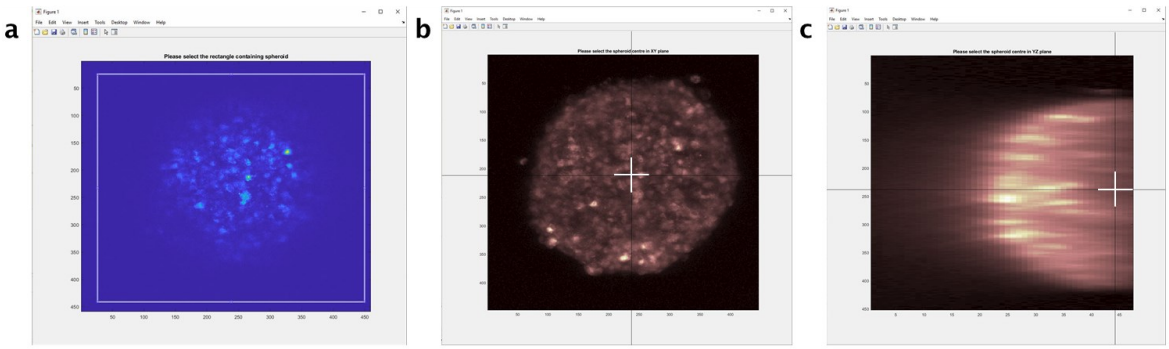

Figure 6 Defining boundaries and center of spheroid data. (a) Selection of the image boundary containing the spheroid. It is recommended to allow a minimum 30% margin from the edge of the visible spheroid. **(b)** Crosshair guide to select center of the spheroid in XY and **(c)** in YZ (in white for ease of visualisation). Note for YZ, only one hemisphere of the spheroid was acquired, thus the center presented is the maximum distance of detectable fluorescence to the right-hand side of the image.

- **User input required:** define the pixel size in XY and Z.

A pop-up dialog box will then ask for input on the pixel size in XY and Z in micrometers (appears as "mum" in the dialog box) (Figure 7). For raw data files, check that the metadata has been correctly imported. For cases where metadata may be unavailable or unrecognized, users have the option to adjust or manually input the pixel size, which is important for correction of the point spread function in Z when calculating radial distances in 3D.

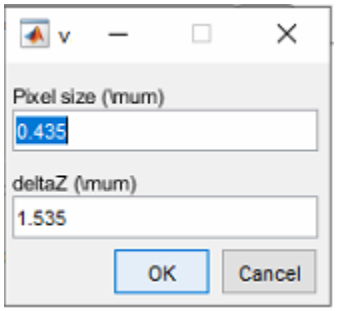

Figure 7 Pop-up dialog box requesting user input and/or validation of metadata for pixel size in XY and Z.

- **User input required**: Enter channel to analyse for nanoparticle uptake.

The final pop-up dialog box prior to initiation of analysis asks for user input to confirm the channel, which contains the nanoparticle fluorescence over time, and on which the DONUTS_3D_T analysis will be applied. By default, this is set to channel 2 (Figure 8).

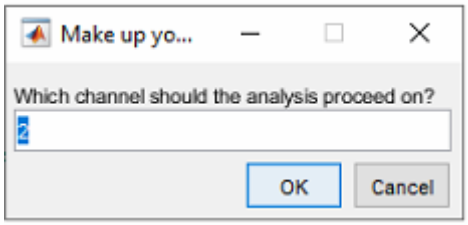

Figure 8 Pop-up dialog box which requests user input and/or validation for which channel to proceed with analysis (default = channel 2).

The script will then scale data (default set to 3 = one third of original size) to reduce computational load, and then initiate parallel pool (Parpool), which divides computational work across multiple "workers" simultaneously. This will be defined by settings within MATLAB which can be accessed using the Parpool link at the bottom, left-hand side of the MATLAB window (Figure 9a). While Parpool accelerates analysis, it will be limited by CPU memory, which is important to account for during user setup and can be adjusted by opening Parpool preferences (Figure 9b). More information on Parpool setup and troubleshooting can be found in Section 3.5.

If no errors are encountered, script will proceed to run analysis. Output is generated as a Microsoft Excel file which contains six tabs of raw and resampled analysis detailed in Table 2.

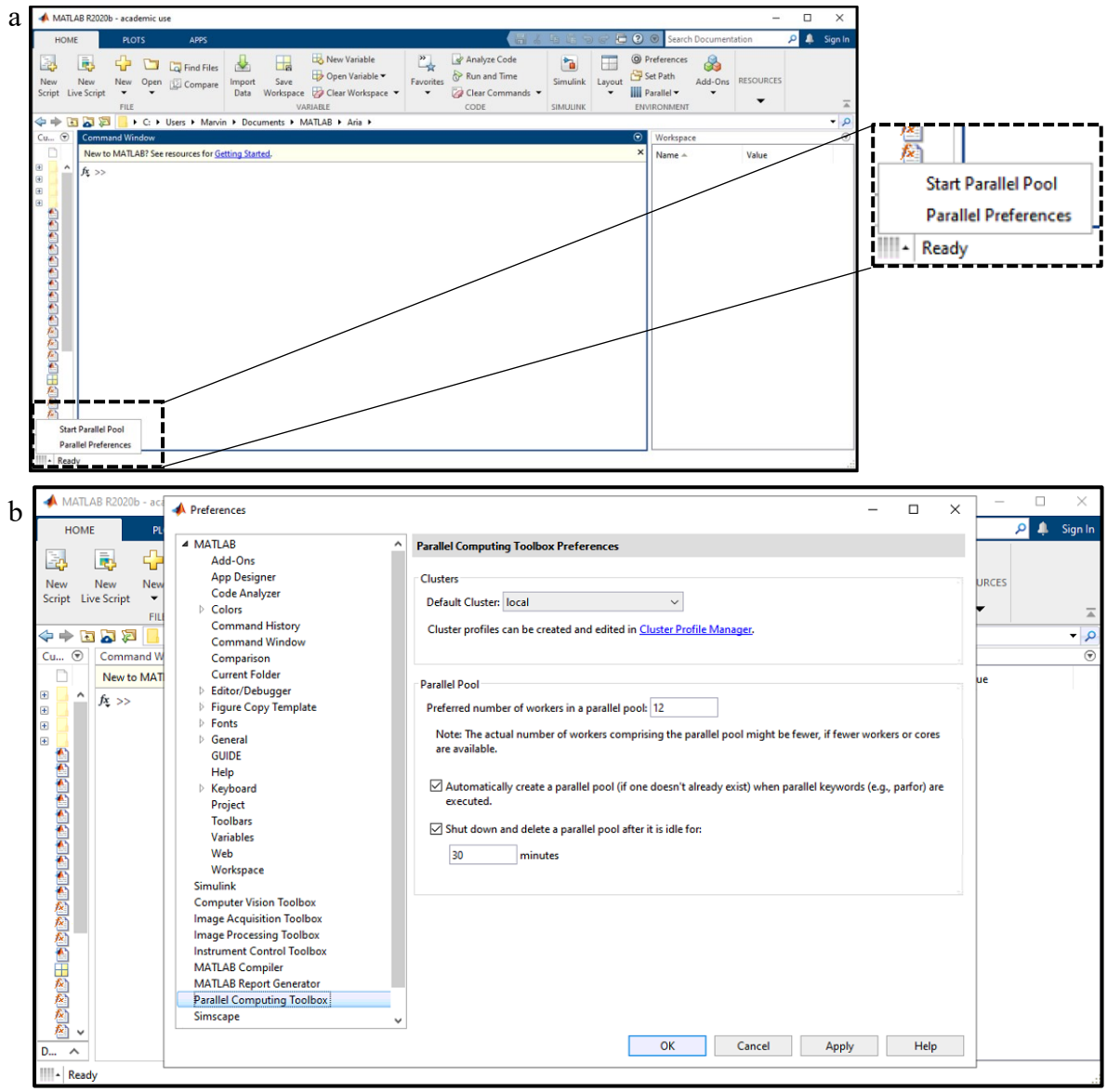

Figure 9 Parallel computing (parpool) access and preferences. (a) Access parpool by left clicking on the four-gradient grey tab in the bottom left-hand corner of the MATLAB window. **(b)** After selecting Parallel Preferences, a pop-up window will open to adjust parpool preferences.

