BioTeSys

Automated FADU

Alternative indicator test for genotoxicity

Automated FADU - supports your risk assessment of possible genotoxic substances.

Genotoxicity tests can be defined as *in vitro* and *in vivo* tests. Mammalian cell-based alternatives open up new opertunities for fast and reliable tests to screen and identify genotoxic potential of substances and possible modifications of their toxicity profile in substance mixtures. Indicator tests, such as FADU assay measuring DNA damage and repair, can provide additional useful information for initial classification.

Genotoxicity tests should detect compounds that induce genetic damage. The induced changes are caused by different mechanisms and/or physiological procedures. The use of S9 fraction enabels in vitro assays to take the physiological activation into account. The increasing demand of chemical safety assessment calls for alternative methods to reduce animal experimentation. Furthermore registration of pharmaceuticals requires also a comprehensive assessment of their genotoxic potential. Results from indicator tests such as detection of DNA damage and repair can provide additional useful information in the context of extended genotoxicity testing.
Even if no single test can cover all the different mechanisms of genotoxical induction, FADU is a valuable part of battery testing. This assay is able to identify DNA strand breaks and by reasonable experimental design also physiologically based alteration. From great importance is the cabability to measure short exposition times (<1min) and the corresponding DNA repair activity. The discrimination between those processes ensures reliable estimation of toxic potential.

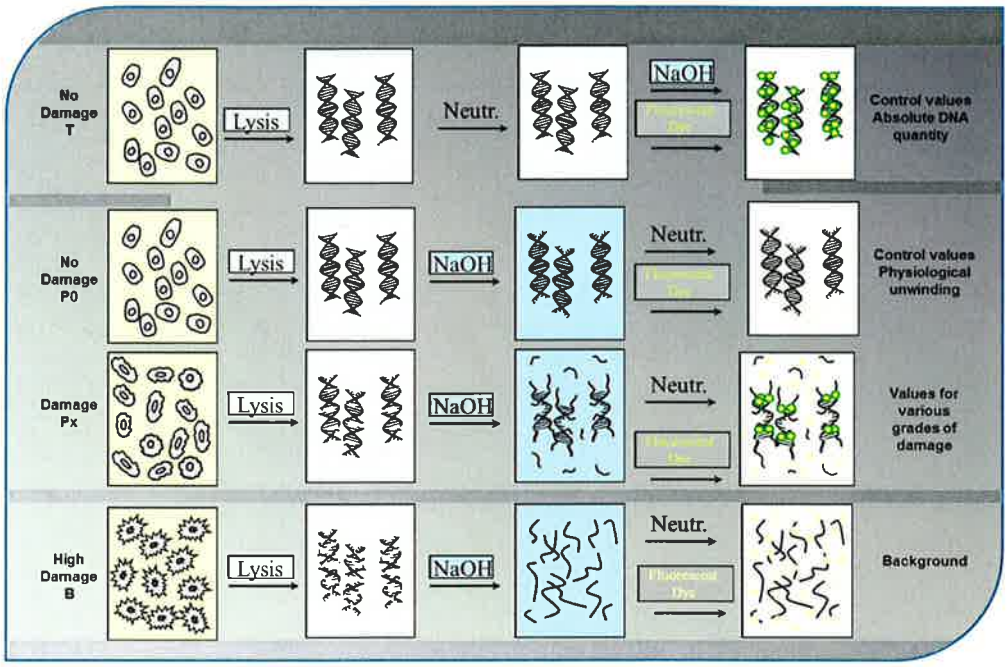
The FADU method was first published in 1981 (Birnboim and Jevcak, 1981) and is a sensitive procedure to quantify DNA strand breaks.
In order
(i) to render the FADU assay more convenient and robust,
(ii) to increase throughput,
(iii) to reduce the number of cells needed,

we have established a modified assay version that is largely automated and is based on the use of a liquid handling device (Moreno-Villanueva et al, 2009).

Our method could consequently reduce the number of false positive or false negative results in genotoxicity testing and provide a proper choice of animal models, thus reducing the number of animals needed in scientific research or safety testing.

