

Definiens Cellenger and Definiens Developer

Probing Hepatotoxicity: Detailed Investigations of Phospholipidosis with High Content Image Analysis

Hepatotoxicity is a major cause of late-stage drug failure or limitation in use. One potential indicator of hepatotoxicity is phospholipidosis, characterized by accumulation of phospholipids in intracellular vesicles. Cell-based assays of phospholipidosis using primary hepatocytes have been developed with the aim of identifying potentially toxic compounds upstream in the drug development process. However, these assays present particular problems for image analysis due to biological and labelling variation within the images. Here we present data generated by Definiens Cellenger and Definiens Developer, which allows highly accurate detection and classification of images despite low-contrast and heterogeneous image input. Moreover, the power of this approach allows investigations of biological phenomena which are impossible using traditional image analysis techniques, thereby revealing novel biomarkers and increasing the value of the assay.

Introduction

Hepatotoxicity is one of the most common causes of withdrawal, non-approval, limitation in use, and clinical monitoring of drugs by the FDA¹. The use of automated, cell-based assays providing directly translatable pathological information on target compounds is desirable early in drug development to avoid late stage attrition. One potential indicator of hepatotoxicity is phospholipidosis, a defect in lipid metabolism resulting in the accumulation of phospholipid in multi-lamellar vesicles.

High-content, cell-based imaging assays examining the uptake of fluorescent phospholipid into cells have been designed to examine any potential phospholipidotic effect *in vitro*. Nevertheless, this assay using cultures of primary hepatocytes presents challenges for accurate analysis, for a number of reasons. Firstly, primary hepatocytes may be mono- or multi-nucleated, so estimation of cell numbers cannot be made on the number of nuclei alone. Secondly, the lipid stain labels the cells in three distinct patterns, depending on whether the cell is viable, is displaying phospholipidosis, or is dead (figure 1). Finally, dead cells present in three different phenotypes: small, bright nuclei; large, granular nuclei or “enucleated”, with no discernible nuclei (figure 2).

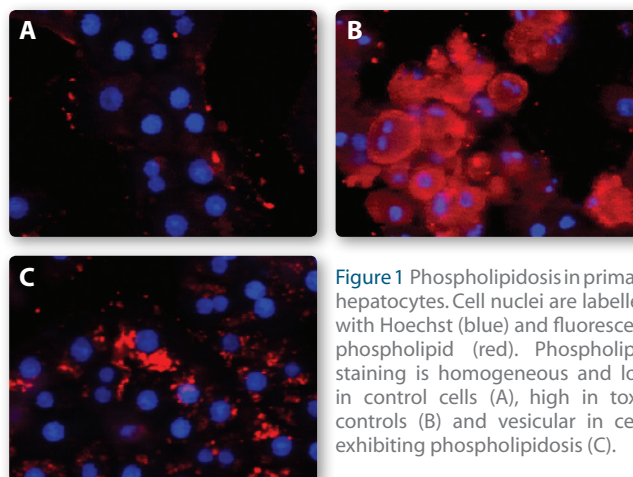


Figure 1 Phospholipidosis in primary hepatocytes. Cell nuclei are labelled with Hoechst (blue) and fluorescent phospholipid (red). Phospholipid staining is homogeneous and low in control cells (A), high in toxic controls (B) and vesicular in cells exhibiting phospholipidosis (C).