Table 2 DONUTS_3D_T output generates a Microsoft Excel file with six tabs, the contents of which are detailed below.

Abbreviated Tab	Full Tab	Detail of Contents
R	Radii *r*	Radii of original data (in pixels) from core to spheroid circumference
rangeR	Resampled radii *r*	Resampled radii (in pixels) according to user defined sampling rate in line 10 of DONUTS. Default = 10 μm
SumCh2	Sum of channel 2	The sum (accumulated) fluorescence signal at each R (per row) for each time point (*t*=1, 2, …, *n*) labelled as tabSum_*t*
SumCh2resamp	Resampled sum of channel 2	The sum (accumulated) fluorescence signal at each RangeR (per row) for each time point (*t*=1, 2, …, *n*) labelled as tab2Sum_*t*
AveCh2	Average of channel 2	The average fluorescence signal at each R (per row) for each time point (*t*=1, 2, …, *n*) labelled as tabAve_*t*
AveCh2resamp	Resampled average of channel 2	The average fluorescence signal at each RangeR (per row) for each time point (*t*=1, 2, …, *n*) labelled as tab2Ave_*t*

2.4 DONUTS_2D_T: Step-by-step walkthrough with comments

In the development of DONUTS_3D_T, authors initially began with a radial averaging script designed to be applied in 2D (XY) to a single z-stack or series of z-stacks, such as that used previously [16]. Like DONUTS_3D_T, DONUTS_2D_T is optimally applied to raw imaging data to calculate nanoparticle uptake across a single or series of z-stack slices over time (*t*).

- **User input required**: Select the script by name 'DONUTS_2D_T.m' and click on Run.
- **User input required:** Choose the file you would like to analyse.

DONUTS_2D_T is designed to read in raw imaging data which can be either in 3D across time (*t*) or on a single 2D (XY) plane across time. Two test datasets (Test Dataset B1 and B2) have been included to trial this script, both from the sample primary dataset (Test Dataset A), at two different depths in Z (core – z120, and adjacent – z100) .

- **User input required**: Enter the z-slice range (first slice to last slice) that you would like to analyze.

Because this script is designed to run on single z-slices, users are required to input the range of z-slices that analysis will proceed on (Figure 10). It is recommended for users to analyse z-slices at the spheroid mid-line (equator) for the most accurate measurement. Each z-slice in this range will be analyzed and generate a separate sheet in a Microsoft Excel file. If running analysis on 2D data across time (*t*), the slice range will be displayed as "1 to 1".

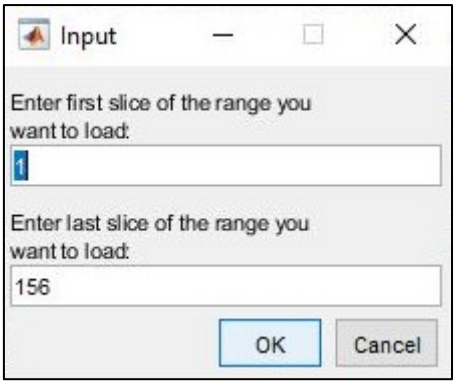

Figure 10 Pop-up dialog box which requests user input to define the z-slice range (first to last) to be loaded for analysis.

- **User input required:** Define 2D center of spheroid.

Like DONUTS_3D_T, this script requires user input to define the center of the spheroid, using a crossline target (Figure 11).

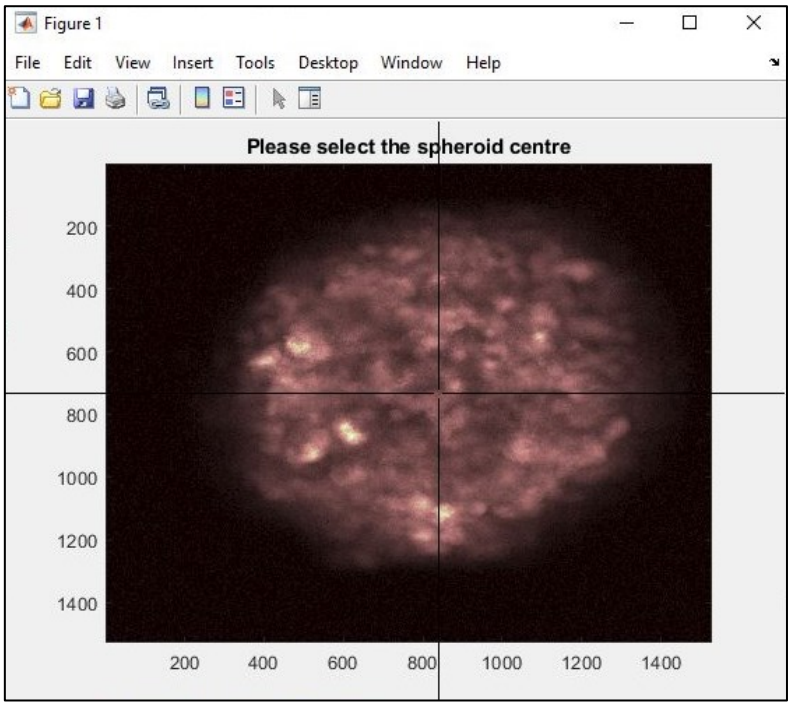

Figure 11 Pop-up dialog box which requests user input to select center of the spheroid in XY using a crosshair guide.

Once selected, script will proceed with analysis. Output data will appear in a Microsoft Excel file, where the sheet labelled channel 1 is distance (in pixels per time *t*), channel 2 is intensity from channel 1 in raw data, and channel 3 is intensity from channel 2 in raw data, both in relative fluorescence units (RFU) per time, *t*. These will be catalogued according to z-slice if multiple slices were selected for analysis.

- **User input required**: Repeat parameter setup for subsequent datasets.

Once one dataset has been analyzed, users will be prompted to select the z-range (Figure 10) and spheroid center (Figure 11) of the next dataset, and so on until analysis on all datasets selected is complete. For files on a computer with specifications as mentioned above, analysis of each slice takes less than two minutes.

2.5 DONUTS_FTCS Package

Downstream from DONUTS_2D/3D_T are several scripts which can be applied to the output previously generated from either Section 2.3 or 2.4 to evaluate nanoparticle penetration kinetics. One of the scripts is a forward in time, central in space (FTCS) diffusion model [18] often employed in fluorescence recovery after photobleaching (FRAP) [19]. Several versions of this script are included in the DONUTS analysis portfolio, which enable standard analysis on one file, batch processing or averaging across several replicates and the processing of an average diffusion coefficient, will be explained below.

