Application Note

Cell Metabolism

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Lipid Accumulation in HepG2 Cells Exposed to Free Fatty Acids

Image-based assay to model nonalcoholic steatohepatitis (NASH)

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Abstract

Nonalcoholic steatohepatitis of NASH is a common liver disease whose major feature is fat in the liver along with inflammation, which can lead to liver damage and cirrhosis. While no definitive cause has been identified, the cellular toxicity of numerous fatty acid metabolites is suspected. The formation of fatty droplets has been proposed as a marker for lipotoxicity caused by fatty acid derived species. This application note examines the production of lipid droplets in HepG2 cells exposed to palmitic and oleic acids.

Introduction

NASH is a common, often "silent" liver disease that resembles alcoholic liver disease, but occurs in people who drink little or no alcohol. While NASH, NASH cirrhosis, and NASH-related hepatocellular carcinoma are becoming more prevalent, the understanding of their causes remains elusive. The major feature in NASH is fat in the liver, along with inflammation and damage (Figure 1).

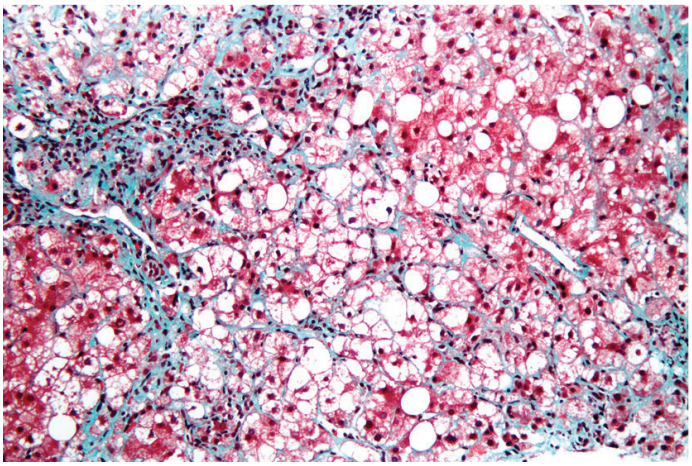

Figure 1. Trichrome stain of liver biopsy depicting nonalcoholic steatohepatitis (NASH). Image depicts balloon degeneration of hepatocytes (large cells with small centrally located nuclei) along with fine chicken-wire fibrosis, hepatocyte necrosis, and inflammation. Image courtesy of Nephron[1], CC BY-SA 3.0.

The main fatty acids in the human body are palmitic and oleic acid.[2] These free fatty acids are absorbed by liver cells through facilitated transport mechanisms and converted to triacylglycerides (Figure 2).[5] Understanding the intracellular absorption of free fatty acids and their role in cellular cytotoxicity is critical towards elucidating the causes of NASH.

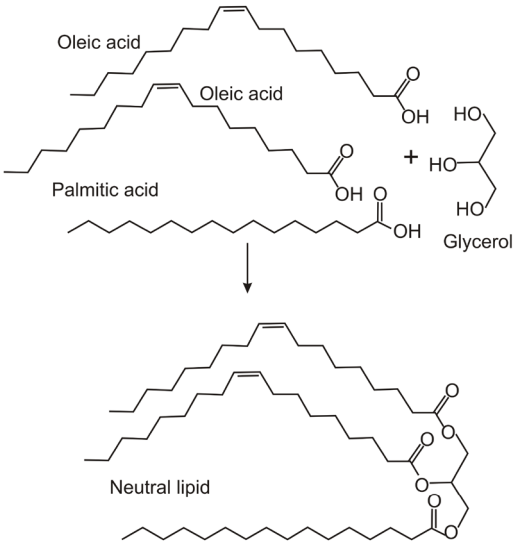

Figure 2. Conversion of free fatty acids to neutral lipids. Free fatty acids such as oleic and palmitic acids are internalized by liver cells and converted to neutral lipid triacylglycerides by esterification with glycerol.

