CHALMERS | UNIVERSITY OF GOTHENBURG

High Content Screening of Cellular Blebbing to Predict Sensitizing Potential of Contact Allergens

Master's thesis in Biotechnology Master Degree Programme

YUANMO WANG

Department of Chemical and Biological Engineering
CHALMERS UNIVERSITY OF TECHNOLOGY
Department of Chemistry and Molecular Biology
UNIVERSITY OF GOTHENBURG
Gothenburg, Sweden 2013
Master's thesis 2013:

High Content Screening of Cellular Blebbing to Predict Sensitizing Potential of Contact Allergens

YUANMO WANG

Department of Chemical and Biological Engineering
CHALMERS UNIVERSITY OF TECHNOLOGY
Department of Chemistry and Molecular Biology
UNIVERSITY OF GOTHENBURG
Gothenburg, Sweden 2013

High Content Screening of Cellular Blebbing to Predict Sensitizing Potential of Contact Allergens
YUANMO WANG

Examiner: Assoc. Prof. Marica B. Ericson, University of Gothenburg
Supervisor: Assoc. Prof. Marica B. Ericson, Ph.D Anna-Lena Stenfeldt, University of Gothenburg

Department of Chemical and Biological Engineering
Chalmers University of Technology
SE - 412 96 Göteborg
Sweden
Telephone + 46 (0)31-772 1000

This thesis work was conducted at the Biomedical Photonics Group, University of Gothenburg, Sweden

Department of Chemistry and Molecular Biology
University of Gothenburg
Box 462
SE 405 30 Göteborg
Phone: 031 786 0000
Web Page: www.cmb.gu.se/

Printed by Chemical Reproservice,
Gothenburg, Sweden 2013

Some parts of this thesis has been omitted due to confidential information subject to patent application.

High Content Screening of Cellular Blebbing to Predict Sensitizing Potential of Contact Allergens

YUANMO WANG

Department of Chemical and Biological Engineering
Chalmers University of Technology
Department of Chemistry and Molecular Biology
University of Gothenburg

ABSTRACT

Contact allergy is one of the most prevalent forms of immunotoxicity found in the Western world. Every day, we are exposed to a plenitude of chemicals. In order to replace animal testing which today is used for screening of skin sensitization, it would be great to develop a potential alternative *in vitro* tool.

This thesis project was trying to develop an alternative, non-animal method to detect and predict contact allergens. It combined cellular cultivation with high content screening microscopy to monitor blebbing of keratinocytes (i.e. HEKn) exposed to chemicals *in vitro*. HEKn cells were seeded in 96-well plates and exposed to four sensitizing chemicals (4-ethoxymethylene-2-phenyl-2-oxazolin-5-one, 1-chloro-2, 4-dinitrobenzene,1, 2-Benzisothiazol-3(2H)-one and benzyl benzoate) at five different concentrations (i.e. 0.05 mM, 0.25 mM, 0.5mM, 1 mM and 2 mM) for 24 hours. This whole process was monitored using a high concent screening microscope.

It was found that HEKn cells began bleb after exposure to chemicals with moderate or higher sensitizing potency. The strongest sensitizer caused the largest bleb-cell ratio. The order of sensitizing potency matched the order of bleb-cell ratio among these four chemicals. In addition, cell viability was investigated. It can be concluded that monitoring bleb formation from HEKn cells exposed to chemicals might be a potential alternative method to evaluate the sensitizing potency of chemicals.

Keywords: high content screening, keratinocytes (HEKn), contact allergy, sensitizers, , blebbing, optical microscopy

ABBREVIATIONS

ACD	Allergic contact dermatitis
BIT	1,2 - Benzisothiazol - 3(2H) - one
DC	Dendritic cell
DMSO	Dimethylsulfoxide
DNCB	1 - chloro - 2,4 - dinitrobenzene
EDTA	Ethylenediaminetetraacetic acid
HEKn	Human epidermal keratinocytes derived from neonatal foreskin
HCS	High content screening
HPC	Hapten-protein complex
IF	Intermediate filament
KIF	Keratin intermediate filament
LAF	Laminar air flow
LLNA	Local lymph node assay
OXAZOLONE	4 - ethoxymethylene - 2-phenyl - 2 - oxazolin - 5 - one
PBS	Phosphate buffered saline
MLF	Unit length filament

Contents

1 Introduction

Each year thousands of new chemical substances and products are produced by the modern chemical industry (1). About 15% of population is allergic to some of these products through accidental or intentional skin contact. For instance cosmetics such as soap, shampoo, face cream and perfume are the most common cause of contact allergy, containing numerous potential allergens (e.g. fragrances and preservatives) (2). Hence identification of allergens in cosmetics responsible for allergic contact dermatitis (ACD) became a mandatory in European Union (EU) in 1997. ACD is one of the clinical manifestations of contact dermatitis, and it is one of the most common occupational skin diseases. Peoples who get ACD from a certain trigger are most likely to suffer from it for the rest of their lives. That is probably severe and long-lasting, and even become environmental health issues (3). It is estimated that ACD affects approximately 1% of the general population and 15-20% of the adult population in Europe (1, 3). It seems that the only cure is to avoid contacting with chemical which has become sensitized. Therefore, identifying contact allergens can help prevention of ACD.

Currently the assessment of the sensitizing potencies of chemical compounds still relies on *in vivo* methods, which primarily involve animal testing such as murine local lymph node assay (LLNA) (4, 5). The *in vivo* testing of cosmetic and toiletry ingredients in EU has been banned in 2013 (6). Hence, development of alternative, non-animal, *in vitro* assays for predicting the sensitization potential of new compounds has become an urgent need. Several new potential *in vitro* assays, such as GSH binding assay using MALDI-MS and enzyme-linked immunosorbent assay (ELISA), have been presented recently (7). Reduced Local Lymph Node Assay (rLLNA) is an improved method which needs fewer amounts of animals than LLNA (8). However, for now no reliable alternative method has been approved.

Recently, several important mechanisms behind contact allergy have been identified by Ericson's research group within Centre for skin research, SkinResQU, (9, 10). Briefly, the *in vitro* cultured epidermal keratinocytes, i.e., the dominating cell type in the skin, expel membrane blebs containing modified proteins as a response to allergens exposure, which has reveal a potential method to for predicting sensitization, which is the foundation of the spin-off company InnoVitro AB.

This master thesis project is performed in collaboration with InnoVitro AB, with the aim to further develop and explore the methodology for *in vitro* predictive screening. It combines cellular cultivation and cellular imaging using high content screening (HCS) microscopy. This project focuses on evaluating and optimizing different parameters. It is also collaborated with Fraunhofer – Chalmers Institute to develop software which will be capable of analyzing images of blebbing cells.

2 Aims

The main aim of this project is to investigate the importance of different parameters (i.e. z-level, chemical concentration and chemical exposure time) and find out the relationship between cell blebbing and these parameters, thereby assisting to develop a novel, *in vitro* method for predicting sensitizing potency of chemicals. The specific tasks are to:

1. Carry out cell blebbing experiments via exposing HEKn cells to different sensitizers, to investigate the reproducibility of the InnoVitro method.
2. Test the sensitivity of InnoVitro software to analyze microscopy images of HEKn cells exposed to different sensitizers
3. Set up the protocol of HEKn cell cultivation on 96-well plate. Evaluate the appropriate confluence by optimizing the seeding density and cultivation time.
4. Implement bleb monitor using HCS microscope to investigate parameters:
   - Magnification of objective lens
   - Z-levels
   - Bleb monitoring time
5. Analyze the correlation between chemical sensitizing potency and blebb response and [redacted]:

3 Background

3.1 ACD - Allergic Contact Dermatitis

Allergic Contact Dermatitis is an inflammatory skin reaction caused by chemical exposure (11). The clinical manifestations are characterized by red rash, blister, localized swelling, itching and pain. There are more than 4000 contact allergens that can cause ACD (12). According to the North American Contact Dermatitis Group (NACDG), metal (e.g. nickel and gold), fragrance, preservative (e.g. formaldehyde) and antibiotic are the most common allergens (13).

ACD is delayed-type hypersensitivity (type IV). It is mediated primarily by allergen-specific T-cells (11). The immunological mechanisms of ACD involve two main phases (**Figure 1**): **sensitization** and **elicitation** phases. The sensitization phase begins with the exposure to contact allergens with dendritic cells (DCs) in the epidermis (14), leading to proliferation of a-specific T-cells and generation of effector and memory T-cells (11).

Contact allergens (a.k.a. haptens) are usually small molecules whose molecular weight is less than 500 Da (11, 15). The haptens are too small (16) to directly elicit an adaptive immune reaction unless they bind to the bigger carrier proteins, form hapten-protein complexes (HPCs) via covalent linkage. Theses complexes serve as the complete allergens (antigen) for stimulating the immune system (17, 18). In the sensitization phase, HPCs are recognized and internalized by dendritic cells (DCs) (19). DCs subsequently migrate via afferent lymph vessels to the draining lymph nodes where they present HPCs to naïve T lymphocytes (20). The reactions between HPCs and naïve T lymphocytes induce the proliferation of antigen-specific T-cells and formation of memory T - cells, which then leave the lymph nodes and enter the circulation in blood and lymph vessels (20).

The elicitation phase is initiated by the subsequent exposure to the same allergens which will lead to elicitation of inflammatory reactions. Once the same haptens penetrate the epidermis again, memory T-cells are recruited to the site of hapten exposure from the circulation by cytokine and chemokine secreted by epidermal cells (e.g. keratinocytes) (19, 21). And more aggressive immune responses (e.g. inflammatory process) are elicited subsequently (22).

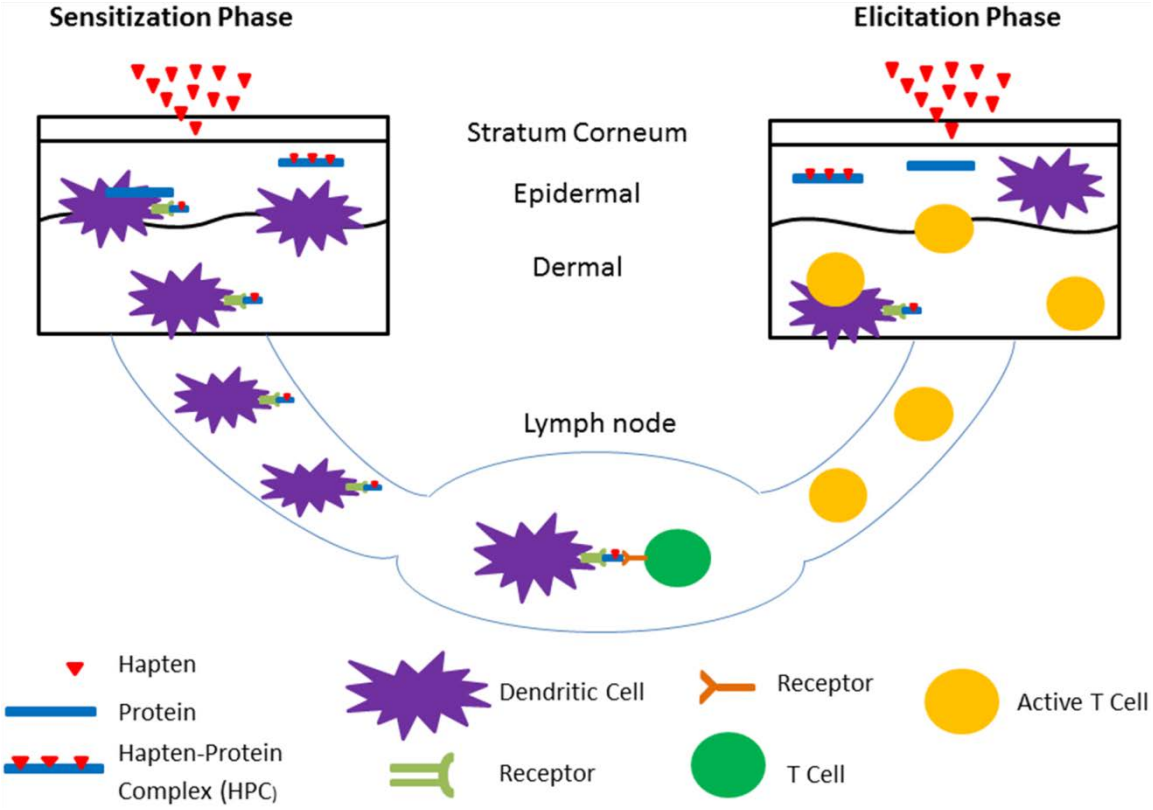


Figure 1 Schematic illustration of pathophysiology of allergic contact dermatitis (ACD).

A mechanism behind the pathophysiology of ACD has recently been suggested by researchers in SkinResQU. Haptens are taken up by keratinocytes, bind to keratins, and subsequently are expulsed as HPCs in membrane blebs to surroundings. These blebs subsequently are taken up by skin resident DCs and hapten-keratin complexes are presented as neoepitopes to T cells in the draining lymph nodes, triggering the acquired

immune response (9, 10). This hypothesis has been proved *in vitro* and *ex vivo* (9, 10). It might play a role during hapten sensitization *in vivo* and should be subject to further investigations.