There are also two versions in the FTCS package compatible with simulated data. These are explained under the simulation packaged in Section 2.7.1.

2.5.1 DONUTS_FTCS_Standard: Step-by-step walkthrough with comments

The script DONUTS_FTCS_Standard is the standard script for analysis of nanoparticle diffusion by the FTCS model of one output excel file from either DONUTS_2D_T or DONUTS_3D_T.

- **User input required**: Confirm sheet names for data and radial distance.

Prior to initiating the script, users should note the sheet names for both the data (Line 10, default = ch3resamp) and radial distance (Line 11, default = rangeR) and adjust to match the sheet names for the data to be analyzed (Figure 12). For instance, if using the resampled output from DONUTS_3D_T, sheet name for data should be adjusted to AveCh2resamp, the sheet containing the averaged, resampled data for channel 2, while the correct sheet name for radial distance will remain rangeR.

```
dat=(num.data.ch3resamp)        %sheet name for data
r=(num.data.rangeR(:,1));       %sheet name for distance
```

Figure 12 User input required to adjust sheet name for data and radial distance in the DONUTS_FTCS package, with text to adjust highlighted in bold above.

- **OPTIONAL User input required:** Transpose and transform distance data

Prior to initiating DONUTS_FTCS_Standard and associated scripts in the DONUTS_FTCS package, users are also required to transpose the distance data in the rangeR sheet or equivalent from a single row to the first column in the sheet. It is also recommended to transform this data from pixels to micrometers so that the diffusion coefficient (in pixel size^2 / t) is calculated in the correct units (μm^2 / t).

- **User input required**: Select Run and choose the file you would like to analyse.

After adjusting parameters above, users may initiate the script. MATLAB will proceed to take intensity data and calculate a diffusion coefficient according to the FTCS model at each defined radial distance, from the core of the spheroid to the circumference. Details of these calculations are available in the methods of the primary article for this work (see Ahmed-Cox, *et al* 2021, J Control Release). On a computer with the specifications defined in Section 2.3, MATLAB will complete analysis in less than two minutes, and write an output file

(Microsoft Excel, approximately 5 Kb) to the same location as the input file labelled as "Diffusion Coefficient_[input file name]".

Information on the data exported is detailed in Table 3. In brief, data under Variable 1 (Var1) will correspond to the diffusion coefficient (in pixel size^2 / t or μm^2 / t) at each radial interval (default = 10 μm), from the core of the spheroid (r = 0, to the circumference $r = n$). Var2 corresponds to the goodness of fit (R^2 value) and Var3 to the adjusted goodness of fit (AdjR^2) for the adjacent diffusion coefficient in column Var1.

Table 3 DONUTS_FTCS_Standard output generates a Microsoft Excel file with three columns, the contents of which are detailed below.

Abbreviated Column	Full Column	Detail of Contents
Var1	Variable 1	The diffusion coefficient (in pixel size^2 / frame time t or μm^2 / t) according to the initial input of the radial distance (r) and time interval (t).
Var2	Variable 2	The goodness of fit (R^2) value of the diffusion coefficient in the adjacent row.
Var3	Variable 3	The adjusted goodness of fit (AdjR^2) value, which is the R^2 corrected by the degrees of freedom and sample size of the diffusion coefficient in the adjacent row.

2.5.2 DONUTS_FTCS_Batch

The DONUTS_FTCS_Batch script is identical to the standard script, with the single extension that it allows users to multi- (batch) select files for analysis. This is useful in instances where users may have several data files from DONUTS 2D / 3D_T that all require processing for diffusion coefficients.

- **User input required**: Confirm identical sheet names for data and radial distance and transpose or transform radial distances.

Users are required to follow the initial setup of sheet names (data and radial distance, *r*) as well as transposition and transformation of sheet containing data for *r* as described in Section 2.5.1. It is important to note that the sheet names for all files should be identical.

- **User input required**: Select Run and choose the files you would like to analyse.

This will initiate the analysis and output one file for each input file, as described above. Output will be identical to that defined for FTCS_Standard (Table 3), with one Microsoft Excel file for each input file analyzed.

2.5.3 DONUTS_FTCS_loadFilesApplyToAverage

As part of the validation of DONUTS_2D / 3D_T, raw data from our proof-of-concept study was analyzed iteratively (minimum of three times) to reduce variation from user selection of the coordinates of the spheroid core in XY and Z. This version of the DONUTS_FTCS script is designed to load multiple files, average the intensity data and apply the FTCS diffusion model to the average.

- **User input required**: Confirm identical sheet names for data and radial distance and transpose or transform radial distances.

As with other versions of the DONUTS_FTCS package, user input is required to transpose and transform data in *r* as well as confirm the sheet names for data and radial distance *r* and adjust if required. Like DONUTS_FTCS_Batch, this script requires the sheet names for data and radial distance to be the same between separate files and has the same requirement for the selection of multiple files.

Output from DONUTS_FTCS_loadFilesApplyToAverage will generate only one Microsoft Excel file with variables as defined in Table 3.

2.6 DONUTS_NumericalFittingScript: Step-by-step walkthrough with comments

As demonstrated in the primary paper related to this work, the FTCS model had poor fit to the fluorescence data of nanoparticle uptake in spheroids derived from scripts in Section 2.3 and Section 2.4. To address this, a data-driven, computational diffusion model was designed using MATLAB, here termed DONUTS_NumericalFittingScript. Details on the design of this analysis can be found in the Methods of Ahmed-Cox, *et al* 2021, J Control Release.

- **User input required**: Open analysis script in MATLAB and confirm sheet names for data and radial distance.

Like the DONUTS_FTCS package of scripts, this script requires user input to manually adjust the sheet names for data and radial distance. These can be found under `inputData` (Line 36) and `coordinates` (Line 37), respectively. By default, the sheet names for the data and radial distance are 'AveCh2resamp' and 'rangeR', respectively.

- **User input required**: Select Run and input parameters requested from dialog boxes.

Once users are satisfied with hard written parameters, you can select Run. MATLAB will then prompt for the particle size (nm), the number of time points and the time interval (Figure 13). For users, note that Test Datasets A or B1, B2 contain data with 30 nm particles.

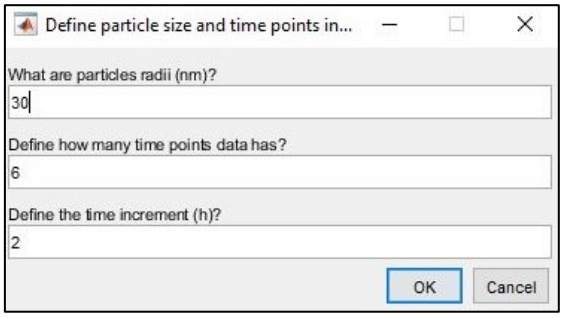

Figure 13 DONUTS_NumericalFittingScript user input required to define parameters including the diameter of the particles (nm), the number of time points and the time increment.

Once parameters have been confirmed, MATLAB will proceed with numerical fitting using the biological data to derive diffusion kinetics. Of note, there are several hard written parameters, including maximum spheroid size and number of time points which can be adjusted. Users are welcome to contact authors for assistance if needed.