As these lipids accumulate, they form droplets that can be detected using lipid specific stains such as Nile Red or BODIPY 493/503. These lipophilic stains readily cross membranes of paraformaldehyde fixed cells where they partition into neutral lipid droplets and exhibit a significant increase in fluorescence (Figure 3).[4,6]

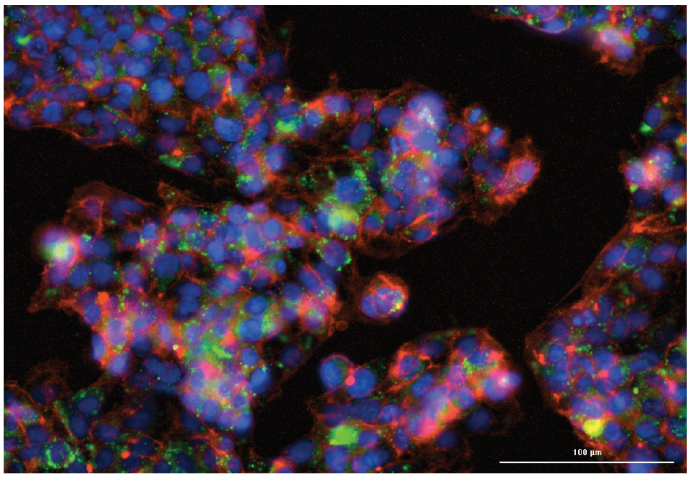

Figure 3. Neutral lipid staining of HepG2 cells. HepG2 cells challenged with 0.5 mM mixture of oleic and palmitic fatty acids for 24 hours were fixed with 4% PFA and stained with BODIPY 493/503 (green), which stains neutral lipids; DAPI (blue), which stains nuclei; and Texas Red phalloidin (red), which stains actin. Three-color 20x images captured using an Agilent BioTek Cytation 5 cell imaging multimode reader. The scale bar denotes 100 µm.

Materials and methods

Cell culture

HepG2 cells were cultured in Advanced DMEM supplemented with 10% fetal bovine serum and penicillin-streptomycin at 37 °C in 5% CO_2. Cultures were routinely trypsinized (0.05% Trypsin-EDTA) at 80% confluency. For experiments, cells were plated into Corning 3904 black-sided clear-bottom 96-well microplates at 10,000 cells per well depending on the experiment.

Fixing and staining

All cell washes and reagent additions for cell fixation and fluorescent staining were carried out using an Agilent BioTek EL406 washer dispenser. Cells were washed two times with 200 µL of PBS (NaCl 137 mM, KCl 2.7 mM, Na_2HPO_4 10 mM, KH_2PO_4 7.4 mM) after which 100 µL of 4% para-formaldehyde (PFA) solution was added. Cells were fixed for 10 minutes at room temperature followed by two washes of 200 µL using PBS. Cells were stained for 30 minutes with 50 µL of working solution of DAPI and either Nile Red or BODIPY 493/503 stain at room temperature followed by two washes with 200 µL of PBS. After aspiration, 100 µL of PBS was added to all wells and the plates were sealed using an optically clear TopSeal-A adhesive plate sealer (PerkinElmer, Waltham, MA). Prepared plates were kept at 5 °C and protected from light prior to imaging.

Imaging

Experiments were imaged using an Agilent BioTek Cytation 5 cell imaging multimode reader configured with DAPI, GFP, and RFP and light cubes. The imager uses a combination of LED light sources in conjunction with band pass filters and dichroic mirrors to provide appropriate wavelength light.

The DAPI light cubes used a 337/50 excitation filter and a 447/60 emission filter. The GFP light cube used a 469/35 excitation filter and a 525/39 emission filter. The RFP light cube used a 585/29 excitation and 624/40 emission filters. The light cube used a 586/15 excitation and a 647/57 emission filter.