Figure right: Scheme of the principle of the FADU Assay

On the left (yellow boxes) the cells are represented. In the middle the double stranded DNA with increasing levels of damage (grey boxes) and increasing extent of unwinding (blue boxes) is represented. The small circles (dark grey boxes) represent the fluorescent dye (yellow = no fluorescence signal; green = fluorescence signal). T, P_0 und B are controls to be run in parallel with the experimentally treated cells. In P0 samples alkaline unwinding is allowed and represents the DNA strand breaks under physiological conditions (*i.e.* without exogenous DNA damage). In T samples the neutralisation follows the lysis and therefore unwinding cannot take place; T-samples provide a measure of total DNA content and yield a fluorescence signal defined as 100 %. In B-samples the cellular DNA has been unwound completely as a result of very high damage and the resulting fluorescence represents background. P_x-samples (P_1,P_2,P_3,P_4....P_x) are the different extent of damage to be measured.



The main advantages of this assay is high reproducibility and sensitivity, easy handling, automation, high-throughput, speed (2 hours), robustness and low cost.

The detection of DNA strand breaks and repair is based on progressive DNA unwinding (denaturation) under highly controlled conditions of alkaline pH, time and temperature. The starting points for the unwinding process are DNA "open sites" like replication forks or chromosome ends, but also DNA strand breaks induced by reactive oxygen species (ROS), irradiation or chemical compounds either directly or as an intermediate of DNA repair acitivities. For monitoring DNA unwinding a fluorescent dye is used as marker for double stranded DNA, and a decrease in the fluorescence intensity indicates an increase of DNA unwinding and consequently a greater number of DNA strand breaks present before starting the unwinding phase.

FADU vs. Comet Assay

There is a large literature describing the use of the comet assay but the results obtained by different research groups are controversial.

The European Centre for the Validation of Alternative Methods (ECVAM) is still working on validation of the comet assay, and the European Comet Assay Validation Group (ECVAG) reported an inter-laboratory coefficient of variation (CV) of 47% (Forchhammer et al, 2010). Many steps of the comet assay protocol (including slide preparation and electrophoresis) affect both intra-assay variability and inter-assay reproducibility (Møller et al, 2010)

Data obtained using the automated FADU assay is very similar when compared with results coming from the comet assay. Based on the dramatic differences in the workflow and runtime of the two assays displayed in the synopsis shown below we are convinced that the automated FADU is superior. Assay and

workflow was successfully presented at CAAT board meeting. Preliminary data of nanoparticle also indicate its feasibility and doesn't interfere with particles.

Automated FADU was awarded by DFG "Ursula M Händel-Tierschutzpreis 2011" not least because the automated FADU assay shows good comparability to standard methods and is highlighted by its simplicity and operability (see original text in http://www.dfg.de/gefoerderte_projekte/wissenschaftliche_preise/haendel-tierschutzpreis/2011/team_moreno_villanueva_buerkle/index.html).

"Sie würdigt zugleich, dass das bereits publizierte Modell Daten hervorbringt, die eine gute Vergleichbarkeit mit Standardverfahren bieten. Nicht zuletzt besticht es, wie es die Jury formulierte, durch seine „Simplizität und gute Durchführbarkeit".

From DFG-Präsident Professor Matthias Kleiner
Ursula M. Händel-Tierschutzpreis 2011

COMET-ASSAY		FADU-ASSAY	
Steps	**Time [min]**	**Steps**	**Time [min]**
Buffer preparation: aliquots possible	5	Buffer preparation: aliquots possible	5
Agarose preparation: Distribution in Eppendorf tubes	20	Equipment: Switch on the robot	2
Equipment: assemble gel electrophoresis device	15		
Eppendorf tubes with agarose at 37°C	15		
DNA damage infliction	30	DNA Damage infliction	30
Embedding cells in agarose on the slides	60	Automatic steps: Addition of suspension buffer, transfer of samples to the 96-well plate, lysis buffer, alkaline buffer, neutralization buffer, Fluorescent Dye	120
Cool down agarose	10		
Lysis buffer	30		
Alkaline buffer	60		
Electrophoresis	30		
70% Ethanol	5		
Drying of the slides	60		
SybGreen	15		
SybGreen incubation	> 240		
Microscopy	> 60	Fluorescence reader	2
Data analysis	> 60	Analysis of data	10
Total time	>715	Total time	174