Once complete, MATLAB will generate data of numerical fit versus biological data, and the diffusion (in units^2 / t) over the spheroid distance (r), from core to circumference. Diffusivities can be found in the excel spreadsheet, and output MATLAB file for graphing externally, should the users desire. Total run time on a computer with specifications detailed above is less than five minutes. Details of DONUTS_NumericalFittingScript can be found in Table 4.

Table 4 DONUTS_NumericalFittingScript output generates two graphs (.png), an excel spreadsheet for kinetic data and saves the MATLAB workspace (.mat), the contents of which are detailed below. Each file is saved under the input file name with a suffix as indicated.

Output File	File pre/suffix	Detail of Contents
Image (.png)	Out1	Plot of the raw input versus numerical solution in fluorescence intensity over distance (r).
Image (.png)	Out2	Plot of the diffusion kinetics calculated from analysis (in units^2 / t) over the spheroid distance (r).
Excel (.xlsx)	NumFit	The diffusion kinetics calculated under `diffusivity`, and spheroid distance (r) under `radius`.
MATLAB workspace (.mat)	Output	The total workspace, with diffusion kinetics calculated under `diffusivity`, and spheroid distance (r) under `spheroidGrid`.

For interest, batch processing is built into this script, but users should note that only files of the same particle size should be batch analyzed, as the particle size is integrated as a parameter during numerical fitting. The sheet names for data and radial distance will also need to be identical.

2.7 Additional analysis and validation packages

To support the quantification of nanoparticle uptake and penetration kinetics in tumor spheroids as described above, authors have included two accessory scripts in the DONUTS platform: 1) a simulated dataset package and compatible versions of both DONUTS_3D_T and DONUTS_FTCS and 2) the analysis script used to quantify nearest neighbor distances of adjacent nuclei in cleared tumor spheroids, stained with DAPI and imaged using lightsheet microscopy. The uses of these scripts are detailed below.

2.7.1 DONUTS simulation package

As briefly mentioned above, a simulation package was designed and included in Ahmed-Cox, *et al* 2021, J Control Release as an approach to both validate DONUTS analysis on biological data and complement evaluation of particle kinetics in convolved cell models. To simulate spheroids and generate initial configuration of cells in spheroid we use scripts developed by Persson and Strang [20] and have included them in the Appendix.

- **User input required:** Open DONUTS_SimulationGenerator and select Run.

To generate a simulated dataset of particle uptake in a spheroid "object" users must first initiate the simulation generator above.

- **User input required:** Select the directory to which simulation data will be saved.

Users are then prompted to select a folder or location where MATLAB will save the simulation data and any associated outputs (Figure 14). It is recommended to save this on a local drive to prevent data loss due to possible network connectivity changes.

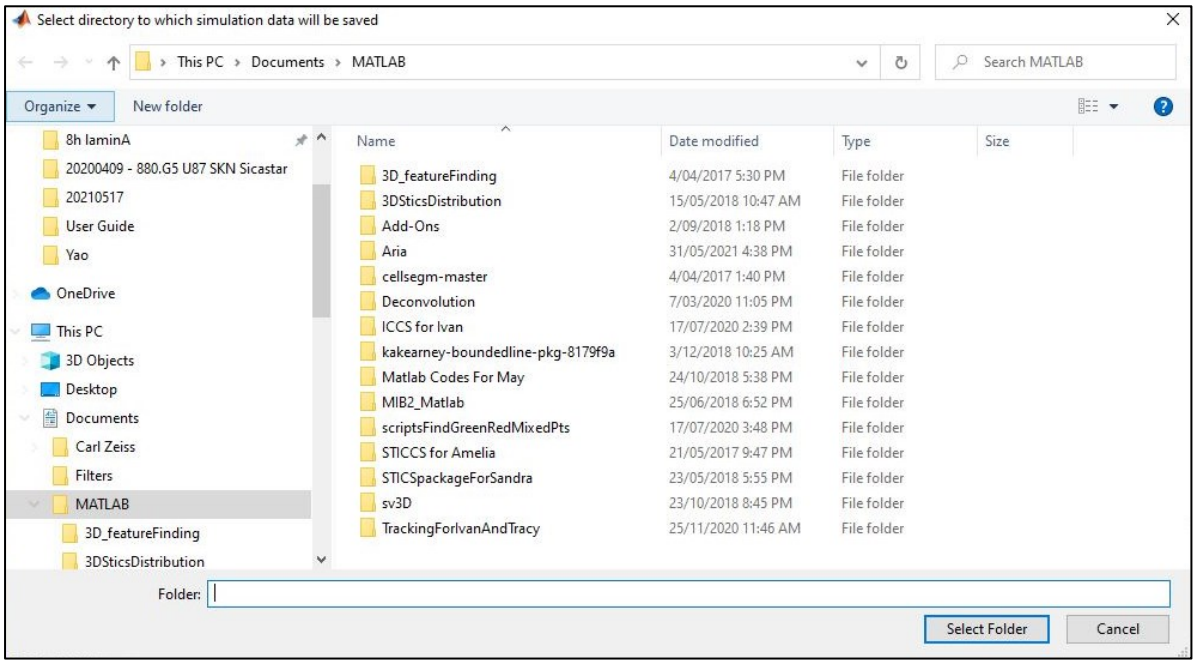

Figure 14 User prompt to select the location where simulation data will be saved, once generated.

- **User input required:** Parameter setup for simulations.

Following data-write preferences, users will then be prompted to edit an array of parameters which will define simulation, all with defaults as detailed below (Figure 15). These include basic parameters such as the image size, number of z-slices, number of time points, interval between each time point (frame time), pixel resolution, and point spread function (PSF) in both lateral (XY) and axial (Z) dimensions (Figure 15a).

Additional parameters are then used to define the features of the spheroid cell "objects" including the size of the spheroid relative to the image size, the cell density fraction (seeding location) of each cell, and the cell radii, relative to the spheroid (Figure 15b). This is followed by parameters used to define particle behavior, particularly the diffusion coefficients of

particles inside or outside cells, and their probabilities of moving between these two environments (Figure 15c). Final options enable users to export a video of their simulation (in either mp4 or avi format) with a specified frame rate in frames per second (fps) (Figure 15d).