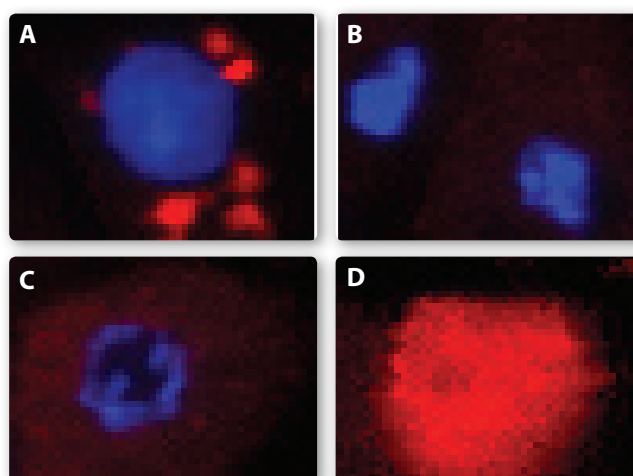


Figure 2 Nucleus and dead cell morphology. A) Healthy nuclei are homogeneous in staining. Dead cells possess small, bright nuclei (B), large, granular nuclei (C) or no nuclei (D)

KEY WORDS

In-vitro toxicology –
Hepatotoxicity – HCA –
Biomarker – Cell-based assay –
Definiens Cellenger –
Definiens Developer

In this application note, we show how Definiens Cognition Network Technology® overcomes these obstacles to return highly accurate data. Moreover we demonstrate unique capabilities of the software, allowing indepth interrogation of the samples, which could in turn identify novel biomarkers in assay systems.

Analysis Method

Primary rat liver hepatocytes in 96-well plates were treated with test compounds and loaded with a fluorescently labelled synthetic phospholipid, along with a DNA marker. Images were acquired with an IN Cell Analyzer 3000 (GE Healthcare, Cardiff, UK), prior to import into Definiens Developer image analysis software equipped with the Cellenger application extension.

Analysis with Definiens software took the following steps (figure 3).

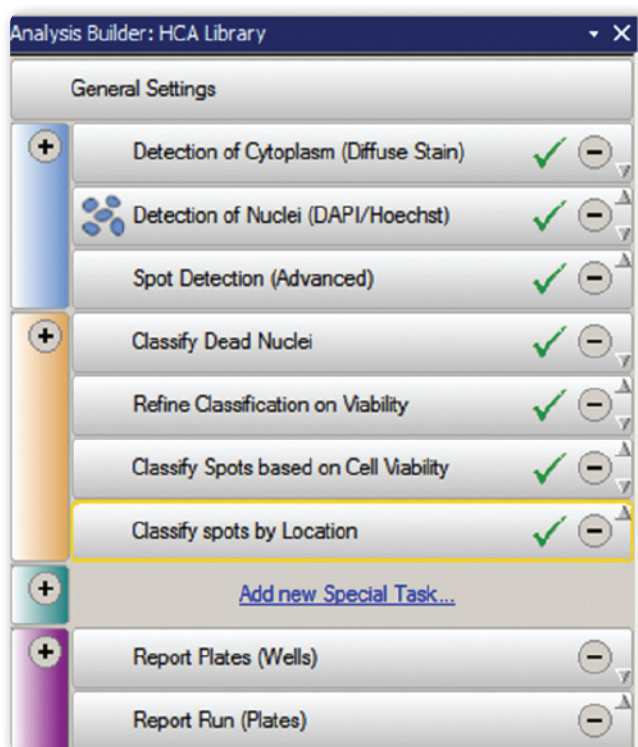


Figure 3 Robust detection actions can be configured using inbuilt algorithms, as can classification of nuclei as live or dead. Further processes (refining classifications and specific classification) can be custom-written using Definiens Developer and package as individual actions.

1. Segmentation and preliminary classification of objects of interest (nuclei, cells, cytoplasm, vesicles), using built in modules. In this instance, detection of cytoplasm took place first, due to the presence of enucleated cells.
2. Classification of objects, including advanced classification techniques. The in-built dead nuclei classifier was used (using brightness and morphology criteria to identify dead nuclei). Customized routines

written in Definiens Developer were added to refine classifications and classify vesicles based on location inside cells.

3. Measurement and interrogation of biological effects and data export.
4. EC50 and LD50 data for selected compounds were calculated using Microsoft Excel Solver plug-in.