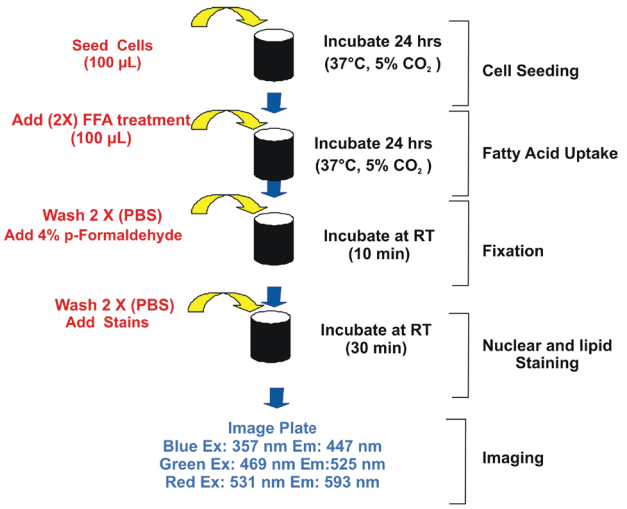

Figure 4. Lipid accumulation and staining process. Cells were routinely seeded into 96-well microplates using an Agilent BioTek MultiFlo FX multimode dispenser and allowed to attach overnight. The following day, cells were challenged with a mixture (3:1) of oleic and palmitic free fatty acids at various concentrations and for various exposure times. Cells were fixed for 10 minutes with 4% para-formaldehyde, then stained with DAPI (nuclei), Texas Red-phalloidin (actin) and either BODIPY 493/503 or Nile Red (neutral lipid) for 30 minutes. Cells were then imaged using an Agilent BioTek Cytation 5 cell imaging multimode reader; images were analyzed with Agilent BioTek Gen5 microplate reader and imager software.

Fluorescence microplate reader measurements

The fluorescence of cells stained with DAPI and Nile Red or BODIPY 493/503 were determined by a Cytation 5 using its spectral filter/dichroic microplate reader optics. DAPI fluorescence was measured with an excitation of 360/20 nm and an emission of 460/20 nm, while Nile Red fluorescence was determined using a 530/15 nm excitation and a 570/15 nm emission. To account for variations in cell location in the well, the fluorescence was measured with a 4 × 4 area scan and the results were averaged. Differences in cell number were corrected by using DAPI fluorescence to normalize the Nile Red signal in each well. The data represent the mean of eight determinations.

Image analysis

Primary and secondary mask analysis of the captured digital images was used to determine the percentage of lipid-positive cells. Primary mask analysis of the DAPI channel identifies individual cells by their nuclei. The secondary mask is denoted as the region up to 30 µm surrounding each nucleus and represents the cell cytoplasmic region (Figure 5). Depending on the experiment, either threshold analysis was used to identify the percentage of lipid-positive cells or the total fluorescence (integral) of this region was used to calculate changes in cellular lipid content. The percentage of lipid-positive cells was calculated using a subpopulation dual mask object analysis. The primary mask identified individual nuclei, with the secondary mask spatially linked to the primary mask. The lipid-positive subpopulation was quantified with a fluorescence threshold of 6×10^6. Data are expressed as a percentage of total cell count obtained with the primary mask. The data represent the mean of eight determinations.

Multicolor images were taken on the same wells with a 10x objective using a 3 × 3 format such that nine discrete images were located at different places across each well.

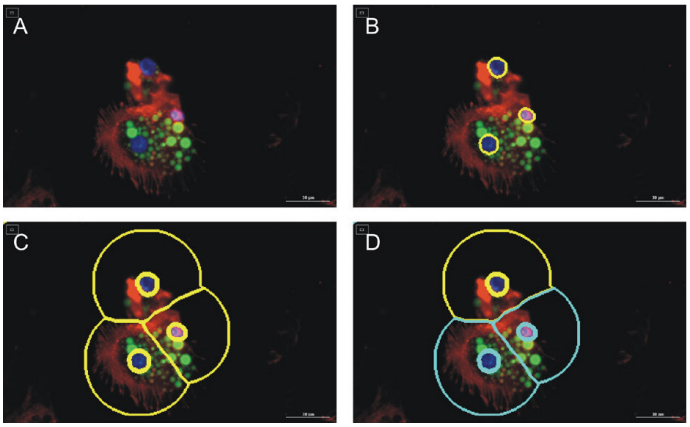

Figure 5. Quantitative analysis of image data. Primary mask analysis of the DAPI channel identifies individual cells by their nuclei. Secondary mask is identified by the space up to 30 µm surrounding the primary mask. (A) Raw image; (B) Primary mask identified by yellow trace; (C) Primary and secondary masks identified by yellow trace; (D) Primary and secondary masks with lipid-positive cells identified with blue trace. Scale bar indicates 30 µm.

Results and discussion

Incubating HepG2 cells with increasing concentrations of free fatty acid mixtures results in the formation of intracellular neutral lipid. Nile Red, which is significantly more fluorescent in a lipid environment as compared to aqueous[3,4], can be detected using either PMT-based fluorescence measurements or image-based subpopulation analysis (Figure 6).