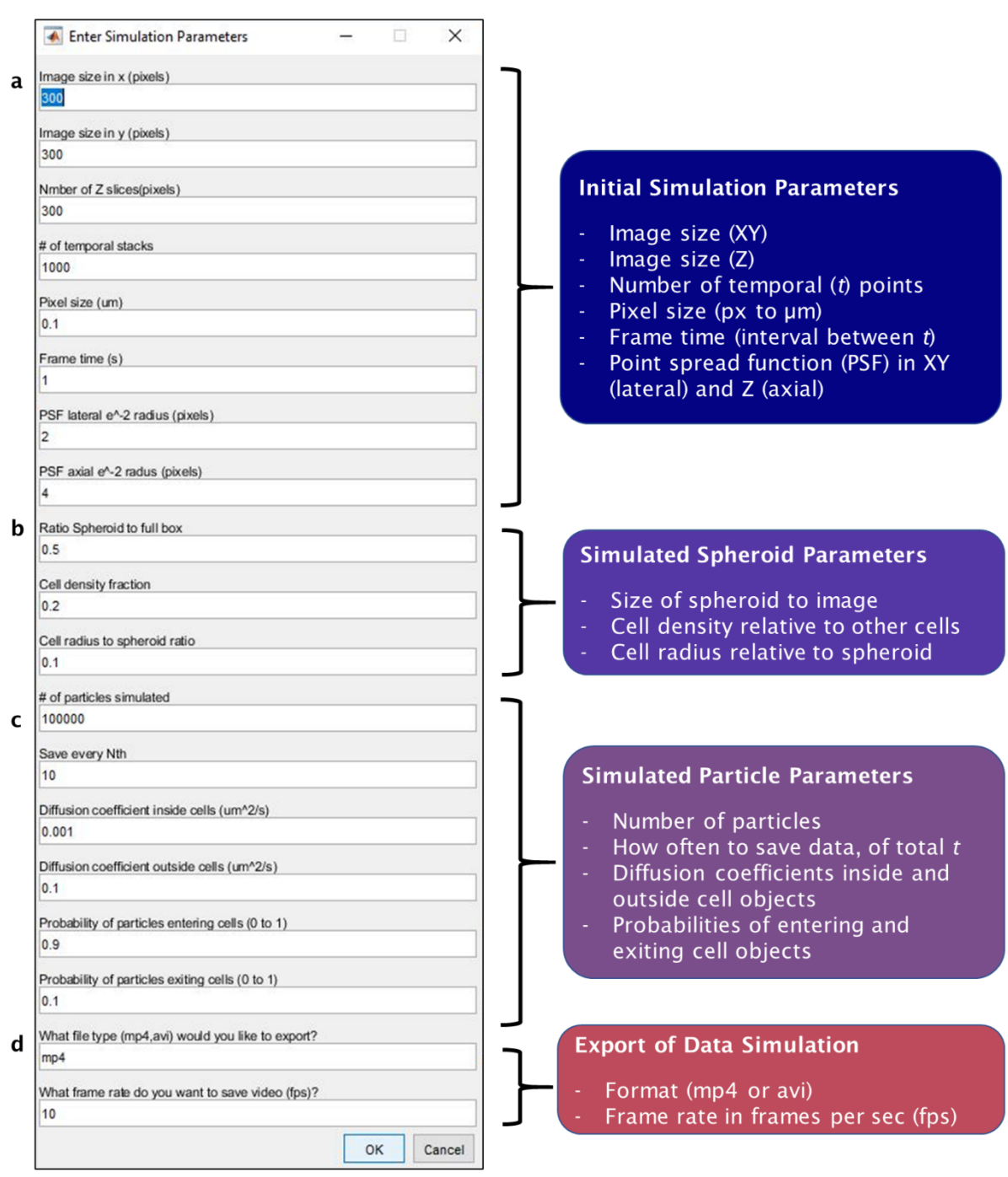

Figure 15 User input for DONUTS_SimulationGenerator which covers parameters for **(a)** initial simulation, **(b)** spheroid generation **(c)** particle convolution and diffusion, and additionally **(d)** export of simulated dataset as a video (either mp4 or avi format).

For verification, users can refer to the Methods of Ahmed-Cox, *et al* 2021, J Control Release generate any one of the nine simulations included according to the authors parameters.

Once data has been simulated, MATLAB will save the simulation as a .mat file and proceed to processing for video export as per user selection above. The processing time to complete simulations is most dependent on the cell density fraction (CDF). For a CDF of 0.2 on a computer with specifications outlined in Section 2.3, simulations may take up to 10 minutes. For simulations of CDF = 0.05, simulations may take up to 60 minutes. Videos are recommended at 50 – 100 fps and may take an additional 30 minutes to export.

Once simulations are exported, users can proceed to DONUTS analysis.

- **User input required**: Open DONUTS_3D_T and select Run.

Users will be prompted to load a file that contains the simulation dataset, as per Figure 5. In this case, users should select the MATLAB file (.mat) which was exported during DONUTS_SimulationGenerator.

- **User input required:** Follow MATLAB prompts according to walkthrough for DONUTS_3D_T (Section 2.3).

Except for loading a MATLAB file rather than raw imaging data, DONUTS_3D_T_SimulationFormat runs identically to the standard DONUTS_3D_T script detailed in Section 2.3. Users may refer to this section from Figure 6 onwards as a guide to analyse simulated data. DONUTS_3D_T_SimulationFormat output includes a Microsoft Excel file with the radial distances from the spheroid core (labelled 'R' in the Excel file) and average intensity profile across time (AveIntProfile). MATLAB will also save a PNG image of the average intensity profile across time over *r*, as well as the raw .mat file with the full workspace. On a computer with specifications as defined above in Section 2.3, analysis takes less than 30 minutes.

Users have the final option as part of the DONUTS simulation package to input the .mat files generated during DONUTS_3D_T_SimulationFormat and load these into MATLAB using the simulation compatible script of DONUTS_FTCS, as either the Standard or Batch processing version.

- **User input required**: Open DONUTS_FTCS_SimulationFormat and select Run to load the MATLAB output from DONUTS_3D_T_SimulationFormat.

Once a file or files are selected, MATLAB will proceed with FTCS analysis and output data as a single or series of Microsoft Excel files. Like the standard DONUTS_FTCS scripts, analysis typically takes less than two minutes to complete.

The overall output of the DONUTS simulation package is detailed in Table 5. For validation, a folder labelled Test Dataset C contains all outputs from the DONUTS simulation package generated using the default parameters in Figure 15, with the exception of the following parameters which were adjusted to reduce computational load: cell density fraction (CDF = 0.1), cell radius to spheroid ratio (recommended to be CDF/2 = 0.05), save every Nth (100), and frame rate per second (fps = 1).

Table 5 Output from the DONUTS Simulation Package from the SimulationGenerator, through particle uptake over distance and time (3D_T) to diffusion kinetics (FTCS_Standard and _Batch).

Output File (extension)	Detail of Contents
Script: DONUTS_SimulationGenerator	
MATLAB workspace (.mat)	Workspace containing all data on simulated spheroid with particle diffusion over time. Filename according to variables for D(in), D(out), P(in), P(out), spheroid radius (pixels), CDF and cell radius.
Video (.mp4 or .avi)	Video of simulated spheroid with particle diffusion over time according to user defined parameters. Filename matches MATLAB workspace above.
Script: DONUTS_3D_T_SimulationFormat	
Microsoft Excel File (.xlsx)	Quantification of particle average fluorescence (AveIntProfile) over R (in pixels) from core of spheroid to circumference. Labelled as AA3D_[MATLAB Filename]
MATLAB workspace (.mat)	Workspace containing all data on quantification, labelled as AA3D_[Filename].
Image (.png)	Export of figure for visualisation of AveIntProf over distance (R) and time (*t*).
Script: DONUTS_FTCS_SimulationFormat* (+Batch)	
Microsoft Excel File (.xlsx)	Quantification of particle uptake kinetics ($\mu m^2/s$) over r (μm) from core of spheroid to circumference. Labelled as Diffusion Coefficient_AA3D_[MATLAB Filename] from DONUTS_3D_T_SimulationFormat output.

2.7.2 DONUTS Nearest neighbor distances (NND) of nuclei in spheroids

For further investigation into differing patterns of nanoparticle uptake in tumor spheroids which appeared tumor model dependent, authors also analyzed the density of tumor spheroids. This was conducted using a MATLAB script to calculate the nearest neighbor distances between adjacent nuclei, using fixed and optically cleared spheroids which were stained with DAPI and imaged on a Zeiss Lightsheet Z.1. Further detail on the methods for sample preparation and image acquisition can be found under the Methods of the primary paper (Ahmed-Cox, *et al* 2021 J Control Release).