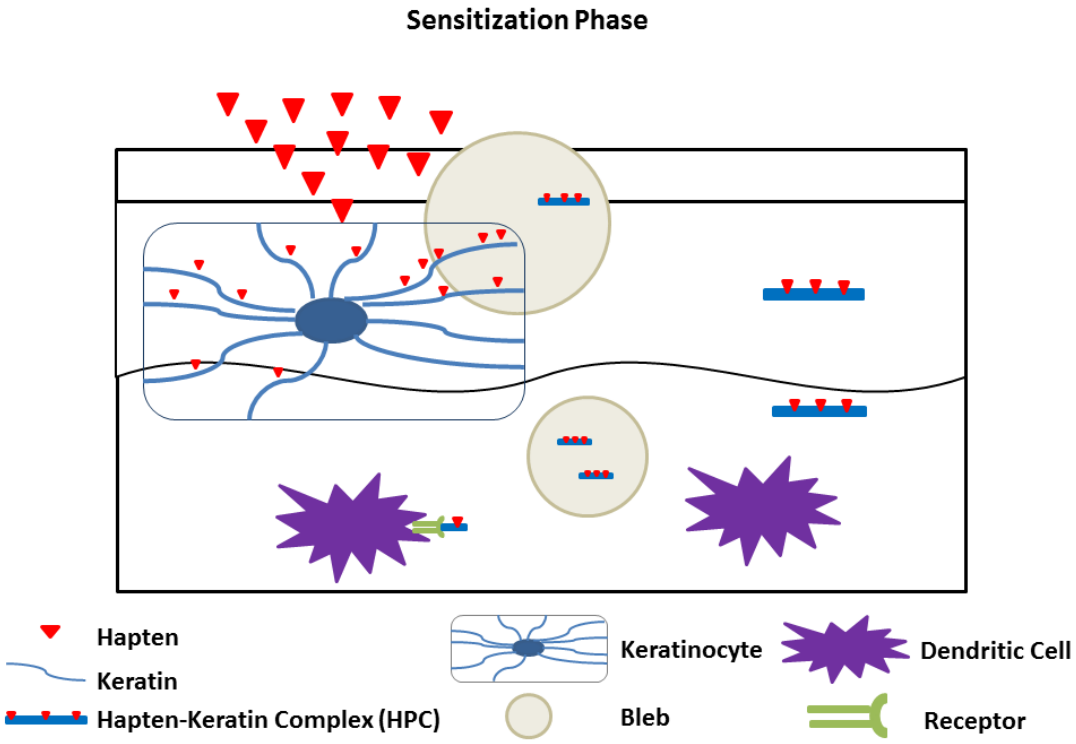


Figure 2 Mechanism behind the pathophysiology of ACD proposed by Ericson's group (9, 10).

3.1.1 Contact Allergen - Hapten

"Hapten", is derived from Greek, meaning "to fasten" (23), and was termed by Landsteiner and Jacobs in their research (17). Most contact allergens are electrophilic, they can form the protein – hapten complex (HPC) **Figure 3** (19). Our skin can be considered as a nucleophilic environment, because it is composed by a large amount of water and proteins with nucleophilic functional groups (20). Nucleophilic functional groups such as thiols (-SH) and amines ($-NH_2$) present in the side chains of the amino

acid cysteine and lysine, respectively, they are two major targets of electrophilic haptens.

Figure 3 Haptenation of hapten and protein with higher molecular weight

3.2 Chemical exposure *in vitro* using HEKn keratinocytes

Keratinocytes, the principle epidermal cell type, are making up approximately 95% of the cells in the epidermis. Epidermis forms the outer layer of the skin, and is organized into four layers, the basal layer; spinous layer; granular layer and cornified layer (**Figure 4**) (15, 24, 25). Keratinocytes are formed from the undifferentiated stem cells located on the lower part of the basal layer (26, 27). These cells divide, differentiate and migrate through each layer and reach the outer layer of the epidermis in the form of corneocytes (26, 27). The major role of corneocytes is serving as a barrier to protect an organism from toxins, pathogens (i.e. bacteria, fungi, viruses), UV radiation , and moisture and heat loss.

HEKn cells are the normal human epidermal keratinocytes isolated from neonatal foreskin. In this thesis, HEKn cells were used for the chemical exposure experiments. Micrometer-sized cell membrane blebs were developed as the response of keratinocytes to hapten exposure as illustrated by **Figure 5**.

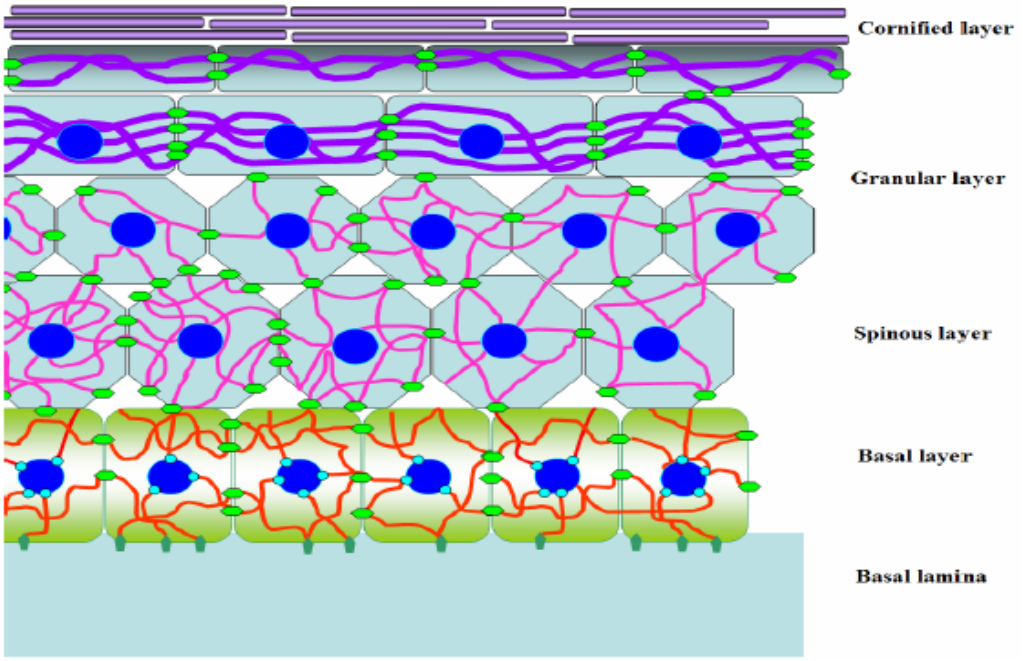


Figure 4 Diagram of the structure of epidermis and the keratin filaments networks in skin epithelial tissue.

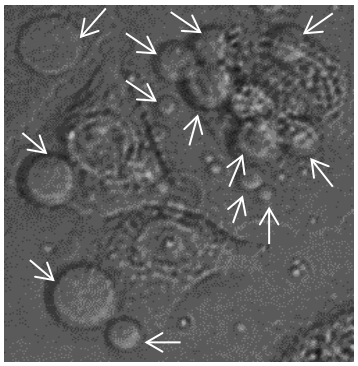


Figure 5 Blebbing HEKn cells were exposed to BIT at 0.05mM. The blebs are high-lighted by white arrows.

HaCaT (Ha = human adult, Ca = calcium, T = temperature) is a well-known spontaneously immortalized human keratinocyte cell line which was developed via a long-term primary culture of normal human adult skin keratinocytes at decreased calcium and increased temperature. HaCaT cells are frequently and extensively utilized as substitute for normal human keratinocytes due to their highly preserved differentiation and

proliferation capacity *in vitro* (28, 29). HaCaT cells possess mutations in both alleles of *p53* gene (30), and it is hypothesized that HaCaT immortalization involves a coordinated action of *p53* gene mutation and elevated culture temperature which act as the major inducers of accumulated genetic alterations (31). In this thesis, HaCaT cells were also cultured. Hopefully, they can be used for the chemical exposure experiments instead of HEKn cells in the next stage.

3.3 Keratin

It has recently been shown that keratin is modified during chemical exposure (9, 10). Keratins are the largest subgroup of intermediate filament proteins and divided into six types (32), they are structure proteins present in the integument in vertebrates and particularly the major constituents of epithelial cells in mammals (33-35). Keratins belong to type I (acidic) and type II (basic) intermediate filament proteins based on their size, sequence homology and charge properties (24, 36, 37). The networks in epidermal keratinocytes are formed by keratin intermediate filaments (KIFs). KIFs are regarded as "bones" which spin between the nucleus to the cell membrane as illustrated by **Figure 4**. KIFs bind to the linker proteins of desmosomal and hemidesmosomal attachment complexes which are cell–cell junctions located on plasma membrane (24, 34, 38). Therefore, this feature implies that keratins play an important functional role in mechanical stability and integrity of epithelial cells and tissues (39) . Keratins can also be classified into "hard" and "soft". Soft keratin has less sulfur compared to hard keratin, and is almost exclusive in epidermis (33).

All IFs and KIF protein chains (i.e. monomers) share the common structural characteristics: a long central α-helix rod domain of about 310 amino acids contains four segments (i.e. 1A, 1B, 2A and 2B) as illustrated by **Figure 6** (32, 34, 35, 40). These four segments are separated by three short non-helical linker regions (i.e. L1, L12 and L2) and

flanked by variable non-helical amino-terminal head domain and carboxyl-terminal tail domain (Figure 6) (32, 34, 35, 40).

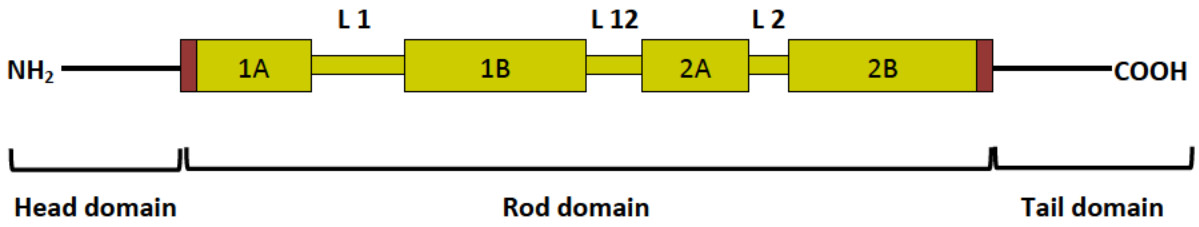


Figure 6 Schematic representation of the tripartite doamin structure shared by all keratin and other IF proteins.

3.4 Optical Microscopy and Live Cell Imaging

Optical microscope (i.e. light microscope) is an instrument which uses visible light and magnifying lenses to examine small samples. The modern optical microscopes share the same basic components of the light path: ocular lens (i.e. eyepiece), objective turret, objective lenses, coarse adjustment, fine adjustment, stage, light source, condenser and mechanical stage (41, 42).

For imaging live cells, their life need be maintained using necessary medium. When light travels through medium other than vacuum, the interaction between light with this medium causes changes in amplitude and phase. However, human eye cannot observe these changes in phase easily under optimal bright field illumination. In fact, changes in phase often carry much important information; they are dependent on refractive index and thickness of the medium. This is the hardest part for observation of live cells (43) .

Generally, cells and other semi-transparent biological specimens are visualized in brightfield microscopy by artificial staining techniques to improve contrast. Unfortunately, the process of staining require tissue fixation which kills these specimens. With the invention of phase contrast microscopy by Fritz Zernike in the 1940s,

observation of unstained living cells in detail became possible (44). In the 1950s, the theory for the second poplar phase contrast microscopy method — differential interference contrast (DIC) microscopy was published by Georges Nomarski. Both these methods can aid the imaging of transparent biological samples.

3.4.1 Phase Contract Microscopy

The phase contrast microscope is a technique used for observing transparent and colorless specimens by enhancing their contrasts based on phase changes caused by the samples. Phase contrast microscopy works by utilizing an optical mechanism to transform minute changes in phase within cellular components and between unstained cells and their surrounding medium into detectable changes in amplitude, which can be visualized as differences in image brightness (i.e. contrast) (42).

The basic principle behind visualization phase changes in phase contrast microscopy is to separate the background light from the specimen scattered light. In order to achieve this, a condenser annular aperture is placed in the front focal plane of the condenser and a matching phase plate is installed in the back focal plane of the objective (**Figure 7** on the left). However, phase contrast technique has two main disadvantages, the specimen must be very thin and a halo is produced in the images (42).

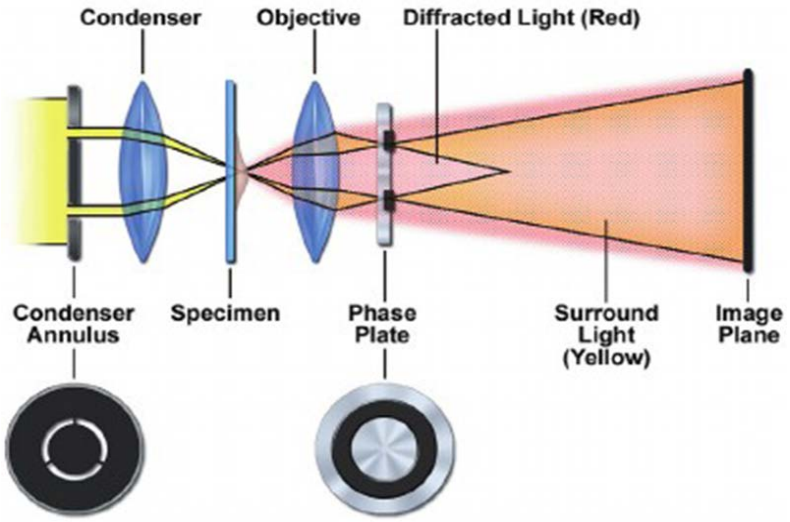


Figure 7 Optical components of a phase contrast microscopy and its Working principle (42)

3.4.2 Differential Interference Contact (DIC) Microscopy

DIC microscope produces high-contrast images also by visually displaying differences in the light refraction within transparent specimens and between the specimens and their surroundings. The difference from phase contrast microscopy is that light path length gradients created by DIC system are primarily responsible for contrast. Its working principle is illustrated by **Figure 8** (42).

In a DIC system, light from the illumination source is passed through a polarizer between the light source and the condenser. Next, this beam is separated into two beams with a slight path difference by a two-layered modified Wollaston prism. When separated beams enter and pass through the specimen, their wave paths are altered in accordance with the variation of the specimen's thickness, slope and refractive index. As the gradient of optical path difference grows steeper, image contrast is dramatically increased. Later, the beams are recombined by the second Wollaston prism and a second polarizer or analyzer, this causes amplitude variations which can be visualized as difference in brightness (42).

DIC system does not produce halo effect, and it can be applied to obtain very clear images of thick specimens. Three-dimensional appearing image is the characteristic of DIC techniques, yet a drawback is that the three-dimensional image of a specimen may not be accurate (42).