Results

1. Segmentation of noisy data

Definiens software generates a hierarchical network of image objects using locally adaptive processes, which allows accurate segmentation of images despite weak signals or noisy data. Such was the case with this assay, as only the weak signal in the nucleus channel could be used for cellular segmentation, as the data from the lipid channel did not always yield cell boundary information (figure 1). Despite the weak Hoechst signal, accurate cytoplasmic segmentation was obtained (figure 4).

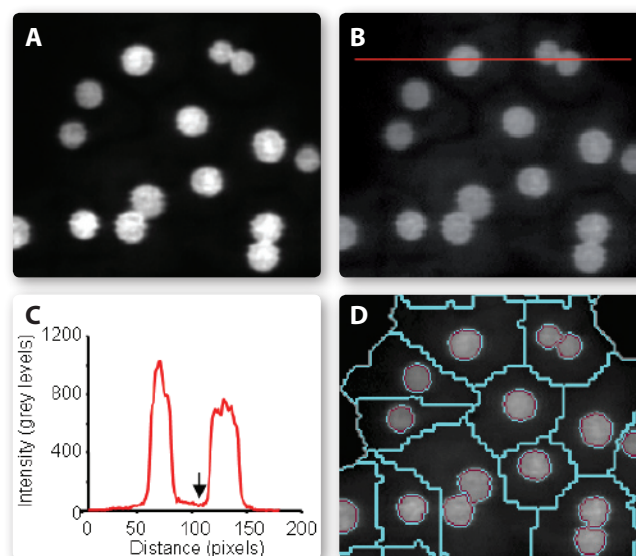


Figure 4 Cellular segmentation using the Hoechst channel. A) Linear image equalization. Nuclei are well defined, but cytoplasmic information is not evident until viewed with different gamma settings (B). Some noisy information is available on cell boundaries (arrow, B and C) but this information can be used to generate accurate cellular segmentation data (D).

2. Advanced classification of image objects

To extract biologically relevant information, semantic labels must be applied to the objects generated in order to impart the knowledge of the biology expert. Definiens Cellenger software permits hierarchical classification of image objects, allowing sophisticated interrogation of the image data. In this example, we used information about nucleus, cytoplasm and vesicle objects and their relationships to each other and to the background to generate labels which were used for data extraction.

Figure 5 shows an excerpt from the image hierarchy, together with example of how spots were classified based on their proximity to cellular membranes adjacent to other cells, or the background.

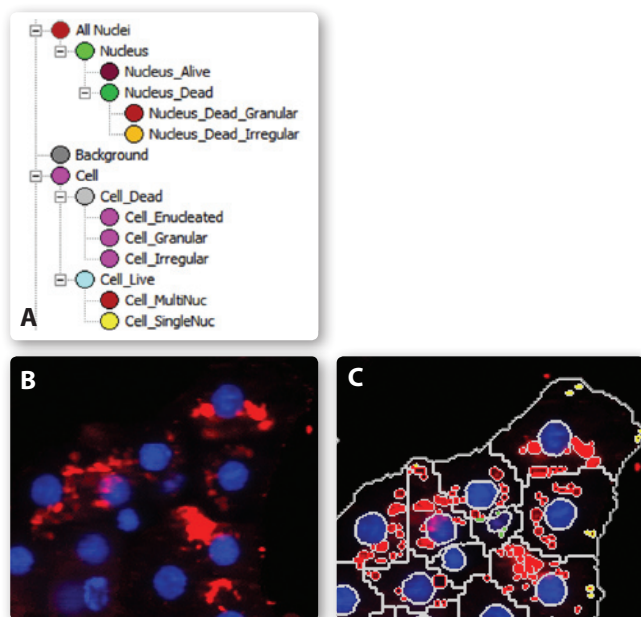


Figure 5 Advanced Classification

(A) Image objects generated by processing were assigned hierarchical labels depending on their properties, thereby enabling detailed investigation of image data. (B-C) Example of detailed classification: the software measured the position of vesicles in relation to membranes backing onto adjacent cells (arrow) or open background space (arrowhead) and classified them accordingly (outlined in red or yellow, panel C). Vesicles in dead cells were not required for the analysis so were labelled differently (outlined in green, panel C)