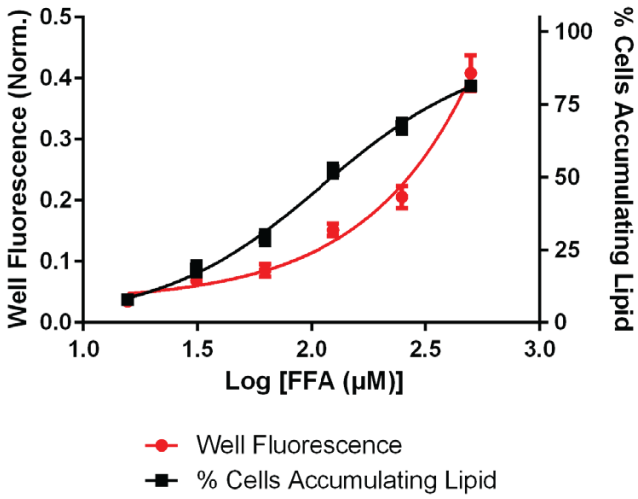

Figure 6. Lipid accumulation in Hep G2 cells treated with various concentrations of free fatty acids. Lipid droplet formation after 24-hour exposure was assessed using Nile Red fluorescence using either PMT-based microplate reader optics (well fluorescence) or image-based subpopulation analysis (% cells accumulating lipid).

It is evident in Figure 6 that image-based subpopulation analysis determining the percentage of lipid positive cells in the field of view provides a greater sensitivity in assessing lipid accumulation than PMT-based well fluorescence. A 3:1 mixture of palmitic and oleic acid was used in these experiments to mimic *in vivo* conditions. If one examines the role of the two free fatty acids separately in regards to neutral lipid formation, it is apparent that oleic acid is primarily responsible. Challenging cells with 50 nM oleic acid alone results in significant lipid accumulation; but using the same concentration of palmitic results in no discernible lipid production (Figure 7). However if the same 50 nM oleic acid is used in conjunction with a greater dose of palmitic acid typical of *in vivo* conditions, a significant boost in lipid production is evident. This effect is lost if the palmitic concentration is reduced to equal that of the oleic acid, however.

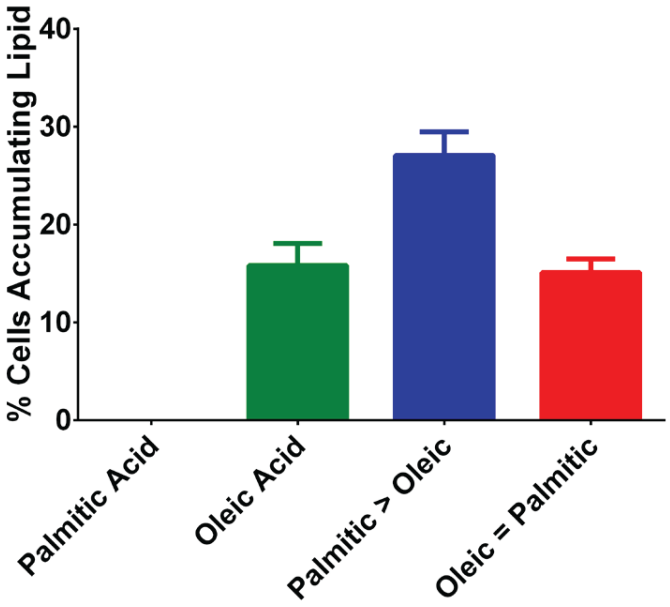

Figure 7. Role of oleic and palmitic acids with lipid accumulation. HepG2 cells were challenged with oleic and palmitic acids either alone at 50 nM or oleic acid at 50 nM with either a higher or equal dose of palmitic acid.

Multiple dyes are capable of detecting neutral lipid droplets within an aqueous environment. This study compared two such lipophilic dyes, Nile red and BODIPY 493/503, in regards to their ability to identify and quantify lipids within cells. When FFA challenged cells are fixed and stained with both Nile Red and BODIPY 493/503 dyes, images indicate that the two dyes co-localize to the cytoplasm (Figure 8). Fixed HepG2 cells stained with DAPI to identify nuclei, as well as the lipophilic dyes exhibit lipid staining only in the cytoplasm. (Figure 8B and 8C). Overlaid images of cells stained with both dyes indicate that regions stain the same regions within the cytoplasm (Figure 8D).