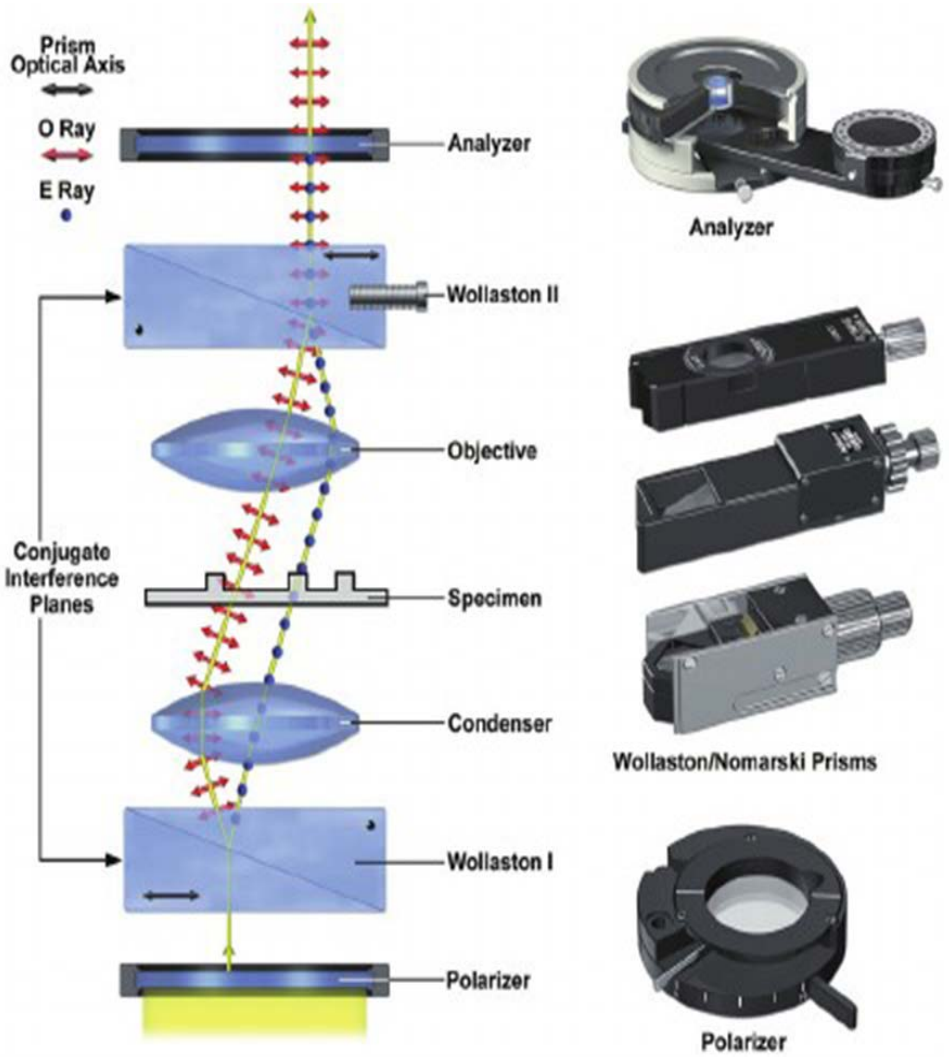


Figure 8 Optical components of a DIC microscope and its working principle (42).

3.4.3 Live-Cell Imaging and High Content Screening (HCS) Microscopy

With the breakthrough in optical microscopy, investigations of living cells and tissue are increasing with the help of live-cell imaging techniques. In order to maintain live cells in the live-cell imaging system, specimen chambers are an integral and crucial part. Oxygenation, humidity, osmolarity and pH are the most important environmental variables that must be addressed (43).

High content screening (HCS) microscopy is a combination of modern cell biology with automated high resolution microscopy (**Figure 9**). It allows for the evaluation of multiple biochemical and morphological parameters in intact biological systems by simultaneous readout of a large number of data. In a HCS system, cells are usually incubated with the substance within a modified culture chamber. These culture chambers can be multi-well plates, single slides, Petri dishes or custom-built arrays. After a period of time, investigated information is collected by an optical microscope equipped with camera, this is the second crucial component of HCS system. The third important component is the exclusive software installed into a computer. It is designed for automated process and analysis of data.

4 Materials and Methods

The project has been performed within the Biomedical Photonics group. Mainly two different facilities have been utilized: the cell culture facility at Dept. of Chemistry and Molecular Biology (GU), and the Centre for Cellular Imaging (CCI) at Sahlgrenska Academy (GU). Economic support was provided by InnoVitro AB. The materials and the methodological procedures are described in details in the following sections.

4.1 Chemicals

All chemicals and substances used in this project are as follows: 4-ethoxymethylene-2-phenyl-2-oxazolin-5-one (Cat. No. E0753; Fluka by Sigma-Aldrich, USA); 1-chloro-2, 4-dinitrobenzene (Cat. No. 237329; Aldrich by Sigma-Aldrich, USA); 1, 2-Benzisothiazol-3(2H)-one (Cat. No. 561487; Aldrich by Sigma-Aldrich, USA); benzyl benzoate (Cat. No. B6630, Sigma-Aldrich, USA); Dimethyl sulfoxide (DMSO; Cat. No. D8418; Sigma by Sigma-Aldrich, USA); Phenol red-free EpiLife® Medium (Cat. No. M-EPICF-500; Cascade Biologics, Portland, OR); Calcium chloride (CaCl2; Cat. No.; Cascade Biologics, Portland, OR); Penicillin/streptomycin (Cat. No. P11-010; PAA Laboratories GmbH, Austria); Human keratinocyte growth supplement (HKGS; Cat. No. S-001-5; Cascade Biologics, Portland, OR); Trypsin/EDTA Solution (Cat. No. NC9887893; Fisher Scientific, USA); Trypsin Neutralizer Solution (Cat. No. R-002-100; Gibco® by Life Technologies, USA). The sensitizers are summarized in **Table 1**.

Table 1 Sensitizers used in this thesis and their sensitizing potency

No.	CAS No.	Chemicals compounds	Abbreviations	Sensitizing potency
1	15646-46-5	4 - ethoxymethylene - 2-phenyl - 2 - oxazolin - 5 - one	OXAZOLONE	Strong
2	97-00-7	1 - chloro - 2,4 - dinitrobenzene	DNCB	Strong
3	2634-33-5	1,2 - Benzisothiazol - 3(2H) - one	BIT	Moderate
4	120-51-4	Benzyl benzoate	-	Weak

4.2 Main Equipment

The equipment involved in InnoVitro project included three main components: a 96-well plate, a HCS system and an analysis software (i.e. InnoVitro software) as illustrate by Figure 9. It also shows the main workflow in the laboratory. The 96-well plate was used as a culture chamber for cultivation of cells with different chemicals. The HCS system is able to provide all conditions for keeping cells alive, and monitoring the specimens by imaging automatically using the built-in microscope. The InnoVitro software was used to analyze images by counting blebs and cells, and predict the results (i.e. sensitizing potency of chemicals).

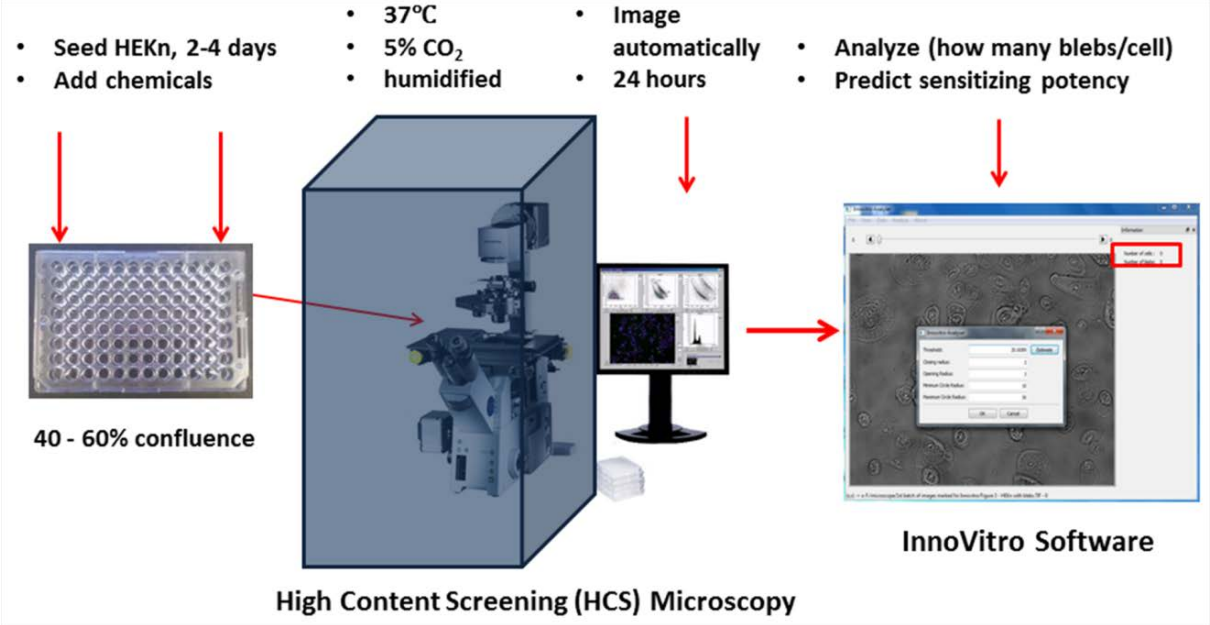


Figure 9 Schemic representation of the main workflow and equipments involved in InnoVitro project.

4.3 Cells

HEKn cell line (Cat. No. C-001-5C; Cascade Biologics, Portland, OR) were used for the primary experiments. They were cryopreserved at the end of the primary culture stage with a medium containing 10% DMSO. According to the specification provided by the manufactures, there are at least 70% of viable cells upon unfreezing and a potential of at least 30% population doublings when subcultured.

4.3.1 Cell Thaw and Culture

HEKn cells were obtained from continuous cell line cryopreserved in liquid nitrogen at -170°C. Cells were thawed in a water bath at 37 °C and transferred into T-25 flasks (25 cm^2; VWR, Sweden). The complete media consists of 50 mL phenol red-free EpiLife® Medium supplemented with 60µM $CaCl_2$, 0.2% penicillin/streptomycin, and 1% (v/v) HKGS. The final concentrations of the constituents in the supplemented medium were as follows: bovine pituitary extract, 0.2% v/v; bovine insulin, 5 mg/mL; hydrocortisone,

0.18 mg/mL; bovine transferrin, 5 mg/mL; human epidermal growth factor, 0.2 mg/mL; gentamicin, 10 µg/mL; and amphotericin B, 0.25 µg/mL.

2500 cells/cm^2 density was chosen to seed 62500 cells for each T-25 flask. The cells then were grown at 37 ℃ in a humidified 5% CO_2 incubator (Thermo Fisher Scientific, USA) with a loosened cap, to allow gas exchange. The medium was changed every third day to maintain cell growth until the culture reached approximately 60% - 70% confluence for use in further experiments. The time between subculturing depends on the seeding density, and it varied from 4 days (i.e. 5000 cells/cm^2 seeding density) to 6 days (i.e. 2500 cells/cm^2 seeding density). The HEKn cells used for chemical exposure experiments varied from passage fourth to passage seventh. The cell subculture, also called cell passaging or splitting, will be further explained in the following section.

4.3.2. Cell Subculture

Before placing cells and necessary equipment into the laminar air flow (LAF) bench, the LAF-bench was started and cleaned with 70% ethanol solution to ensure the sterility. Also, all bags with materials (i.e. flasks, tubes, pipettes, pipette tips and reagent solutions) or their packaging were cleaned using 70% ethanol before placing into the hood. The cells were checked under the optical transmission microscope to evaluate the confluence and the potential contaminations prior to the subculturing procedures.

After removing all of the old culture medium from the flask with HEKn cells, 2ml Trypsin/EDTA Solution was added to wash away residual medium from cells. Then these 2ml of Trypsin/EDTA solution were substituted with 1ml of fresh Trypsin/EDTA solution to detach the cells from the surface of the flasks. The cells were incubated for approximately 8 - 10 min at room temperature and viewed under the microscope to ensure that they had been completely round. Rapping the flask gently against the palm could be conducted to dislodge cells from the surface of the flask. In order to stop the enzymatic activity of Trypsin/EDTA, 3ml of Trypsin Neutralizer Solution were added to

the flask. The detached cells were transferred to a sterile 15ml tube. Another 3ml of Trypsin Neutralizer solution were added to the flask and any remaining cells were transferred to the same 15ml tube. Cells were centrifuged at 150 × g, 17 ℃ for 7min, and the supernatant was carefully removed. The pellet was resuspended in 1ml of 37 ℃ culture medium. 10 µl of cell suspension was taken to the hemocytometer and the number of cells was counted according to **Section 4.3.3**. Finally, the calculated volumes of cell solution corresponding to 62500 cells/flask were added into the new T-25 flasks containing 5ml of complete media and incubated as described in the **Section 4.3.1**.

4.3.3 Cell Counting

The number of cells during the subculture procedures was determined by an ordinary hemocytometer and a standard light microscope. The hemocytometer was cleaned with 70% ethanol and dried carefully prior to each counting. 10µl of the homogeneous cell suspension were added slowly to on both sides of the hemocytometer slowly to ensure the space between the hemocytometer and the cover glass was fully filled.

An optical transmission microscope equipped with 10X magnification objective lens was used to observe the hemocytometer images. At least two squares on the edges of each side were counted. For each square, the cells on the top and left lines were counted, but those on the bottom and right lines were skipped. The average cell number was multiplied by 10 000 for each side (correction factor of the hemocytometer) to obtain the approximate number of cells per ml. This cell number was then multiplied by the volume of cell solution to know the total number of cells.

4.3.4 Seeding Cell in 96-well Plastic Plate

The 96-well plates (Cat. No. 167008; Thermo Scientific, USA) were chosen for chemical exposure experiments since it matches the HCS microscope. The same medium for the

cell culture in 96-well plates was used, but without antibiotics. In biochemistry, it is more similar to the real in vivo environment. Otherwise, antibiotics should be counted as a factor because they may cause unusual biochemical reactions within cells.

Presented in **Figure 10** is the practical plan for chemical exposure experiments with a 96-well plate. Four chemicals (i.e. Benzylbenzoate, BIT, DNCB and Oxazolone) were chosen in this thesis; they were dissolved using DMSO and then diluted in cell media to obtain a concentration gradient (i.e. from 0.05mM to 2mM). The final concentration of DMSO in each well was 1%. HEKn cells were cultured with 150µl of medium in every well, and three wells were prepared as a group for each concentration. In addition, there were one DMSO group and one pure media group as the negative control. This plate was placed in to an ordinary incubator and medium was changed every third day to maintain cell growth. Then, chemical exposure experiments could be carried out when cell confluence reached approximately 40% -60 % after 3 - 4 days of cultivation. This confluence is the optimal one for chemical exposure and particularly image analysis.