3. Intricate analysis and data export

The key to data extraction lies within high quality image segmentation and classification as described in the previous paragraphs. The principle readouts from the phospholipidosis assay are cell viability (giving an indication of the toxicity of a compound) and phospholipidotic effect as measured by the average integrated spot intensity of the cells in an image. The maximal phospholipidotic effect typically occurs at concentrations well below toxic doses of compound. Representative data, from which LC50 and EC50 data can be derived, are shown in figure 6.

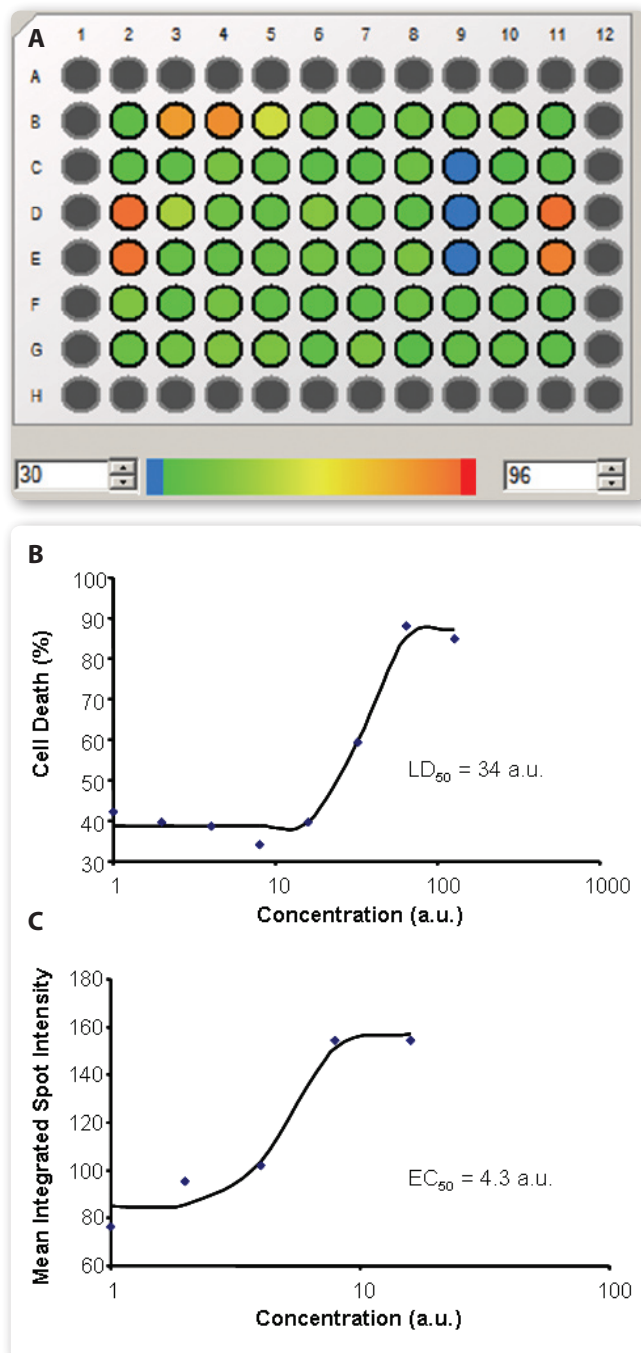


Figure 6 Standard phospholipidosis readouts

(A) Heatmap showing cell morbidity (green-low-red-high). Rows 2 and 11 contain control samples, with wells D2, 11 and E2, 11 cytotoxic controls. Drug dose decreases from rows 2 to 10, with each row representing a different compound treatment. (B) Death curve for compound 1 (row B) and (C) PLD response for the same compound. The maximal PLD response occurs at significantly lower drug concentrations than the toxic dose.