Inter-Laboratory Study with BioTeSys GmbH, Esslingen, Germany; Molecular Toxicology Group, University Konstanz, Germany; Swiss Federal Laboratories for Materials Testing and Research (EMPA), St. Gallen, Switzerland

The inter-laboratory validation is ongoing in collaboration with BioTeSys GmbH in Esslingen, Uni Konstanz and the Swiss Federal Laboratories for Materials Testing and Research (EMPA), St. Gallen (Switzerland).

The formation of DNA strand breaks is being tested for a variety of toxic compounds. The test substances comprise known inducers and non-inducers of DNA strand breaks, respectively, such as alkylating agents, DNA-crosslinking agents, polycyclic aromatic hydrocarbons or their active metabolites, topoisomerase inhibitors, oxidants, various other DNA-damaging agents, spindle poisons, kinase inhibitors, pesticides, aldehydes, alcohols, various other toxins, and solvents.

Each compound is being applied to human cells at different concentrations. The formation of DNA strand breaks will be measured after three different incubation times. At least ten independent experiments will be performed per compound.
Intralaboratory validation resulted in intra-assay CV = 5% and inter-assay CV = 9% for Jurkat cells and 13% for PBMC.

About BioTeSys

BioTeSys GmbH is an entire service provider (certificated acc. DIN ISO 9001:2008) in the field of biological and chemical analysis. The company offers a wide range of services, being a partner for the development and realization of new concepts in the topics cosmetics, nutrition and Consumer Health Care. The examination and evaluation of effects of biological active ingredients is its core competence.

BioTeSys activities in the field of in vitro test systems comprises efficacy testing of active ingredients in different fields, e.g. inflammation, resorption, gene expression. We encourage the confirmation to the 3R test strategy and are a member of In Vitro Testing Industrial Platform (IVTIP). IVTIP is an informal forum of European companies with a genuine and active interest in in vitro testing to be used in regulatory/safety testing or for early decision-making in compound discovery and development. IVTIP is in close contact to EU, ECVAM, Cosmetics Europe and other organisations.

Automated FADU - also contributes valuable information to a wide range of toxicological, epidemiological, pharmacological, and occupational health studies

Besides risk assessment the investigation of DNA damage and repair mechanisms is a field of interest in toxicology, pharmacology, epidemiology and medical sciences. The five points resumed below elucidate the different research areas in which the automated FADU assay is or has been applied successfully.

Toxicology

Genotoxicity tests are essential to identify compounds that have a potential to compromise not only the environment but also human and animal health, including compounds that increase the risk of cancer. We have detected DNA strand break formation after treating cells during very short time periods (10, 20, 30 minutes) drug exposure, with low concentrations of well-known genotoxic compounds (Moreno-Villanueva et al, 2011).

In vitro and *ex vivo* research in human primary cells

DNA repair was investigated in human peripheral mononuclear cells (PBMC). It was found that there are no significant differences between the DNA repair capacity using synthetic medium or autologous plasma/serum in cells from healthy young donors. However, the variation in the repair response was higher in medium supplemented with 10% foetal calf serum (FCS).

Human studies

FADU is used in several human studies, i.e. (i) DNA repair capacity in PBMC from patients suffering from post-traumatic stress disorder (PTSD), (ii) effects of aging and cognitive decline on DNA repair, and (iii) DNA repair capacity in PBMC of 3,700 volunteers from 7 different European countries. For the later protocols have been developed to enable the successful use of FADU assay on cryopreserved PBMC

Basic research: DNA damage and repair pathways

The putative role of XPC in the removal of oxidative damage is supported by in vitro data, in which $Xpc^{-/-}$ mouse embryonic fibroblasts exhibit a severe decrease in survival when cultured at 20% oxygen compared with 3% oxygen pressure (Melis et al., 2008). A current study is measuring the accumulation, within the passages, of DNA strand breaks and the DNA repair capacity in DNA repair deficient cells under 21% compared to 3% oxygen cell culture conditions