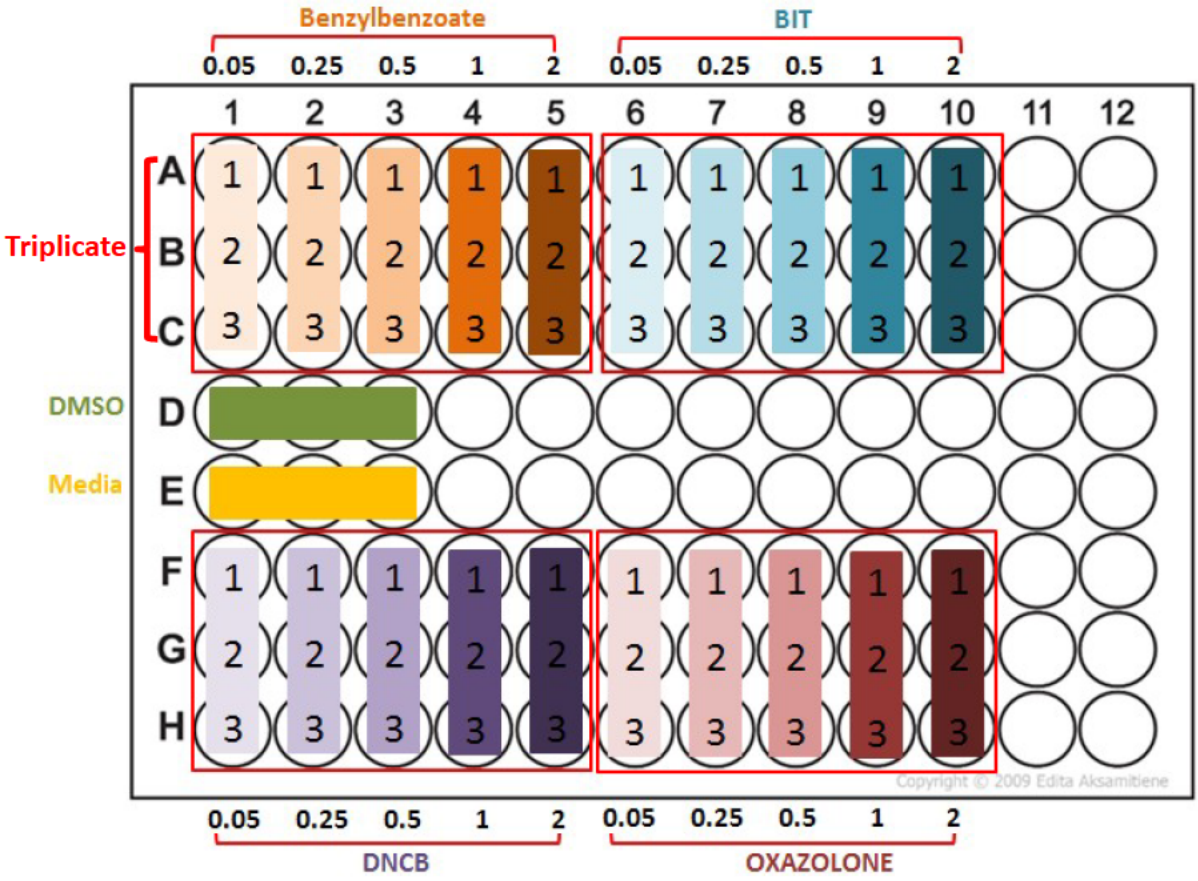


Figure 10 Schematic representation of 96-well plate and the plan of chemical exposure experiments. Concentration unit is millimolar (mM).

4.4 Chemical Exposure Experiment

Presented in Figure 11 is the primary process of chemical exposure experiment, HEKn cell used in this project was cultured in 96-well plate and then incubated with different chemicals. When cell confluence reached 40% - 60%, media in each well were substituted by 100µl of fresh media (without antibiotics) that had been warmed using 37°C water bath in advance. Then the plate was placed back into incubator. This process was mainly conducted on the LAF-beach.

Next, chemicals were weighed and dissolved into DMSO to obtain the desired concentration. Cell media was used to dilute the chemical DMSO solution further. Then,

50µl of diluted chemical solution was added into each corresponding well that had contained 100µl of media. The final concentration of all chemicals was diluted 100 times. These chemical dissolution and chemical exposure processes were performed in a fume hood.

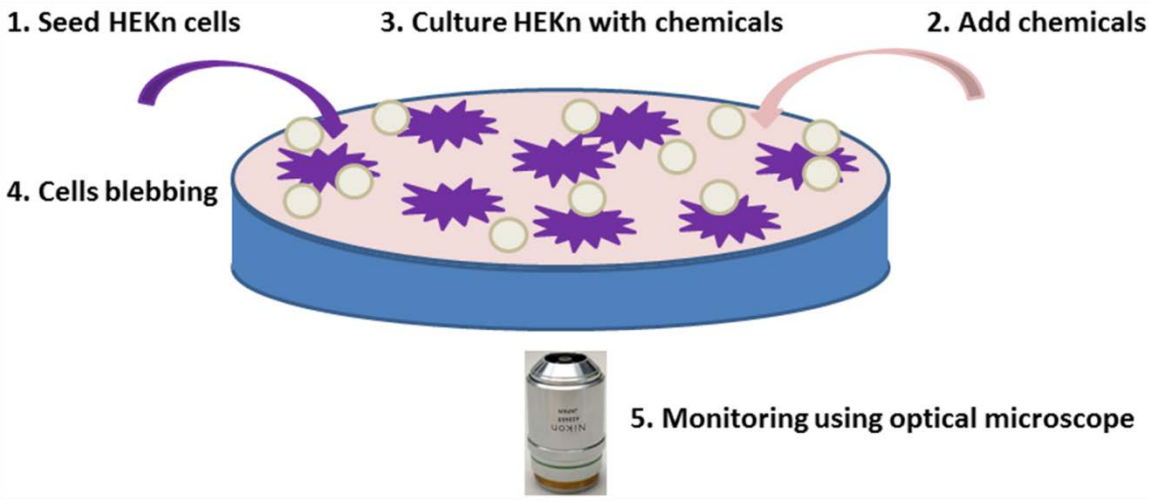


Figure 11 Schematic representation of the primary processes of chemcial exposure experiments.

4.4 Cellular Imaging and High Content Screening

For Section 5.1 - 5.4, the HEKn cells and the blebs were visually examined using an optical transmission microscope Nikon Eclipse TE300 (Nikon Inc., Shinjuku, Tokyo, Japan). For each well, images at three different positions were selected and taken by a Kappa DX20 H-FW digital camera (Kappa optronics GmbH, Germany). Since the HEKn cells and their blebs were not always at the same focus planes of the objective, two images of the same position might be taken, one focused on cells and one focused on blebs. The selected images were visualized on the screen of computer using the software Kappa ImageBase (Kappa optronics GmbH., Germany) with a 20× objective lens. The captured

images were further organized using the software Image J (National Institutes of Health, USA).

For Section 5.5, HEKn cells with blebs were monitored using HCS system as illustrated by Figure 12. The HCS system utilized in this thesis is based on a highly automated imaging system which combines an Olympus IX81 motorized microscope with software called scan^R (EMBL, Heidelberg, Germany) and a Hamamatsu C8484 CCD camera. This system is also equipped with several types of objectives: high NA objectives with and without Phase Contrast for "thin" and thick sample carriers, Long Working Distance Phase Contrast objectives for "thick" sample carriers, high resolution Water Immersion objective; hardware and software autofocus. It is provided by Centre for Cellular Imaging (CCI) at Gothenburg, Sweden.

CO_2 and humidified air are injected into a stage culture chamber to provide a humidified 5% CO_2 environment. The temperature is also kept at 37 ℃ by a heater. This makes the HCS system possible to screen living specimens (e.g. living cells).

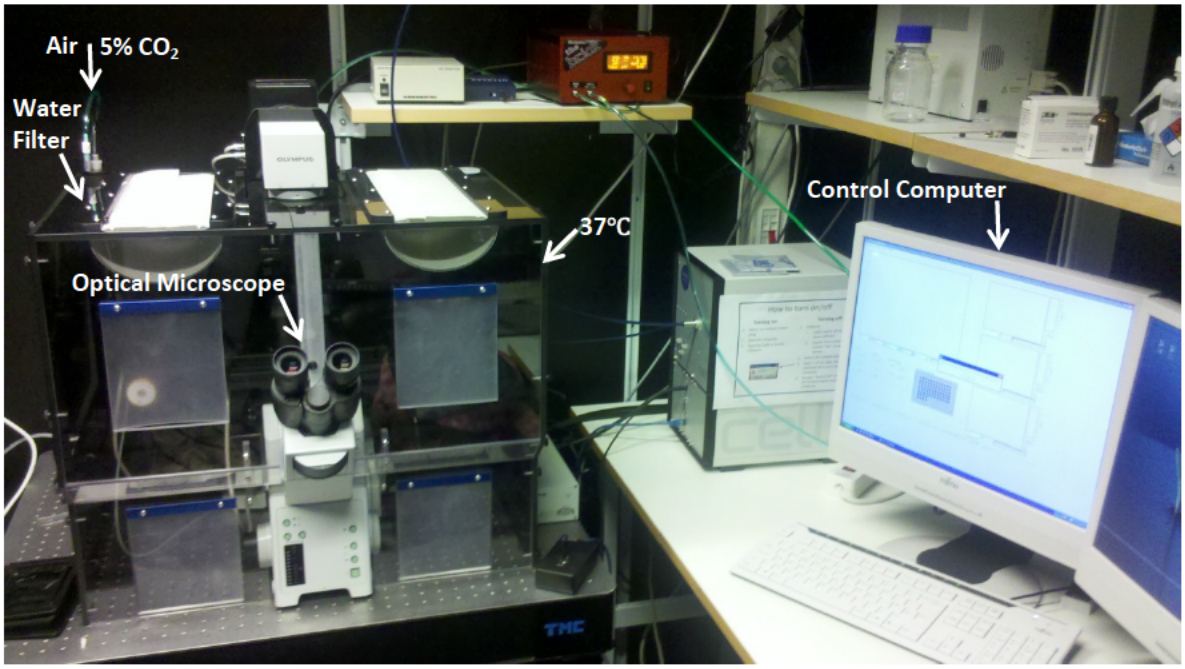


Figure 12 Schematic representation of the crucial components of a HCS system at Centre for Cellular Imaging (CCI), Gothenburg, Sweden.

Scan^R is a modular microscope-based imaging platform designed for fully automated process and analysis of large amounts of data from living specimens. Scan^R can handle many different chamber formats e.g. multi-well plates, single slides, Petri dishes or custom-built arrays. This makes it possible to screen both large and small numbers of samples. Scan^R also possesses fully automated image acquisition that allows the user to select the number of images per well, the number of wells and the different filter sets.

In this thesis, settings of HCS system is pre-programmed by Scan^R. The specific settings are as follows:

- Magnification：LUCPLFLN 20× objective lens
- Illumination：Transmission
- Exposure time: 10
- Intensity: 50%
- 66 wells are chosen for imaging according to **Figure 11**.
- 5 positions (i.e. one at the center, four around the center) are chosen for imaging in every well as illustrated by **Figure 13**.
- For each position, cells are monitored at 5 different z-levels as presented in **Figure 14**. The distance between every two z-levels is 3μm. Because blebs may locate at different z-levels of focus, they may not be observed together with HEKn cells.

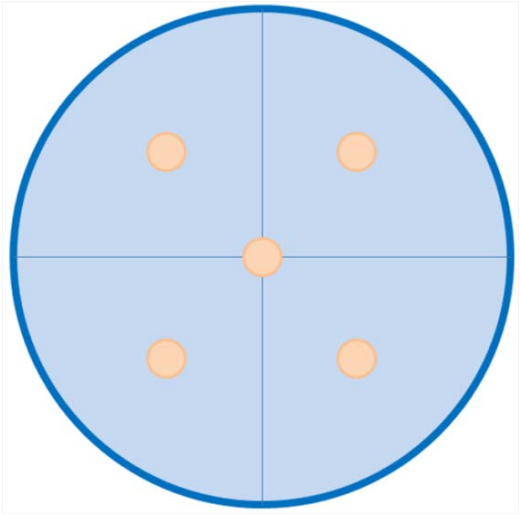


Figure 13 Schematic representation of cellular imaging at 5 positions of one well in a 96-well plate.

- Images are obtained every one hour. The whole chemical exposure time lasts 24 hours; there are 25 time points in total.

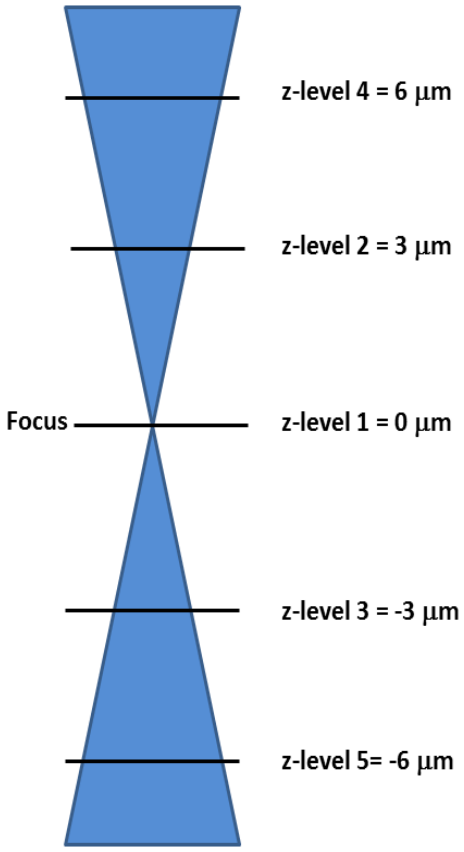


Figure 14 Sechmatic representation of the z-levels around the focus. 5 different z-levels were tested

4.5 Image Analysis

In this thesis, the whole data obtained from HCS system contains more than 4000 images. They are named by well, position, z-level and time point. For instance, B8--W00020--P00002--Z00001--T00000--Trans means this image is taken at well B8 (i.e. well 20), position 2, z-level 2 and the 1st time point using transmission optical objective lens. These images are sorted and organized into different file folders named by well, position and z-level. For example, A1-P2-Z0 means well A1, position 2 and z-level 1. Finally, each folder contains 25 images at 25 continuous time points.