This final section of the User Guide is designed to assist validation and general use of this accessory script, titled DONUTS_NearestNeighborDistancesinSpheroids. Like DONUTS_2D/3D_T, this script is designed to process raw imaging data of nuclei in 3D, at a single timepoint. Single cell resolution is required for accurate segmentation and analysis.

- **User input required:** Select Run and choose the file you would like to analyse.

Once a file is selected, MATLAB will load z-stack data and prompt users to select the boundary of the spheroid as in Figure 6a.

- **User input required:** Outline 10 nuclei for average radii calculations.

Users will then be prompted by a dialog box to identify 10 nuclei via a click and drag interface (Figure 16). This will provide an average radius for MATLAB to use in downstream segmentation. MATLAB will then commence analysis, smoothing data, creating a binary mask to define the spheroid boundary, and initiating feature finding of nuclei [21] and subsequent triangulation of nuclei distances in 3D. Users can follow analysis stages using comments embedded in the script.

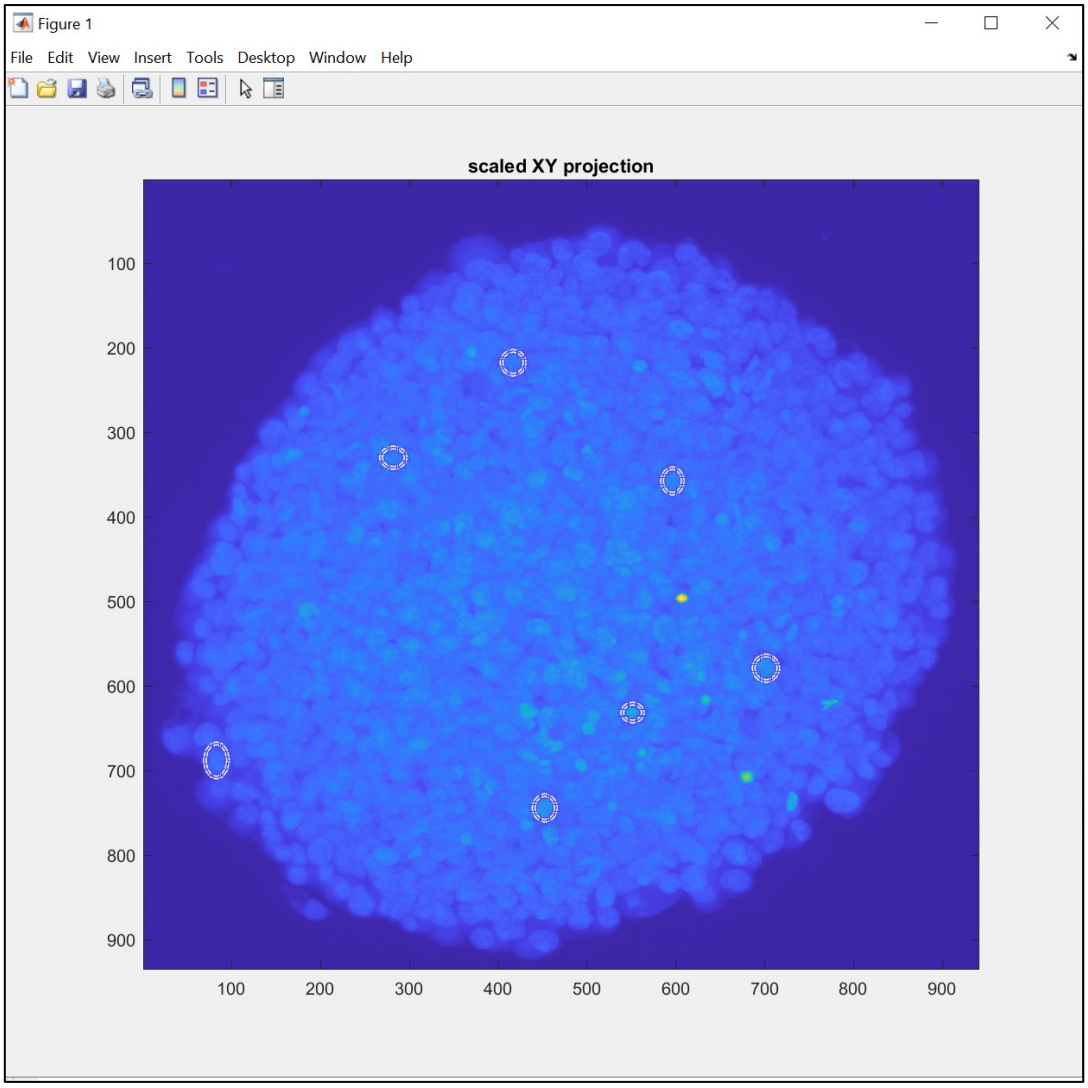

Figure 16 MATLAB figure prompting user to define nuclei. User is required to click and drag to define the bounds of 10 nuclei which will be used to calculate an average radius and initiate spheroid segmentation.

Approaching completion, MATLAB will display several figures which are also saved for future reference. These include a histogram plotting the frequency of nearest neighbor distances (NND), alongside the average NND and standard deviation as well as several slices of the spheroid which are superimposed with nuclei counts from that slice. This was used by authors to verify automated nuclei counts against external manual counts as a measure of quality control.

For convenience, both the histogram and quality control superimposed z-slices are exported and saved as PNG files. Other outputs include a Microsoft Excel spreadsheet detailing the coordinates (X, Y, Z) and radius of each nuclei as well distance to center, total and peak intensity and NND. MATLAB also calculates global measurements for the spheroid, including its volume, based upon the mask defined in early stages of analysis, and hence the calculated density of nuclei (both in mm^3). MATLAB also saves the workspace as a .mat file but requires clearing of large matrices prior to saving to reduce file size. More information on the output from DONUTS_NearestNeighborDistancesinSpheroids is detailed in Table 6. For reference, data in primary paper includes analysis of spheroids imaged at 1920 x 1920 pixels (XY pixel size of 0.285 µm) and 1400 z-slices (Z voxel size = 0.39 µm). The analysis required a computer of similar specifications to the reference in Section 2.3 (dual 2.60 GHz processors and a 64-bit operating system) but with additional RAM (256 Gb) to complete analysis. On average, each spheroid took approximately 45 minutes to complete analysis and export data as detailed in Table 6. For user ease, the Test dataset (Test Dataset D) for this script has been reduced to a spheroid hemisphere of dimensions 960 x 960 pixels (pixel size of 0.571 µm) with 318 z-slices (voxel size of 0.780 µm). This covers an image of sizes in X: 548.16 µm, Y: 547.89 µm, Z: 248.04 µm. This dataset can be run on a computer with 128 Gb RAM and complete in less than five minutes.

Table 6 DONUTS_NearestNeighborDistancesinSpheroids output generates several analysis files alongside the .mat file, the contents of which are detailed below.