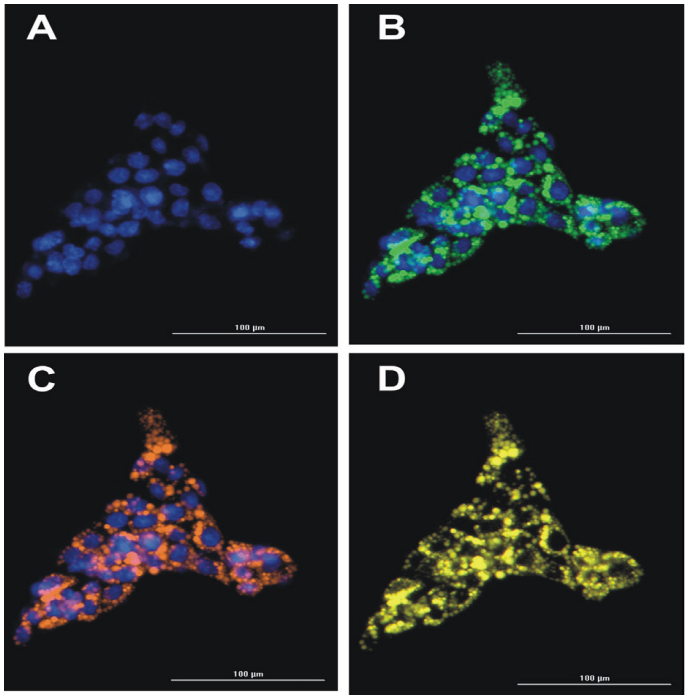

Figure 8. Co-localization of lipid staining dyes. HepG2 cells challenged with 0.25 mM FFA for 24 hours then fixed with 4% PFA and stained with DAPI (nuclei) and both Nile red and BODIPY 493/503 lipid stain. (A) DAPI image only; (B) DAPI and Nile Red; (C) DAPI and BODIPY; (D) Nile Red, and BODIPY.

Further image analysis also shows that either dye is appropriate for quantitative analysis. Using subpopulation analysis, lipid-positive cells are identified from a mixed pollution with either dye. While dye intensities require that different threshold values be used, the percentage of positive cells for both dyes agrees well when HepG2 cells are challenged with FFA and monitored over time (Figure 9).

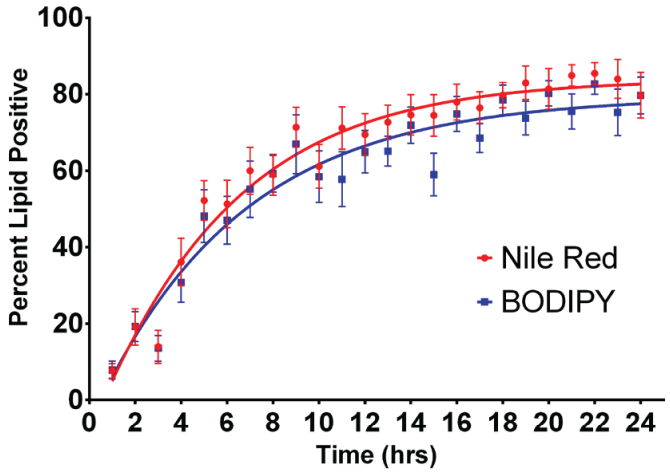

Figure 9. Comparison of lipid dyes to detect increases in lipid content. HepG2 cells challenged with 500 nM FFA and the percent of lipid positive cells plotted over time cells fixed and stained with DAPI to identify nuclei and either BODIPY 493/503 or Nile red to identify neutral lipid. The percentage of positive cells was calculated using a subpopulation dual mask object analysis. The primary mask identified individual nuclei, with the secondary mask spatially linked to the primary mask. The lipid-positive subpopulation was identified with a threshold of 1.2×10^6 and 2.5×10^6 for BODIPY 493/503 and Nile red stains, respectively.