The image data is analyzed using InnoVitro software which is under development by Fraunhofer-Chalmers Research Centre. It was installed on November 28th, 2012. For now, its' biggest use is to count and document cells and blebs manually (Figure 15).

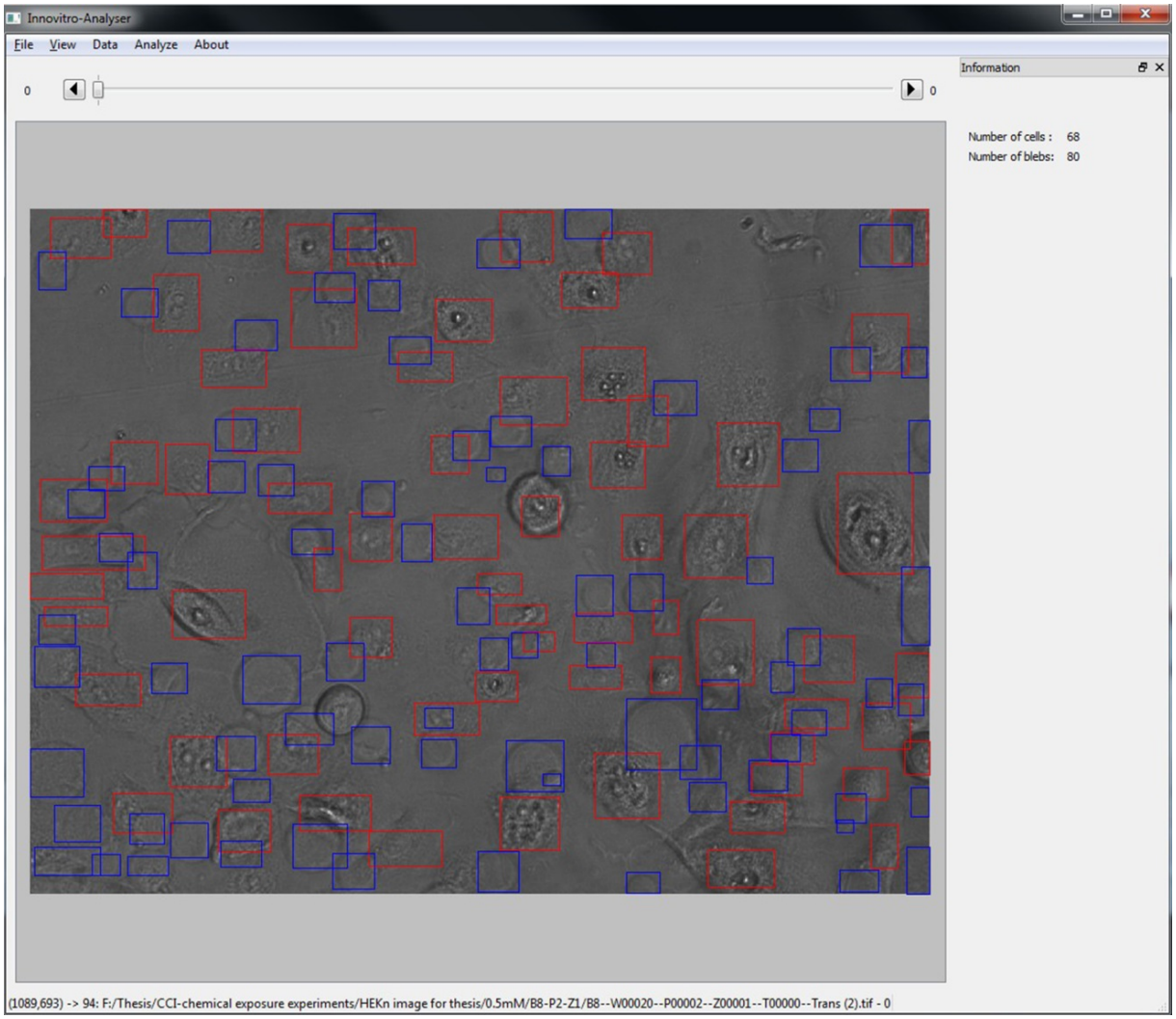


Figure 15 Schematic representation of InnoVitro software. The red squares mark cells and blue squares mark blebs. The number of cells and blebs is presented at the top right corner. HEKn cells exposed to BIT at 0.5mM.

Threshold is the level at which something starts to happen or have an effect. In image processing field, thresholding is the simplest method to segment image (45). It was categorized into six groups based on the information the algorithm manipulates by

Sezgin and Sankur (46). InnoVitro software was designed to be able to detect the most amounts of cells and blebs in one selected image by finding an optimal threshold.

5 Results

5.1 Optimizing Magnification of Objective Lens

Figure 16 shows two images of HEKn cells seeded at the same concentration (i.e. 2000 cells/well) for 2 days. The images were obtained from the same position of the same well by two different objective lenses: 10X and 20X. For these two images, neither of them showed much preference. However, Figure 14 b) obtained by 20X objective lens is analyzed easily by InnoVitro software. Thus, 20X objective lens was chosen for the further experiments.

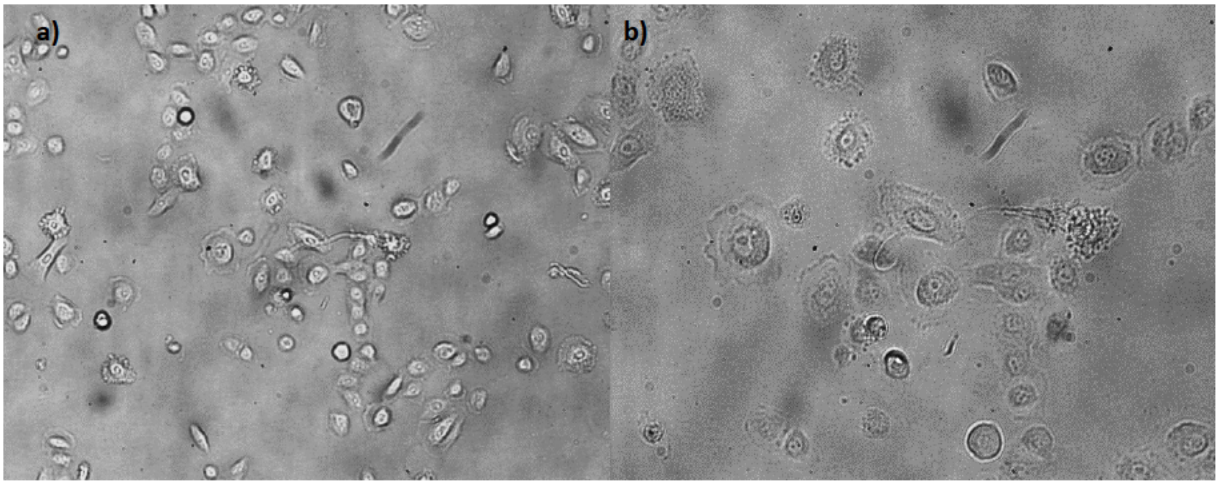


Figure 16 HEKn cells seeded at density of 2000 cells/well and obtained using optical transmission microscope with different objective lenses: a) 10X; b) 20X. Images taken in the same position of the same well.

5.2 Optimizing Seeding Density of HEKn Cells in 96-well Plates

HEKn cells with a wide range of seeding density (i.e. 4000 cells/well - 20000 cells/well) were cultured for 1 – 4 days in a 96-well plate. Presented in Figure 17 a), b) and c) are HEKn cells with 40% - 60% confluence. These cells were seeded at three different densities (i.e. 6000, 8000 and 10000 cells/well) and incubated for 2 - 4 days. For HEKn

cells, at least 2 days are need for cell proliferation to reach the log - growth phase, and image with 40% - 60% cell confluence in one well is preferred by InnoVitro software. This result shows that seeding density of HEKn cells is flexible, suitable confluence can be obtained by different seeding density (i.e. 6000 cells/well for 4 days, 8000 cells/well for 3 days and 10000 cells/well for 2 days).

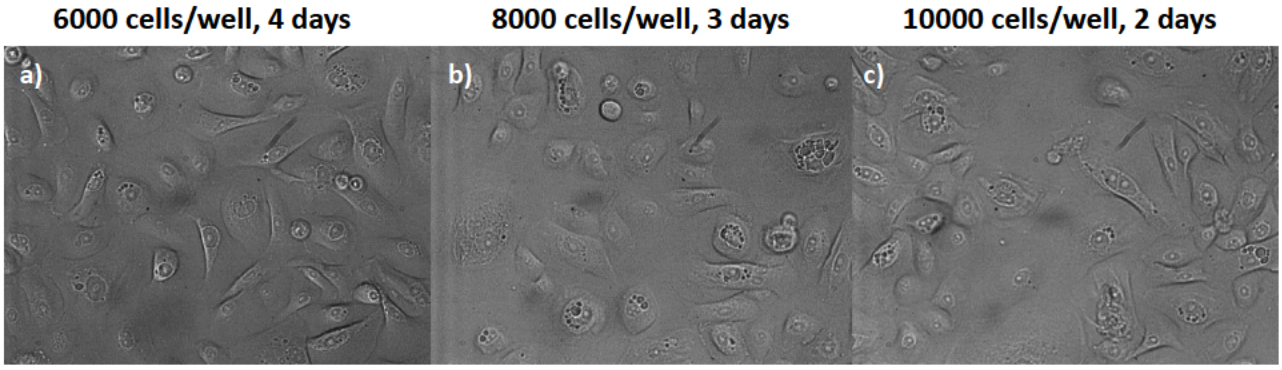


Figure 17 HEKn cells seeded at different density for 2 - 4 days: a) 6000 cells/well, 4 days; b) 8000 cells/well, 3 days; c) 10000 cells/well, 2 days. Images obtained using optical transmission microscope equipped with 20X objective lens.

5.3 Chemical Exposure Experiments

5.3.1 Bleb Monitoring

HEKn cells exposed to four different compounds according to Table 1 (Page 11) for 24 hours are presented in Figure 18. As shown by the Figure 18 a) and b), HEKn cells began to bleb after exposed to OXAOLONE and DNCB, and more blebs were developed compared to c) and d). Some single cells even formed two or more blebs. As illustrated by Figure 18 c) and d), less blebs were developed after exposure to BIT and Benzylbenzoate. This implies that HEKn cells are more sensitive to strong sensitizers by demonstrating a more pronounced blebbing.

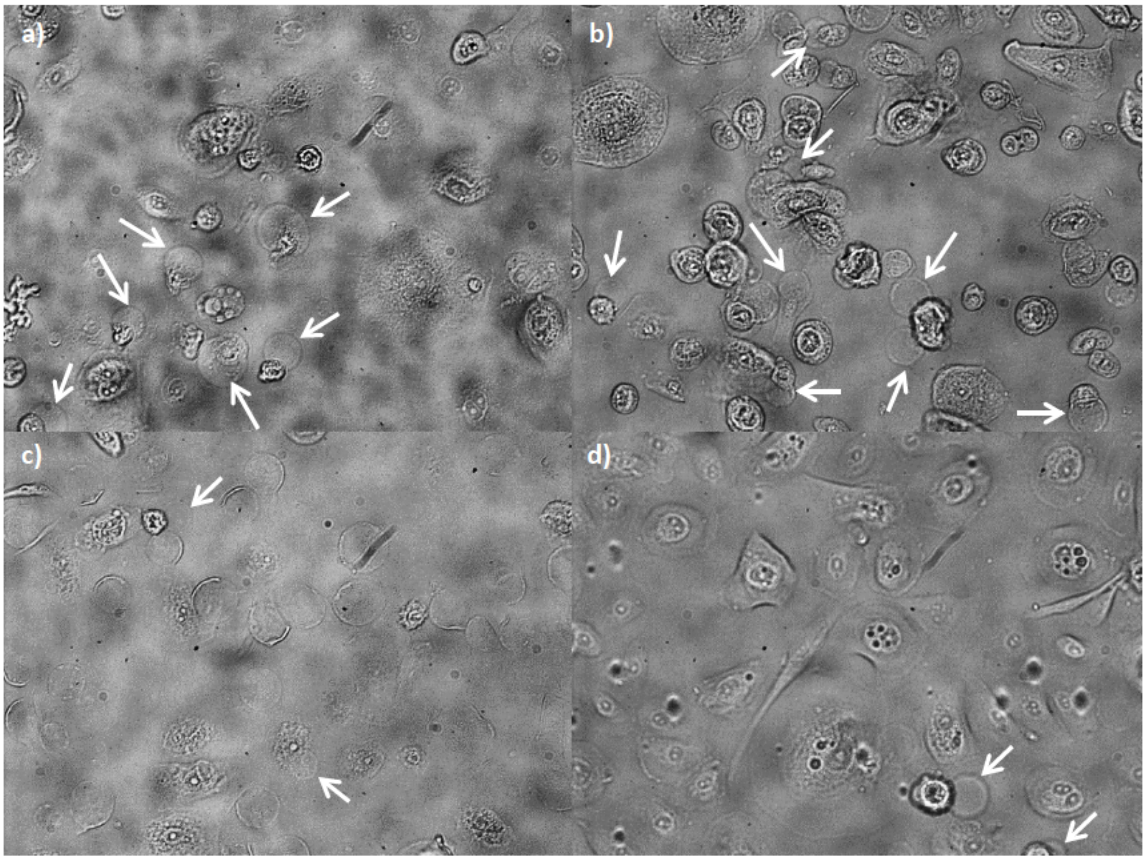


Figure 18 HEKn cells were cultured at concentration 8000 cells/well for 3 days and then exposed to four different compounds: a) OXAOLONE; b) DNCB; c) BIT; d) Benzylbenzoate. The exposure time for all chemicals is 24 hours. The images obtained using optical transmission microscope equipped with 20X objective lens. Arrows highlight the blebs.