PNG Prefix	Full Label	Detail of Contents
HistNNDist	Histogram of Nearest Neighbor Distances (NND)	A histogram plotting the calculated NND relative to their frequency of occurrence, as well as the average NND ± SD ($d_{NN} \pm \sigma_{dNN}$)
Slice(2*x*)31	Z-slice (2*x*)31, where *x* = 0.5, 1, 2 … , *n*.	Several PNG files, each of a single z-slice of raw data as indicated, with superimposed nuclei detection and total nuclei count (#Nuc = *y*). Sampled every 200 slices, starting at z31.
Sheet in Excel File	**Full Label**	**Detail of Contents**
NucleiStats	Nuclei statistics, with one nucleus per row	Coordinates (X, Y and Z) of each nucleus (µm) *TotInt* = total fluorescence intensity *Radius* = radius of nuclei (µm) *PeakInt* = maximum fluorescence intensity *FracVoxAboveThresh* [a] = fraction of voxel of nuclei above threshold *DistToCenter* = distance from core of spheroid (µm)
NNdist	Nearest neighbor distance from each nucleus	The nearest neighbor distance from each nucleus (µm) detailed in NucleiStats
DensityNuclei	Density of nuclei in whole spheroid	*VolumeSpheroInmm* = volume of spheroid (mm^3) *densityNucleiPermm* = calculated number of nuclei (density) per mm^3

[a] When feature fitting in 3D, a threshold is applied to establish what local maxima are important. If the intensity of local maxima is below the set threshold, they are rejected as noise. Once all the maxima are counted and voxels belonging to them are known, this value is divided by total number of voxels in the data. This parameter is exported for reference.

3 Troubleshooting

Section 2 is designed as a step-by-step guide to the core analysis scripts in DONUTS, pertaining to measuring nanoparticle uptake in tumor spheroids in either 2D or 3D, and downstream analysis or complementary validation. Successful analysis assumes all steps execute without error. Below we have discussed solutions to several errors that users may encounter.

3.1 Error opening file and initiating analysis

Errors initiating analysis are likely, particularly when first installing MATLAB and downloading and applying DONUTS scripts. For a file open error, ensure all scripts and associated macros have been copied to the local drive (see Appendix for list), and that the path is correctly mapped (including subfolders). Also ensure all files are unique (no duplications, or files of same name), as this can cause errors in the MATLAB directory. If you are not a first-time user of MATLAB, and have other scripts in your working directory/path, please ensure that none of the DONUTS scripts (including supporting scripts) have the same name as your local scripts. Also check that the embedded script text for specific sheets in a file (as discussed in Sections 2.5, 2.6 and 2.7.1) has been adjusted to match the name of the data to be imported and analyzed. Assuming all MATLAB scripts are in order, it is then relevant to check the file itself. Users are advised to check DONUTS for the appropriate file selection and file extension. For instances, such as in Section 2.7.1, where scripts require input of MATLAB workspaces (.mat files), or Sections 2.5 and 2.6 which require Microsoft Excel (.xlsx files) for kinetic quantification, import of the wrong data file or format will prompt a read error [`file not found`]. For import of raw data in Sections 2.3, 2.4 and 2.7.2, the script "GetOMEData" is compatible with all major imaging file formats (including Nikon, Olympus, Leica and Zeiss) but is not compatible for post-processed images (TIFF, JPEG etc.). In this instance, users are welcome to contact authors for assistance.

3.2 Difficulty seeing outline of spheroid for selection of center in XY and Z

In some cases, for DONUTS_3D_T and DONUTS_2D_T, the channel selected for the spheroid boundary (recommended as a membrane stain) may have low or intermediate fluorescence. In this case it is recommended to open raw data in a secondary imaging program (i.e., Zen) for a quick check of contrast and quality control. If data is acceptable, a logarithm can be inserted in Line 31 of DONUTS_3D_T and applied to the image to amplify the signal in channel 1 (membrane) and assist in defining the core of the spheroid and creating the binary mask of the circumference in 3D. The same method can be done in Line 53 of DONUTS_2D_T, i.e., replace

`imagesc(squeeze(mean(mean(mean(data,3),4),5)));` with

`imagesc(log(double(squeeze(mean(mean(mean(data,3),4),5)))));`

3.3 Metadata error

Depending on the file format for initial DONUTS scripts (Sections 2.3 and 2.4), the metadata typically included by imaging software in the data file may not be extracted correctly or may not even be embedded. In this case, the user has the option to use the dialog box in Figure 7 to manually input the pixel size in XY and Z.

3.4 Memory shortage

Memory shortages, if they occur, are most likely during DONUTS_3D_T and DONUTS_NearestNeighborDistancesinSpheroids. Despite efforts from the authors to manage possible RAM limitations (assisted in 3D analysis by data rescale in Line 11 and parallel pool in Line 130), imaging data, particularly in 4D (3D across time) can result in large matrices, which can prompt a data dump and/or crash in MATLAB. The high resolution of data for nuclei segmentation may cause a similar data dump. There are several possible solutions to assist.

Firstly, MATLAB has settings for Java Heap Memory which can be accessed via the HOME Tab, under Preferences > General > Java Heap Memory. This will display the current and available memory in megabytes (MB) and can be increased to enable greater memory storage temporarily created variables, which is essential to process large files (Figure 17).

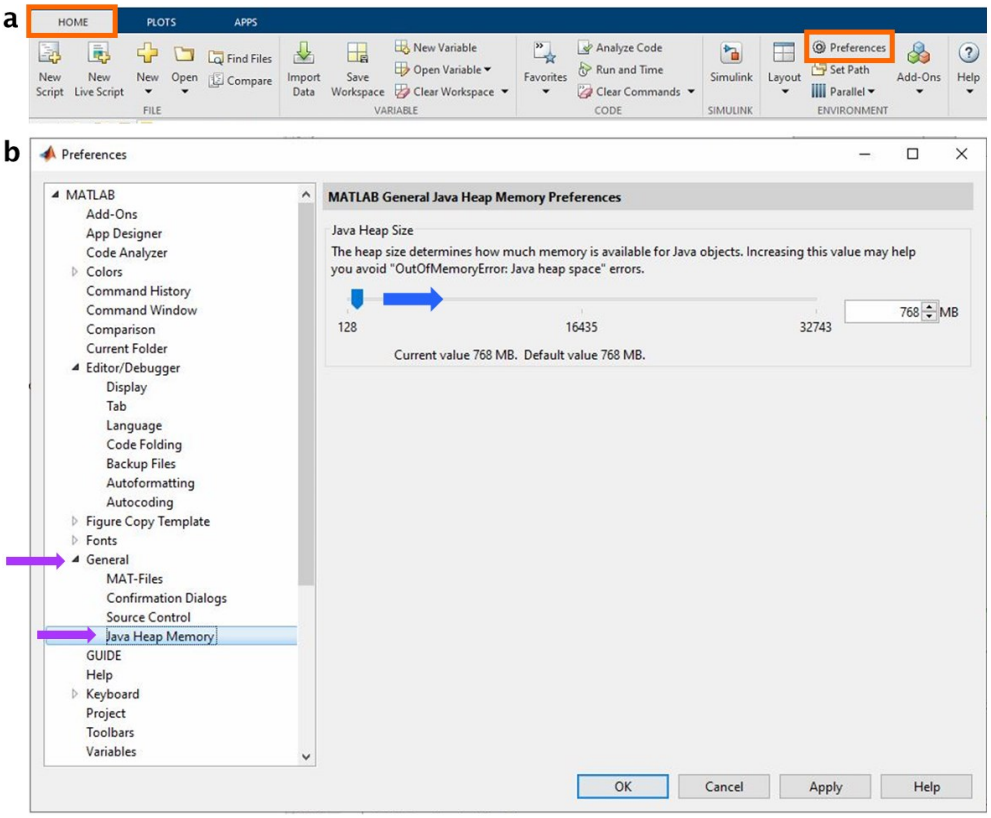

Figure 17 Memory errors and changing Java Heap Memory settings in MATLAB (a) Access Preferences under the HOME Tab (in orange). **(b)** This will open a dialog box which allows users to scroll down to General and select Java Heap Memory (purple arrows). Increasing the memory permissions for Java (blue arrow) will improve speed and storage in MATLAB and may resolve memory errors.