The advanced classification information can be used to probe the data to a much greater level. This has the potential to reveal subtle biological effects, or allow difficult questions to be answered which were previously impossible to address. For instance, one commonly asked question is whether mono-nucleated cells behave in a similar manner to multi-nucleated cells. In order to examine this question, we looked at the maximal phospholipidotic response of cells in phospholipidosis (PLD) controls and test compounds exhibiting a response (1, 2, and 6, from the plate map in figure 6).

Figure 7 shows integrated vesicle intensity data from pooled controls and test samples. In controls, and test samples 1 and 6, there was a slight increase in integrated vesicle intensity in multinucleated cells compared to single nuclear cells. This data may in part be attributed to multinucleated cells covering a larger area (data not shown) and these data not being normalised to cell area (although this is possible). Nevertheless, test compound 2 bucks this trend, with a 15% increase in vesicular intensity in phospholipidotic samples. Such behaviour may indicate a difference in the way cells respond to this compound, and may thus merit further investigation.

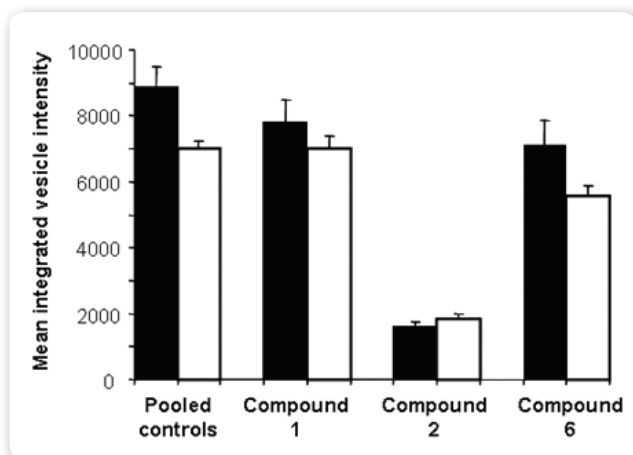


Figure 7 Comparison of single and multi-nuclear cells.

Mean integrated vesicle intensity data is shown for controls (pooled data, 4 wells) and individual test compounds. Black bars: multinucleated cells. White bars: mononucleated cells. Error bars: \pm s.e.m.

Another question that we addressed is whether vesicles preferentially associate with membranes adjacent to other cells, as opposed to those localised along open space. Inspection of the images appears that this appears to be the case (e.g. figure 5B) and this is borne out in the image data of wells exhibiting PLD (figure 8A). However, for accurate analysis, the relative amount of membrane backing onto adjacent cells or open background must be taken into account. The number of vesicles of each type (closer to cell or background membrane) was therefore normalised to the amount of membrane present in the image (figure 8B).