A number of compounds have been shown to affect the formation of fatty liver *in vivo*. This assay was used to test the ability of compounds to influence the formation of neutral lipid droplets in HepG2 cells *in vitro*. Using untreated cells as a negative control and cells treated with 250 nM FFA as the positive control, the influence of four different compounds on neutral lipid formation *in vitro* was tested. As shown in Figure 10, only sodium butyrate was shown to alter lipid formation in these experiments. The presence of 100 µM sodium butyrate decreased lipid formation 70%. Hydrazine produced a slight but statistically insignificant increase in the number of lipid-positive cells compared to the control.

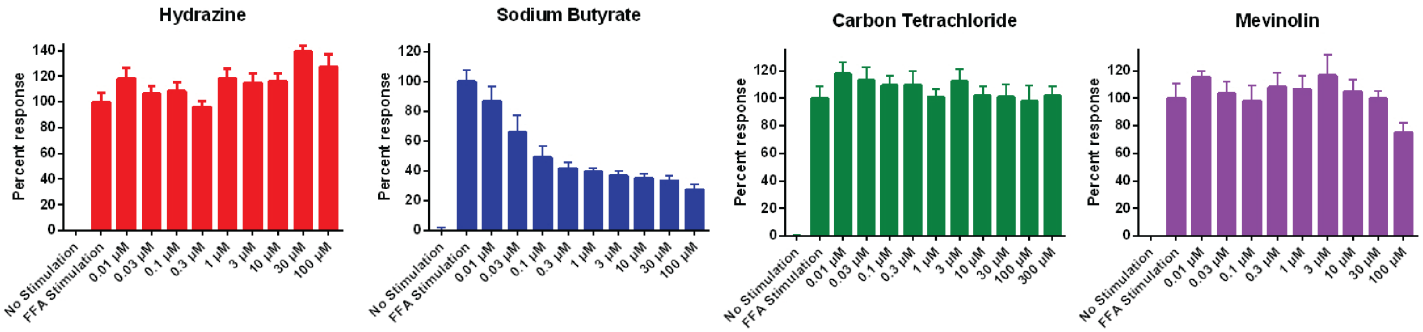

Figure 10. Effect of compounds on neutral lipid formation. Various concentrations of known effectors of fatty liver formation were added simultaneously with 0.25 mM FFA mixture to HepG2 cells. After 24 hours, cells were fixed and stained for lipids. The percent response reflects the ratio of total lipid staining normalized for cell number for each concentration to the FFA stimulated control after the no-stimulation control has been subtracted. The data represent the mean of seven determinations.

Conclusion

Nonalcoholic fatty liver disease (NAFLD) represents a spectrum of diseases ranging from hepatocellular steatosis and nonalcoholic steatohepatitis (NASH) to hepatocellular carcinoma.[7] Both hepatic steatosis and NASH can develop via several molecular pathways. Increased free fatty acid (FFA) uptake, *de novo* lipogenesis and endoplasmic reticulum (ER) stress are mechanisms implicated in NAFLD progression.[8,9] This application note describes a phenotypic-based assay that can be used to monitor these effects *in vitro*. Using two different lipophilic dyes, FFA can be taken up by HepG2 cells and esterified into neutral lipids in a dose- and time-dependent manner. The primary fatty acid responsible for lipid accumulation is oleic acid, although palmitic acid appears to have an enhancing effect on lipid production if it has a significantly higher concentration than its FFA partner. Either dye can be used allowing analysis with different fluorescent color channels depending on experimental needs.

As a proof of concept, this assay format was used to test known and unknown affecters of fatty liver *in vivo*. Sodium butyrate was shown to be protective in regards to preventing lipid formation *in vitro*, possibly by relieving ER stress.

The Agilent BioTek Cytation 5 cell imaging multimode reader has a number of features that enable neutral lipid droplet imaging. Four separate LED positions allow multiplex fluorescence imaging using a number of different magnification microscope objectives. Besides identification of the lipid droplets, counterstaining for cytoplasmic markers or nuclei provide cellular location information. In addition, the imager holds six objectives with magnification up to 60x. Agilent Biotek Gen5 microplate reader and imager software provides autofocusing of cells in microplates, capturing of images with both automatic or user-defined parameters (LED intensity, CCD gain, integration time, etc.) and cellular analysis algorithms that allow cell segmentation and cell counting. The Gen5 software used to control reader function is also capable of performing automated image analysis such as counting cells that meet fluorescence threshold and size criteria.

References

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