In the instance where memory errors are still occurring in 3D analysis, users can increase the rescale in Line 11 of DONUTS_3D_T. Default is 0.3, adjusting to 0.2 (resampling to 20% of data size) is likely to assist both speed and memory. For nuclei segmentation, it may be pertinent to conduct a similar data rescale, while ensuring new pixel to micrometer dimensions remain correct.

In addition, instances of memory shortages can be predicted and monitored through Task Manager (accessed via the Windows shortcut Ctrl_Alt_Delete). Where possible, high RAM computers are always recommended for microscopy analysis.

3.5 Parallel pool error

The most common failure in Parpool is when a new parallel pool loop is initiated while a pre-existing Parpool is already running. In this instance, close the current Parpool using `delete(gcp('nocreate'))` in the Command window, or by selecting Close Parallel Pool in the Parallel Pool Options Icon in Figure 9a. If errors continue, users can monitor memory usage in Task Manager as described above in Section 3.4 and decrease the number of workers if needed using Parallel Pool preferences (Figure 9b).

3.6 Data output issues

In some instances, users may note variances in data output. Some variance is expected for a biological model in 3D (Figure 18), however it is important to also validate the integrity of each sample before presuming model bias. Section 1.3 above provides some examples of quality control measures during sample preparation. Section 2.7.2 on nuclei segmentation also includes embedded quality control images which can be correlated against manual counts to ensure accuracy. For imaging, additional errors may arise during acquisition, for example, a partially embedded spheroid in agarose may become mobile in solution part way through imaging, which would have carry-on effects in analysis if not immediately detected. In some cases, analysis may also run errors if the fluorescence signal is below detectable limits (which will vary depending on wavelength, laser power and detection). In this case, NAN values may cause arbitrary starting points for DONUTS_2D/3D_T, and can be seen in sharp fluctuations, particularly in early timepoints (Figure 18a N=3, t=1 hour). In this case, users can consult the output sum of the analyzed channel, rather than the average, to see whether accumulating fluorescence signal rather than averaging resolves detection error.

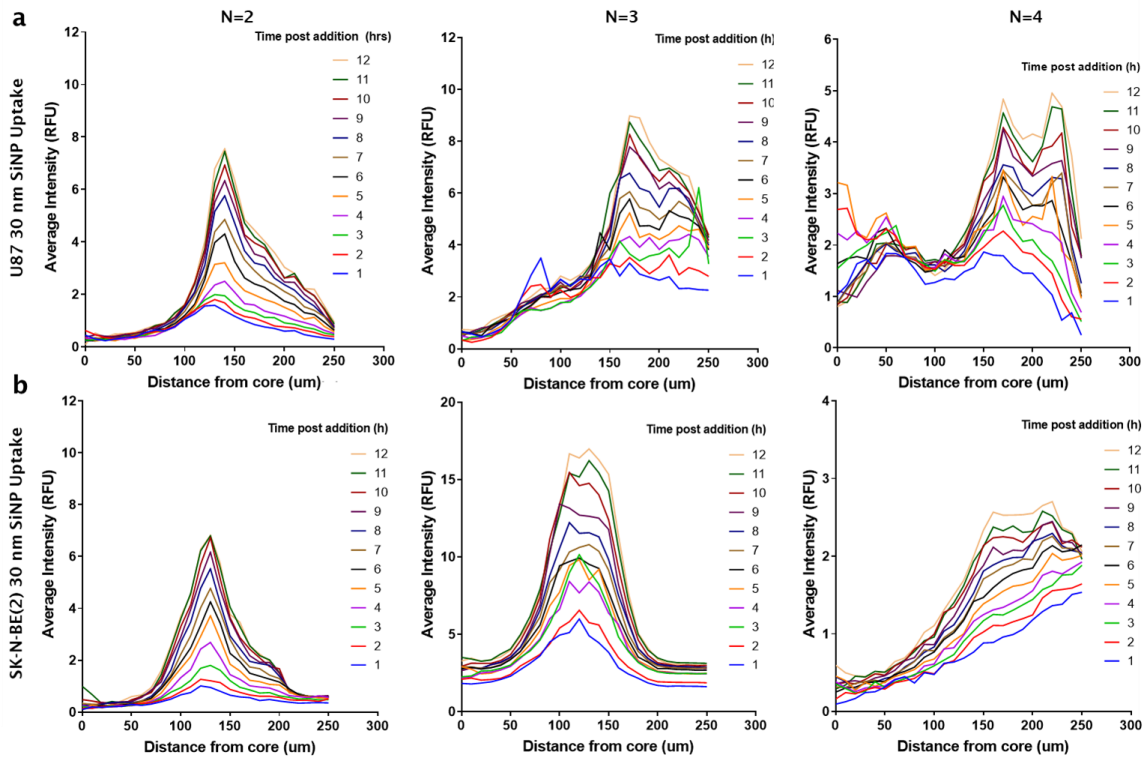

Figure 18 3D Azimuthal quantification of 30 nm SiNP uptake in **(a)** glioblastoma (U87) and **(b)** neuroblastoma (SK-N-BE(2)) cell spheroids. Each graph represents SiNP tumor spheroid uptake of a biologically independent experiment. From Supplementary data, Ahmed-Cox, *et al* 2021, J Control Release.

3.7 Other errors

While we have collated several common errors and discussed reasonable solutions above, this is by no means a comprehensive list of errors that may occur. MATLAB has several online communities (https://au.mathworks.com/matlabcentral/?s_tid=gn_mlc) which may assist on software related errors. Users are also welcome to contact authors for recommended improvements as well as assistance in troubleshooting, if required.

4 Acknowledgments

The analysis package detailed for DONUTS is built upon several previous scripts, for which the authors would like to acknowledge the following contributions:

1) Inhull.m

John D'Errico (2021). Inhull (https://www.mathworks.com/matlabcentral/fileexchange/10226-inhull), MATLAB Central File Exchange. Retrieved May 28, 2021.

2) DistMesh package [20]

3) NaN suite

Jan Gläscher (2021). NaN Suite (https://www.mathworks.com/matlabcentral/fileexchange/6837-nan-suite), MATLAB Central File Exchange. Retrieved May 28, 2021.

4) consolidator.m

John D'Errico (2021). Consolidator (https://www.mathworks.com/matlabcentral/fileexchange/8354-consolidator), MATLAB Central File Exchange. Retrieved May 28, 2021.

5) GetOMEdata.m

Sebastien Rhode (2021). Control ZEN Blue and the microscope from MATLAB (https://www.mathworks.com/matlabcentral/fileexchange/50079-control-zen-blue-and-the-microscope-from-matlab), MATLAB Central File Exchange. Retrieved May 28, 2021.

6) moving_average.m

Carlos Adrian Vargas Aguilera (2021). moving_average v3.1 (Mar 2008) (https://www.mathworks.com/matlabcentral/fileexchange/12276-moving_average-v3-1-mar-2008), MATLAB Central File Exchange. Retrieved May 28, 2021.

7) bfmatlab package for OME import into MATLAB [17]

8) 3D feature finding suite [21]

5 References

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