5.3.2 Z-levels

Figure 19 shows the blebbing HEKn cells exposed to Oxazolone and obtained at the same position of the same well but at different z-levels. a) is focused on the cells and b) is focused on the blebs. Sometimes the cells and their blebs are located at different z-levels, and two images of the same position probably need to be obtained. b) also shows that 4 blebs were developed around a single cell. These results demonstrate that

HEKn cells and their blebs can be located at different z-levels, and that several blebs can be formed by single HEKn cell after exposed to strong sensitizer.

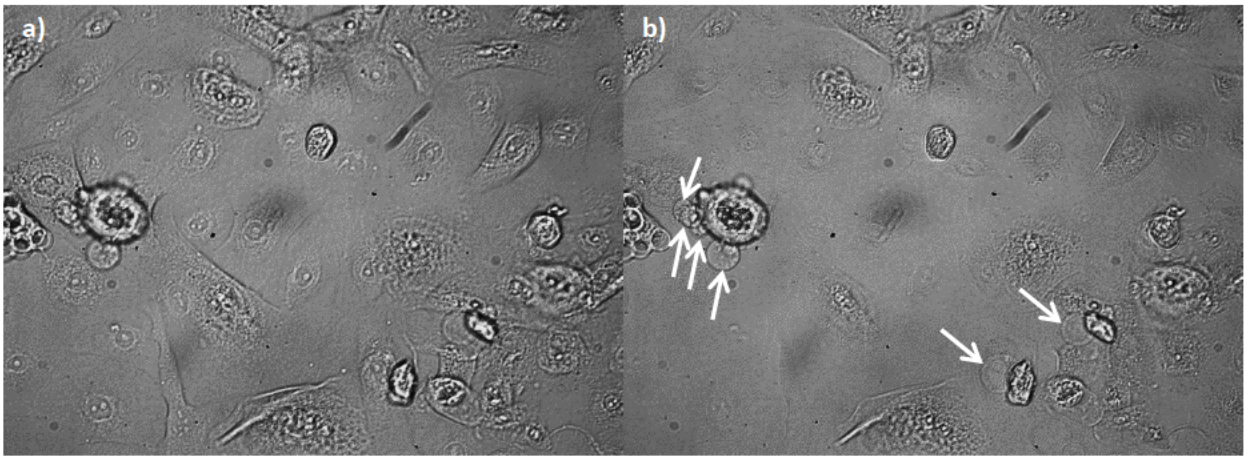


Figure 19 HEKn cells exposed to OXAOLONE at the same area of the same well with different z-levels of focus. The images were obtained using optical transmission microscope equipped with 20X objective lens. Arrows highlight the blebs. Chemical exposure time is 24 hours.

5.4 Image Analysis Using InnoVitro Software

5.4.1 The Importance of Threshold Value

Figure 20 shows the same image of blebbing HEKn cells exposed to strong sensitizer DNCB at 0.5mM, it was analyzed with four different threshold values from low to high using InnoVitro software. It can be seen that almost all cells and cell colonies could be roughly detected by larger rectangles using a relatively low threshold value. However, the individual cells, particular cells with small size are hardly separated from the cell colonies. Several rectangles even overlapped. More cells in b) could be detected when threshold was increased to 60. Then, the number of detected cells was decreasing with the increased threshold value in c) and d). Single cells with smaller sizes in c) and d) could be detected by the software with higher threshold value. However larger cells was ignored at the same time.

These results show that the ability of InnoVitro software to detect individual cells from cell colonies will be enhanced with the increase of threshold value. However, bigger cells will be ignored at the same time. This indicates the importance of finding an optimal threshold value where most cells can be detected.

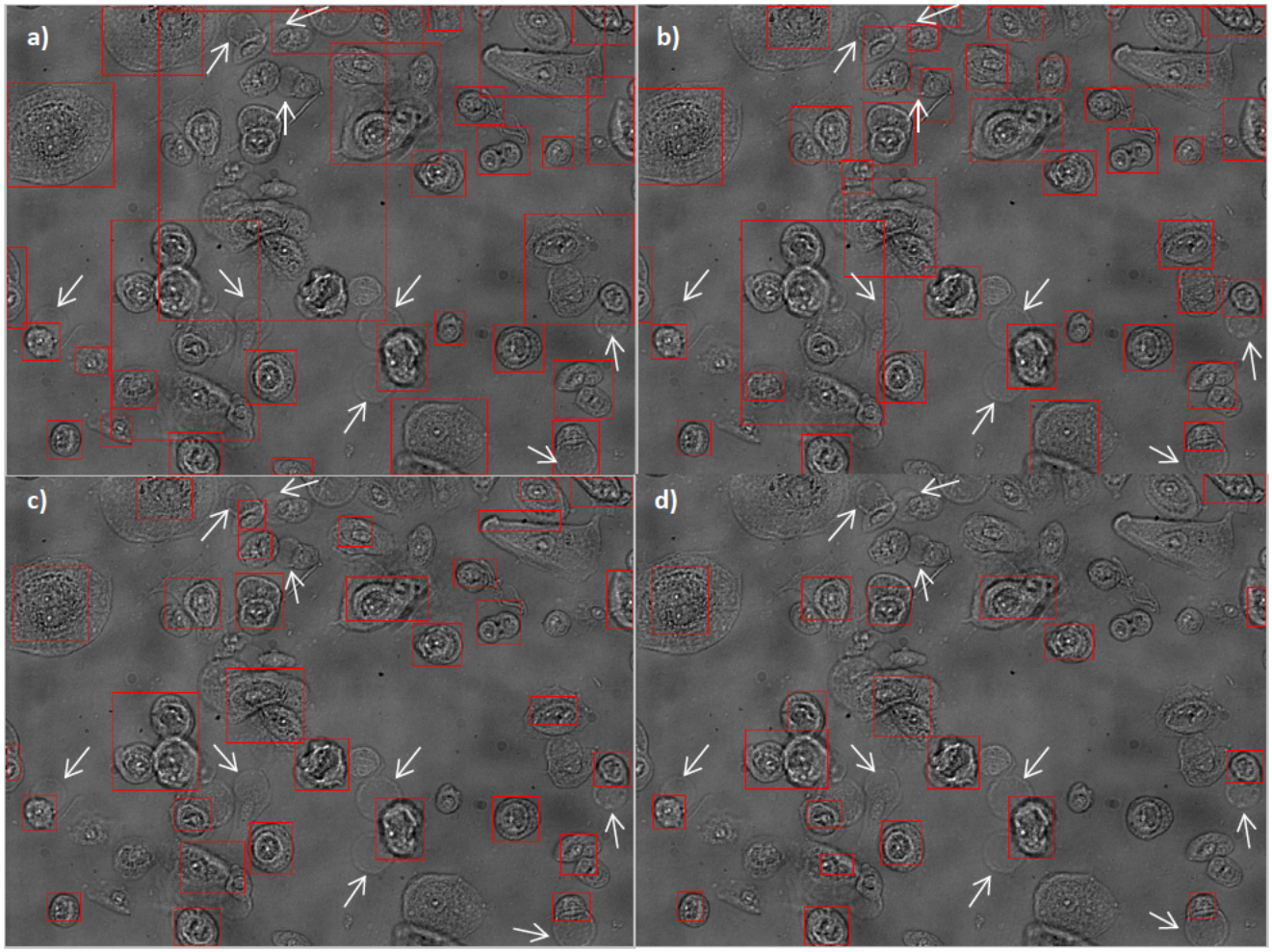


Figure 20 HEKn cells with blebs. HEKn cells were exposed to DNCB at 0.5mM, 1625 cells/well, 4days, and 20X, a) threshold 40; b) threshold 60; c) threshold 80; d) threshold 100. The images were obtained using optical transmission microscope with 20X magnification objective lens. Chemical exposure time is 24 hours. Arrows highlight the blebs.

5.4.2 The Effect of Cell State on Analysis

Figure 21 a) and b) show HEKn cells exposed to strong sensitizers DNCB, c) and d) show HEKn cells without chemical exposure. These images were analyzed using InnoVitro software with respective optimal threshold values. It can be seen that the blebbing cells in a) and b) were easier identified than the cells in the colonies in c) and d).

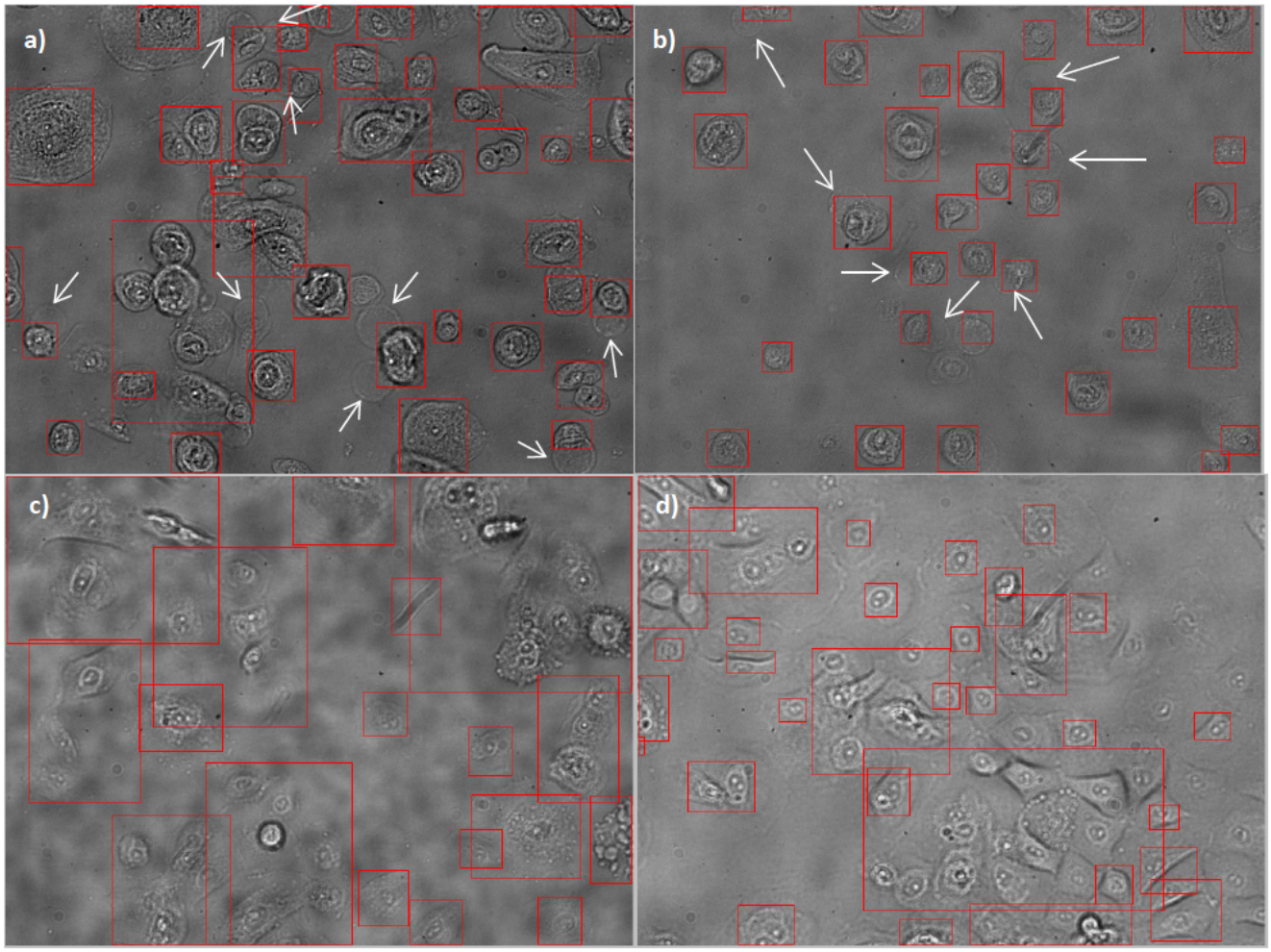


Figure 21 Blebbing HEKn cells exposed to different chemicals and analyzed with InnoVitro software at different optimal threshold values: a) 60; b) 30; c) 30 and d) 45. The images were obtained optical transmission microscope equipped with 20X objective lens. Exposure time for all chemicals is 24 hours. Arrows highlight the blebs.

5.5 High Content Screening (HCS) on HEKn Cells

HEKn cells were cultured into 96-well plate and exposed to different chemicals with the same concentration gradient (i.e. 0.05mM – 2mM). HCS microscopy was used for monitoring the whole process and acquiring images automatically at different z-levels every hour for 24 hours.

5.5.1 The Influence of z-level on Visualization of Blebs and Cells

Figure 22 shows images of blebbing HEKn cells exposed to 0.5mM DNCB (strong sensitizer) of the same position at five different z-levels, 3μm apart. It can be seen that both blebs and cells can be observed clearly in all of these four images, although **a)** and **e)** are slightly out of focus. This result demonstrates that the z-levels do not affect the render of HEKn cells and blebs so much when the main stream of blebbing occurs.