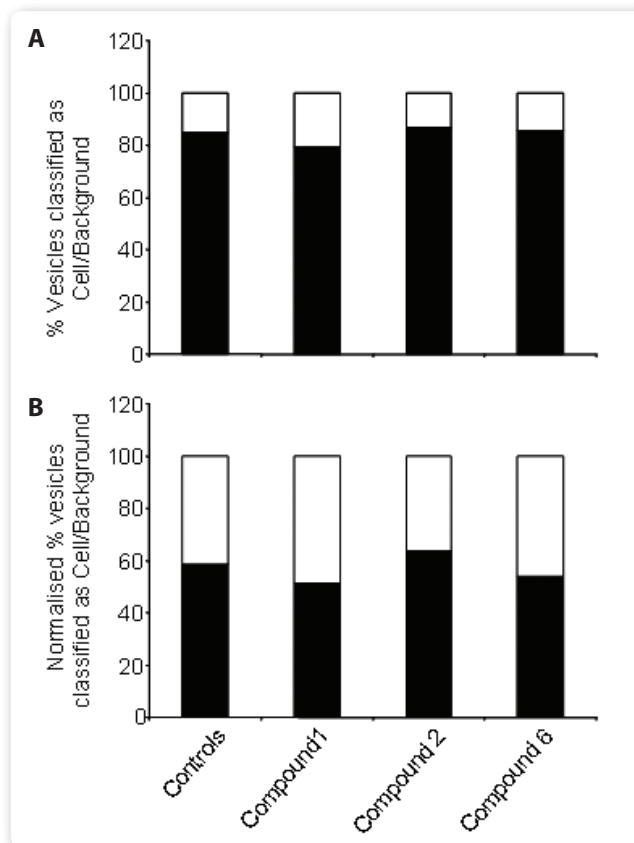


Figure 8 Vesicles location data.

The % of vesicles lying in close proximity to adjacent cells (black bars) as opposed to background (white bars) appears to be significantly greater based on the numbers of classified vesicles (A). However, when the total amount of membrane bordering adjacent cells or background is taken into account, the significance of the trend is markedly reduced (B).

The normalized data indicates that the preferential localization of vesicles observed, while still apparent, is not as profound as might be thought from inspection of the image data alone (figure 7B). This can be explained by the increased quantity of membrane bordering other cells as opposed to background space.

Discussion

It is thought that the introduction of toxicology assays earlier in drug development will have a number of beneficial outcomes. Identification of hit compounds earlier should reduce the time taken in the screening process and the number of candidates reaching late stage development will have higher efficacy and reduced toxic effects. Furthermore, the number of animals used in taking a single drug to market will be markedly reduced, impacting ethical issues surrounding drug discovery. All of these contribute to a huge potential reduction in drug development costs, and the financial loss associated with attrition. In order to derive maximum benefit from these assays, detailed and rigorous analysis techniques must be used alongside the evolving cellular labelling and image acquisition technologies.

In this application note, we have demonstrated how Definiens Cellenger and Definiens Developer image analysis technology has been applied to study phospholipidosis, a potential indicator of drug toxicity. This was done through the steps of very accurate image segmentation despite poor image contrast. The object-based analysis techniques of Definiens software were then used to interrogate the images to going well beyond the capabilities of most existing automated analytical methods and extract information that only hours of painstaking manual analysis would otherwise reveal. For instance, we examined whether mono and bi-nucleated cells responded similarly to drug treatments, in relation to the phospholipidotic response. One compound appeared to behave differently from controls and other compounds causing a phospholipidotic effect. While further investigation is evidently required, this analysis may have revealed a novel biomarker specific to this class of compound, which could be used in further investigations. Similarly other questions, such as whether there is a difference in sensitivity of mono and bi-nucleated cells to the toxic effects of drugs, or even classes of drugs, could easily be answered through export of the relevant data once the appropriate classification has been made.

This approach also enables new hypothesis to be generated and answered relatively quickly. Visual inspection of the images indicate that there may be a preferential localisation of phospholipidotic vesicles along cell:cell boundaries, as opposed to cell:background boundaries. When the vesicles were classified according to their proximity to the different membranes, and the localization normalized to the total membrane in the image, the effect, although it existed, was found to be reduced in the compounds tested. Additional questions could easily be answered by this approach – for instance, further classification of vesicles (including a proximity to nucleus class, for instance), or examination of vesicular clustering with different drugs, concentrations, or restricting the analysis to cells only with membranes backing background. These steps are very easy once the appropriate classification has been made.

The combination of accurate segmentation and hierarchical classification, building a relational network of objects, provides the basis for extensive, detailed, multivariate analysis. Such in-depth interrogation of samples has the potential to generate and answer new hypothesis, reveal novel biomarkers and unlock the full potential of cell-based assays.

Acknowledgements

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