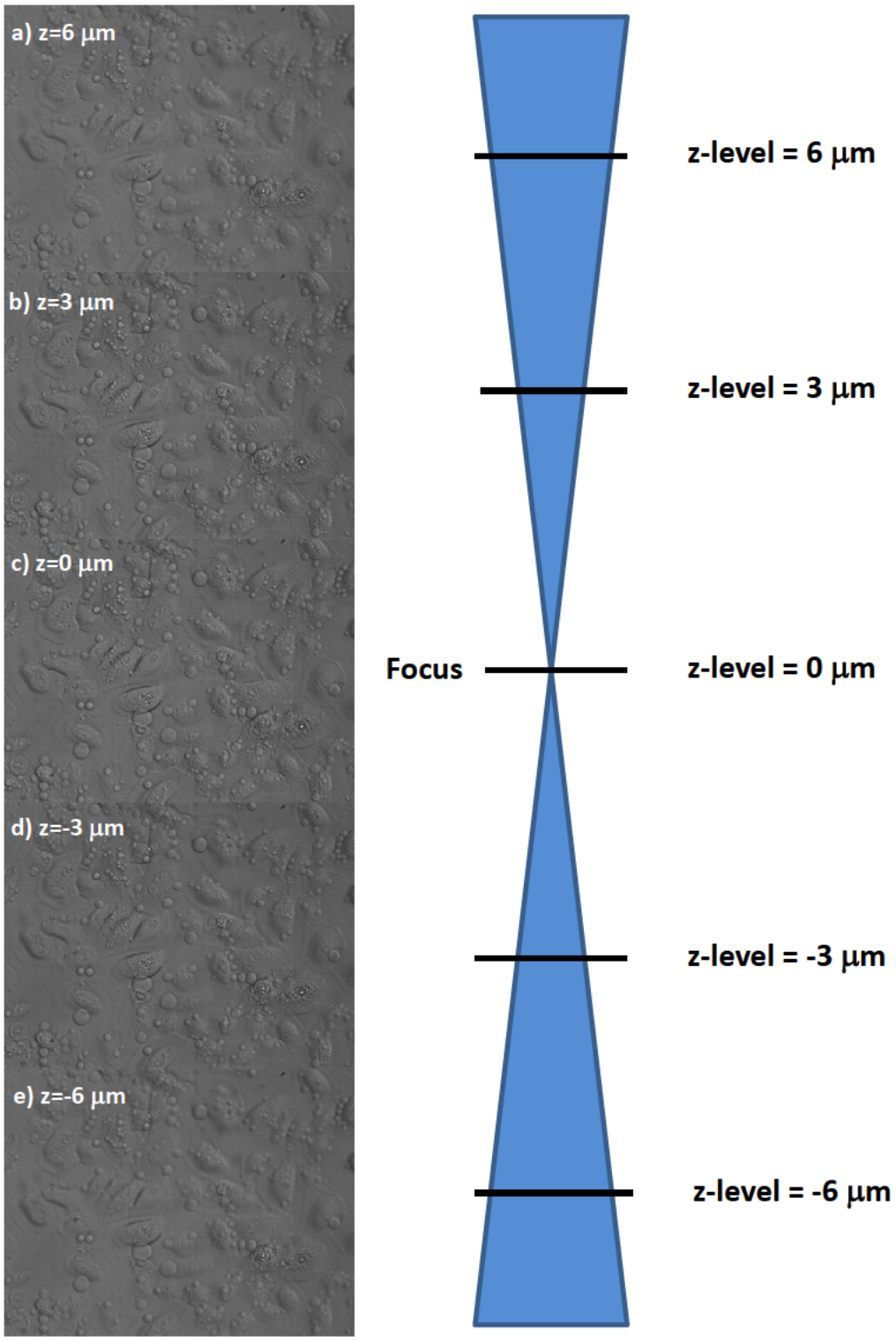


Figure 22 Blebbing HEKn cells exposed to 0.5 mM DNCB for 3 hours and taken at different z-levels: a) z=6µm; b) z=3µm; c) z=0µm; d) z=-3µm and e) z=-6µm. The images were obtained using optical transmission High Content Screening microscope equipped with 20X objective lens.

5.5.2 The Correlation between Onset Time of Cell Blebbing and Chemical Concentration and Chemical Sensitizing Potency

In the HCS system, HEKn cells were monitored for 24h, and images were obtained every one hour continuously during the experiment. **Figure 23** shows that the onset time of cell blebbing when HEKn cells are exposed to four different chemicals with a concentration gradient (i.e. 0.05mM – 2mM).

For weak sensitizer (i.e. Benzylbenzoate), 1-3 blebs are usually formed within one image, as HEKn cells without any chemical exposure. This situation can be regarded as almost no blebs formed. For moderate sensitizer (i.e. BIT), blebbing occurred at 2 hours after exposed to BIT with the lowest concentration 0.0010 (w/v %). The onset time of blebbing decreases with the increasing chemical concentration.

For strong sensitizer DNCB, HEKn cells begin to bleb at 2 hours after exposed to DNCB with all concentrations. For strong sensitizer Oxazolone, blebbing has not occurred until the concentration increases to 0.0106 (w/v %). Blebbing occurred at 1 hour after chemical exposure with all three higher concentrations.

These results imply that the onset time of HEKn cell blebbing is not inversly proportional to the chemical concentration and chemical sensitizing potency. For most chemicals, the HEKn cell blebbing may occur within 2 hours after chemical exposure. It seems that there is a threshold value of chemical concentration to trigger HEKn cells blebbing. Thus, chemical concentration need be considered as an important parameter in chemical exposure experiments.

Table 2 Onset time of blebbing when HEKn cells exposed to different chemicals with different concentration. X means almost no bleb

	0.05mM	0.25mM	0.5mM	1mM	2mM
Benzylbenzoate (Weak)	X	X	X	X	X
BIT (Moderate)	2 h	1h 40min	1h 40min	1h 40min	1h
DNCB (Extremely strong)	2 h	2 h	2 h	2 h	2 h
Oxazolone (Extremely strong)	X	X	1 h	1 h	1 h

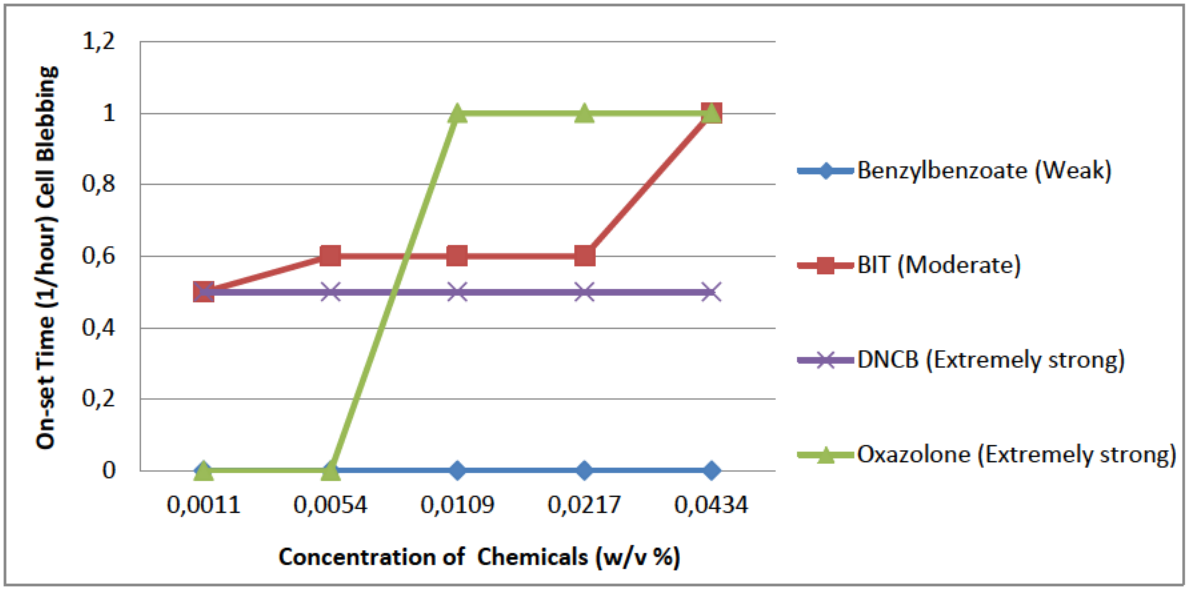
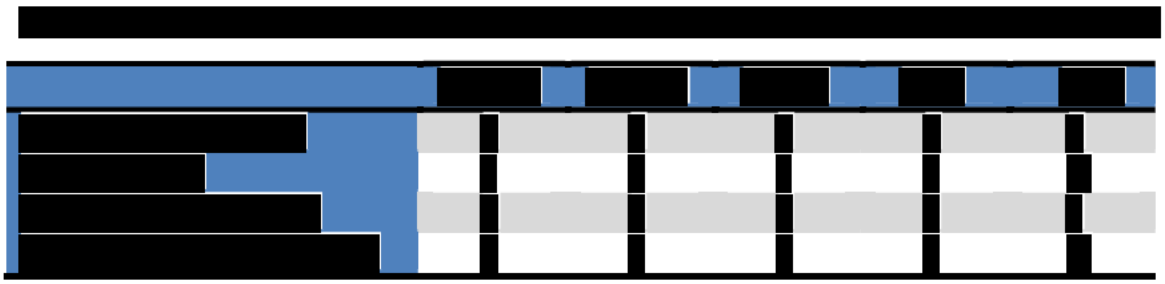


Figure 23 The corraletion between on-set time (1/hour) of cell blebbing and concentration of chemicals. HEKn cells were exposed to four different chemicals with the same molar concentration gradiation (0.05 mM, 0.25mM, 0.5mM, 1mM and 2mM). Benzylbenzoate: 0.0011, 0.0054, 0.0109, 0.0217 and 0.0434 (w/v %); BIT: 0.0010, 0.0051, 0.0102, 0.0203 and 0.0406 (w/v %); DNCB: 0.0011, 0.0053, 0.0106, 0.0212 and 0.0424 (w/v %); Oxazolone: 0.0008, 0.0037, 0.0076, 0.0151 and 0.0302(w/v %).



5.5.6 The Correlation between Bleb Number and Sensitizing Potency

The average bleb-cell ratio per image with 0.5mM chemical concentration is summarized into Figure 19. Six time points are chosen. The peak stands for the biggest bleb-cell ratio for every chemical. Oxazolone leads to the highest peak. The peak caused by DNCB is the second highest. BIT induces the third highest peak of bleb-cell ratio. There are almost no blebs formed because of Benzylbenzoate.

According to LLNA method, Oxazolone shows the strongest sensitizing potency followed in the order by DNCB, BIT, Benzylbenzoate (47). The order of theses ratio peaks in Figure 27 just matches the sequence of sensitizing potency obtained from LLNA. Besides, blebbing of HEKn cells exposed to these four chemicals reached their highest amount within 10 hours. Thus, it is suggested that the time for monitoring blebs could be controlled within at least 15 hours.

These results imply that the largest bleb-cell ratio per image is proportional to chemical sensitizing potency. Thus, quantification of bleb number might be a potential method to grade sensitizing potency of chemicals.

Table 7 The average bleb-cell ratio per image when HEKn cells were exposed to four different chemicals with the same molar concentration (0.5 mM) at six different time points

0.5mM	1 h	1h40m	2 h	7 h	15 h	24 h
Benzylbenzoate (Weak)	0	0	0	0	0	0
BIT (Moderate)	0	0.8	1.1	0.5	0.2	0.1
DNCB (Extremely strong)	0	0.1	0.3	2.2	1.0	0.6
Oxazolone (Extremely strong)	2.4	3.0	3.1	1.8	1.3	1.1

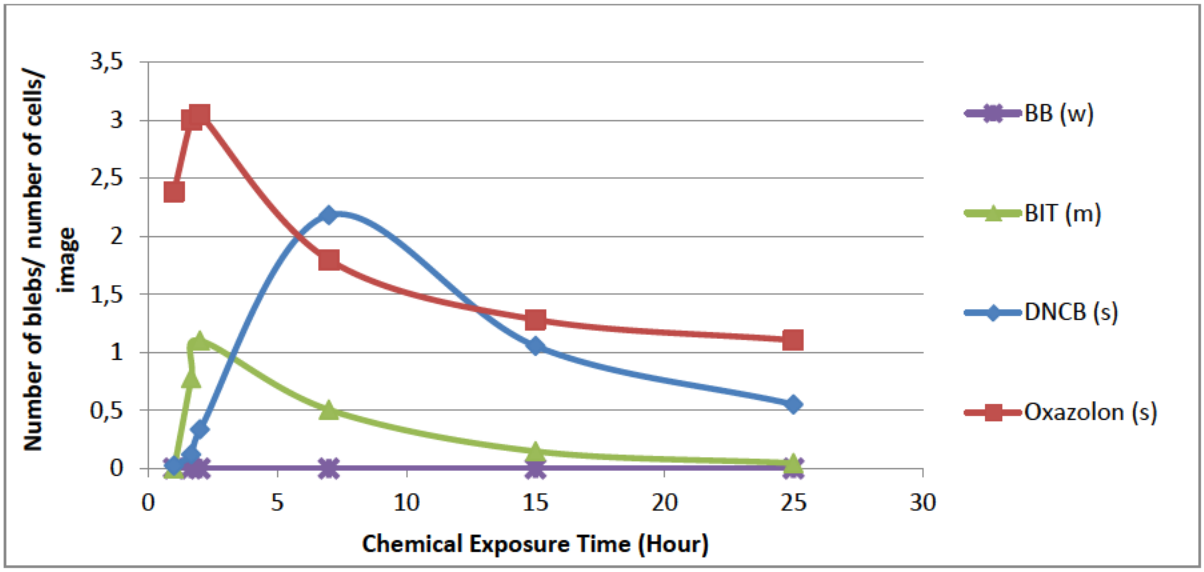


Figure 25 The relationship between the chemical exposure time and number of blebs/number of cells in one image. HEKn cells were exposed to four different chemicals with the same molar concentration (0.5mM). Benzylbenzoate: 0.0109(w/v %); BIT0.0102 (w/v %); DNCB: 0.0106 (w/v %); Oxazolone: 0.0076 (w/v %).

5.5.7 The Correlation between bleb-cell ratio and chemical concentration

The average bleb-cell ratio per image with 1mM chemical concentration is summarized into Table 8. The same six time points were chosen. The largest bleb-cell ratio is 3.5 which caused by Oxazolone. The second largest bleb-cell ratio is 2.2 when HEKn cells are exposed to DNCB. The third largest bleb-cell ratio is 1.1 when HEKn cells exposed to BIT. Benzylbenzoate was found not to induce any bleb response. The amount of blebs formed is the highest almost at the same time as the onset of blebbing Table 7. The largest bleb-cell ratios at 1 mM concentration (Table 8) are similar to the bleb-cell ratios

obtained at 0.5 mM (Table 7). The order of highest bleb-cell ratios in Table 8 also matches the sequence of sensitizing potency obtained from LLNA.

Table 8 The average bleb-cell ratio per image when HEKn cells were exposed to four different chemicals with the same molar concentration (1 mM) at six different time points

1mM	1 h	1 h 40m	2 h	7 h	15 h	24 h
Benzylbenzoate (Weak)	0	0	0	0	0	0
BIT (Moderate)	0	0.9	1.1	0.6	0.3	0.2
DNCB (Extremely strong)	0	0.7	1	2.2	1.2	0.7
Oxazolone (Extremely strong)	1.8	3.5	2.7	1.6	1.2	0.9

The results imply that the largest bleb-cell ratio per image seem related to chemical sensitizing potency regardless of sensitizer concentration in the investigated concentration range.

6 Discussion and Conclusion

In this study, HEKn cells were exposed to four different chemicals with different sensitizing potencies. HEKn cells reacted significantly to moderate, strong and especially extremely strong sensitizers, when significant blegging was observed. [redacted] In contrast, HEKn cells exposed to weak sensitizers had little or no reaction. Thus it can be concluded that cell blebbing is related to sensitixzer potency confirming earlier results.

It was also found that the InnoVitro software worked best for the stronger sensitizers as the cell images could be analyzed more easily. [redacted] The optimized threshold value of InnoVitro software was found to vary for every single image. The threshold depended on the state of cells caused by sensitizers and the cell confluence.

HEKn cells were seeded on 96-well plate for further HCS experiment. It was decided to seed 6000 – 10000 HEKn cells per well of the 96-well plate for 2 - 4 days as the seeding density and cultivation time.

During the HCS bleb monitoring experiment, 20X objective lens was chosen for imaging of HEKn cells and blebs compared to 10X objective lens. Z - level did not significantly affect the observation of cells and blebs simultaneously during the blebbing event. To limit the amount of data, it was decided to take one image at one z-level for each position per well. Since all these four sensitizers can trigger HEKn cells to expulse the largest amount of blebs within 10 hours, the monitoring time might be decreased from 24 hours to at least 15 hours.

[redacted]

It was found that the order of highest bleb-cell ratio using concentration of 0.5mM and 1mM, matched the sequence of sensitizing potency of these four chemicals (i.e. Benzylbenzoate, BIT, DNCB and Oxazolone) using LLNA method. The difference of chemical concentration was not found to affect the amount of blebs developed by HEKn cells to a major extend. Neither were the morphologic and mobility changes affected to any major extent in the investigated concentration range.

In conclusion, quantification of cell blebbing might be an alternative method to grade sensitizing potency of chemicals, as demonstrated by the results of this report. However, further work needs to be undertaken in order to be able to distinguish weak sensitizers from non-sensitizer using this assay.

7 Future Plans

In the future the image data obtained from this project should be further analyzed. Specifically, counting bleb at more specific time points to find out the accurate time when blebs reach their highest amount. The highest bleb-cell ratio may increase at this accurate time point.

In order to examine the difference of the highest bleb-cell ratio at different chemical concentrations, it is suggested to count bleb at other three chemical concentrations (i.e. 0.05 mM, 0.25 Mm and 2 mM). More chemical concentrations are suggested to be tested as well if the chemical solubility is allowed.

Concerning the choice of cell model, it would be advisable to test the immortalized HaCaT cell-line for the chemical exposure experiments. They may be used as the substitute instead of HEKn cells.

[redacted]

For now, limited data are obtained from four sensitizers. Thus, more sensitizers should be tested in the further investigation. Moreover, the chemicals used in this project are restricted to sensitizers, it will good to test irritants in order to understand what response irritants are causing using the in vitro test.

8 Acknowledgements

I would like to thank everyone who gives me kindly help and support.

Firstly, I would like to thank my supervisor and examiner Assoc. Prof. Marica B. Ericson, for providing valuable knowledge and guidance during the last one year. Thanks also for providing resources to support my work in the laboratory for this project.

Ph.D. Anna-Lena Stenfeldt, my co-supervisor. You are a very amiable person. Thanks for your technical assistance and teaching me the key technologies about chemical exposure.

I also very appreciate the tremendous assistance and support from PhD student Vladimir Kirejev. You taught me the major operating skills of cellular cultivation and optical transmission microscope. You were involved in the project like a real supervisor and providing me a lot valuable suggestions. You assisted me in reviewing the thesis.

I would specially like to thank Jenny Almkvist and Lorna Fletcher from GU-Holding. You promoted InnoVitro project and made it smoothly progress.

Ann-Therese Karlberg, you gave me tremendous help to finish the poster of InnoVitro project for OEESC 2013 conference at Amsterdam.

My colleagues and friends in Biomedical Photonics Group:
Ph.D. Stina Guldbrand, Ph.D student Johan Borglin.

I appreciate the necessary resources provided by University of Gothenburg and Chalmers University of Technology.

Thanks to Johan Karlsson, Mats Kvarnström and Mats Jirstrand from Fraunhofer-Chalmers Research Centre.

My colleagues and friends in the dermatochemistry group:

Anna Börje, Johanna Rudbäck, Ida Belogorcev Niklasson and Tamara Delaine.

Financial support for this work was obtained from InnoVitro at GU-Holding. This project was performed within the Centre for Skin Research (SkinResQU) at the University of Gothenburg. I would like to thank all the colleagues in the SkinResQU.

Finally I would like to thank my family, my dear mother, Weihong Yan, and my best friend, Miaomiao Guo. For your endless encouragement and support. I will always love you.

9 Reference

1 Thyssen J P, Linneberg A, Menne T, Johansen J D. The epidemiology of contact allergy in the general population - prevalence and main findings. *Contact Dermatitis* 2007: **57**: 287-299.

2 Timm-Knudson V L, Johnson J S, Ortiz K J, Yiannias J A. Allergic contact dermatitis to preservatives. *Dermatol Nurs* 2006: **18**: 130-6.

3 Robert L. Rietschel J F F, Alexander A. Fisher. Fisher's Contact Dermatitisedn, 5th (ed)^(eds), 2001.

4 Basketter D A, Gerberick G F, Kimber I. Measurement of allergenic potency using the local lymph node assay. *Trends in Pharmacological Sciences* 2001: **22**: 264-265.

5 Oecd. Test No. 429: Skin Sensitization: Local Lymph Node Assay. *OECD Guidelines for the Testing of Chemicals* 2002.

6 Eu. DIRECTIVE 2003/15/EC OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 27 February 2003 amending Council Directive 76/768/EEC on the approximation of the laws of the Member States relating to cosmetic products. *Official Journal of the European Union* 2003: **L 66**: 26-35.

7 Kato H O M, Yamashita K, Nakamura Y, Fukumori Y, Nakai K, Kaneko H. Peptide-binding assessment using mass spectrometry as a new screening method for skin sensitization. *The Journal of Toxicological Sciences* 2003: **28**: 19-24.

8 Oecd. Test No. 429: Skin Sensitization: Local Lymph Node Assay. *OECD Guidelines for the Testing of Chemicals, Section 4* 2010: 20.

9 Carl Simonsson S I a, Anna-Lena Stenfeldt, Jo¨Rgen Bergstro¨M, Brigitte Bauer, Charlotte A. Jonsson, Marica B. Ericson and Kerstin S. Broo. Caged Fluorescent Haptens Reveal the Generation of Cryptic Epitopes in Allergic Contact Dermatitis. *The Journal of Investigative Dermatology* 2011: **131**: 1486–1493.

10 Brigitte Bauer S I a, Anna-Lena Stenfeldt, Carl Simonsson, JöRgen BergströM, Marica B. Ericson, Charlotte A. Jonsson, and Kerstin S. Broo. Modification and Expulsion of Keratins by Human Epidermal Keratinocytes upon Hapten Exposure in Vitro. *Chemical research in toxicology* 2011: **24**: 737-743.

11 R.J. G. Rycroft T M, P. J. Frosch, J.-P. Lepoittevin. Textbook of Contact Dermatitisedn, 3rd (ed)^(eds), 2001: 1114.

12 Groot A, Frosch P. Patch Test Concentrations and Vehicles for Testing Contact Allergens. In: *Contact Dermatitis*edn, P Frosch, T Menné and J-P Lepoittevin (ed)^(eds), Springer Berlin Heidelberg, 2006: 907-928.

13 Zug K A, Warshaw E M, Fowler J F, Jr., Maibach H I, Belsito D L, Pratt M D, Sasseville D, Storrs F J, Taylor J S, Mathias C G T, Deleo V A, Rietschel R L. Patch-Test Results of the North American Contact Dermatitis Group 2005-2006. *Dermatitis* 2009: **20**: 149-160.

14 Robert L. Rietschel J F F, Alexander A. Fisher. Fisher's Contact Dermatitisedn, 6th (ed)^(eds), 2008: 862.

15 Kimber I, Basketter D A, Gerberick G F, Dearman R J. Allergic contact dermatitis. *International Immunopharmacology* 2002: **2**: 201-211.

16 Spiewak R. Immunotherapy of allergic contact dermatitis. *Immunotherapy* 2011: **3**: 979-996.

17 Landsteiner K, Jacobs J. STUDIES ON THE SENSITIZATION OF ANIMALS WITH SIMPLE CHEMICAL COMPOUNDS. *The Journal of Experimental Medicine* 1935: **61**: 643-656.

18 Martin S F, Esser P R, Weber F C, Jakob T, Freudenberg M A, Schmidt M, Goebeler M. Mechanisms of chemical-induced innate immunity in allergic contact dermatitis. *Allergy* 2011: **66**: 1152-63.
19 Divkovic M, Pease C K, Gerberick G F, Basketter D A. Hapten-protein binding: from theory to practical application in the in vitro prediction of skin sensitization. *Contact Dermatitis* 2005: **53**: 189-200.
20 Karlberg a-T, Bergstrom M A, Borje A, Luthman K, Nilsson J L G. Allergic contact dermatitis-formation, structural requirements, and reactivity of skin sensitizers. *Chemical research in toxicology* 2008: **21**: 53-69.
21 Barker J. ROLE OF KERATINOCYTES IN ALLERGIC CONTACT-DERMATITIS. *Contact Dermatitis* 1992: **26**: 145-148.
22 Rello S, Stockert J C, Moreno V, Gámez A, Pacheco M, Juarranz A, Cañete M, Villanueva A. Morphological criteria to distinguish cell death induced by apoptotic and necrotic treatments. *Apoptosis* 2005: **10**: 201-208.
23 Itai Chipinda J H, and Paul D. Siegel. Haptenation: Chemical Reactivity and Protein Binding. *Journal of Allergy* 2011: **2011**: 11.
24 Lee C-H. structure of intermediate filaments. In: *BioWave*edn(ed)^(eds), 2007.
25 Hashimoto K. Regulation of keratinocyte function by growth factors. *Journal of Dermatological Science* 2000: **24, Supplement 1**: S46-S50.
26 Douglas J D. Allergic Contact Dermatitis: A Focus on Nickel. *Dermatology nursing* 2009: **21**: 354 - 357.
27 Douglas J D. Allergic Contact Dermatitis and Para-Phenylenediamine. *Dermatology Nursing* 2010: **22**: 31-33.
28 Schurer N, Kohne A, Schliep V, Barlag K, Goerz G. Lipid composition and synthesis of HaCaT cells, an immortalized human keratinocyte line, in comparison with normal human adult keratinocytes. *Exp Dermatol* 1993: **2**: 179-85.
29 Schoop V M, Mirancea N, Fusenig N E. Epidermal Organization and Differentiation of HaCaT Keratinocytes in Organotypic Coculture with Human Dermal Fibroblasts. 1999: **112**: 343-353.
30 Lehman T A, Modali R, Boukamp P, Stanek J, Bennett W P, Welsh J A, Metcalf R A, Stampfer M R, Fusenig N, Rogan E M, Harris C C. P53 MUTATIONS IN HUMAN IMMORTALIZED EPITHELIAL-CELL LINES. *Carcinogenesis* 1993: **14**: 833-839.
31 Fusenig N E, Boukamp P. Multiple stages and genetic alterations in immortalization, malignant transformation, and tumor progression of human skin keratinocytes. *Molecular Carcinogenesis* 1998: **23**: 144-158.
32 Fraser R D B, Macrae T P, Parry D a D, Suzuki E. Intermediate Filaments in α -keratins. *Proceedings of the National Academy of Sciences of the United States of America* 1986: **83**: 1179-1183.
33 Mckittrick J, Chen P Y, Bodde S G, Yang W, Novitskaya E E, Meyers M A. The Structure, Functions, and Mechanical Properties of Keratin. *Jom* 2012: **64**: 449-468.
34 Uitto J, Richard G, Mcgrath J A. Diseases of epidermal keratins and their linker proteins. *Experimental Cell Research* 2007: **313**: 1995-2009.
35 Coulombe P A, Omary M B. 'Hard' and 'soft' principles defining the structure, function and regulation of keratin intermediate filaments. *Current Opinion in Cell Biology* 2002: **14**: 110-122.
36 Fuchs E. KERATIN GENES, EPIDERMAL DIFFERENTIATION AND ANIMAL-MODELS FOR THE STUDY OF HUMAN SKIN DISEASES. *Biochemical Society Transactions* 1991: **19**: 1112-1115.

37 Albers K M. Keratin biochemistry. *Clinics in Dermatology* 1996: **14**: 309-320.
38 Magin T M, Vijayaraj P, Leube R E. Structural and regulatory functions of keratins. *Experimental Cell Research* 2007: **313**: 2021-2032.
39 Moll R, Divo M, Langbein L. The human keratins: biology and pathology. *Histochemistry and Cell Biology* 2008: **129**: 705-733.
40 Gu L-H, Coulombe P A. Keratin function in skin epithelia: a broadening palette with surprising shades. *Current Opinion in Cell Biology* 2007: **19**: 13-23.
41 Mertz J. *Introduction to Optical Microscopy*. Greenwood Village, Colo., Roberts and Company Publishers, 2010.
42 Murphy D B, Davidson M W. *Fundamentals of Light Microscopy and Electronic Imaging (2nd Edition)*. Somerset, NJ, USA, Wiley-Blackwell, 2012.
43 Baker M. CELLULAR IMAGING Taking a long, hard look. *Nature* 2010: **466**: 1137-1142.
44 Society B, Uk. C Z. *Celebrating 50 Years of Live Cell Imaging*. Biochemical Society, 2003.
45 Hornby a S. Oxford Advanced Learner's English-Chinese Dictionary (Seventh Edition), 7th Edition edn, S Wehmeier (ed)^(eds), Oxford University Press, The Commercial Press, 2009: 2346.
46 Sezgin M, Sankur B L. Survey over image thresholding techniques and quantitative performance evaluation. *Journal of Electronic Imaging* 2004: **13**: 146-168.
47 Van Och F M M, Slob W, De Jong W H, Vandebriel R J, Van Loveren H. A quantitative method for assessing the sensitizing potency of low molecular weight chemicals using a local lymph node assay: employment of a regression method that includes determination of the uncertainty margins. *Toxicology* 2000: **146**: 49-59.