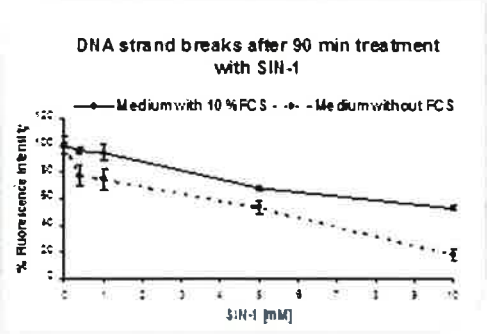
Experimental design: Cell line standardisation

Besides the identification of genotoxic potential FADU is able to be used in standardisation of cell lines with respect to their ability to repair induced damage. This will be useful to identify cell lines or passage numbers with defined repair capability to avoid heterogeneity leading to possible great standard deviation in the results.

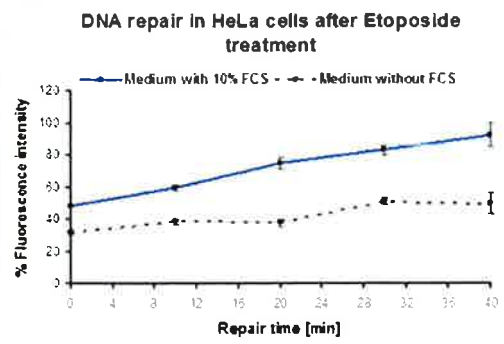
DATA

Cell culture conditions

Effect of foetal calf serum on DNA damage and repair in cultured cells



DNA strand break induction by treatment of Jurkat cells with the peroxynitrite-forming agent SIN-1. Cells were incubated with SIN-1 in presence (solid lines) or absence (dashed lines) of FCS. DNA strand breaks formation increases with increasing the doses in both cases but the number of DNA strand breaks formed is higher in cells incubated without FCS.



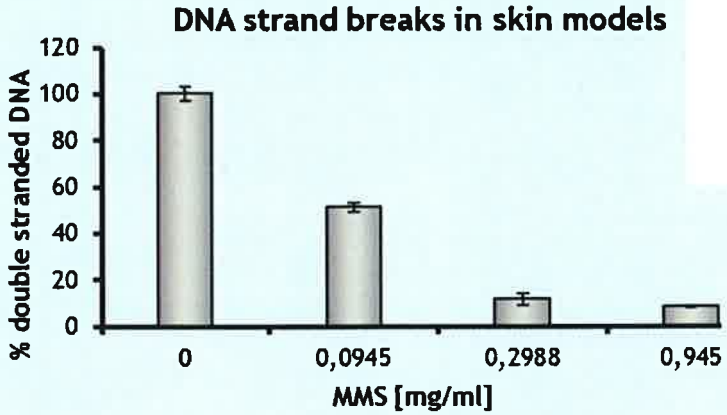
Repair of DNA strand breaks in HeLa cells after treatment with etoposide. in medium with (solid line) or without (dashed line) FCS. Not only the damage (time point 0) but also the repair shows less fluorescence intensity in medium without FCS.

Presence of FCS during DNA repair

For analysing DNA strand breaks and repair in cell lines we have been using commercially available medium **without FCS**, since in some cases we have observed a protect-tive effect probably due to the antioxidant character of serum. Not only with regards to DNA damage but also concerning DNA repair we observed in some cases a positive in-fluence of FCS.

Choice of proper cell type and DNA damage in skin models

Working with tissues and organs is more challenging than working with suspension cells



DNA strand break formation in skin models.
EST-1000 skin models (CellSystems, Germany) were treated with three different concentrations of methylmethanesulfonate (MMS) diluted in aceton or pur aceton. Subsequently the skin models were washed with phosphate buffered saline followed by Trypsin/EDTA. The reaction was stopped by addition of 10% FBS. The suspension was centrifuged and the resulting pellets were resuspended in cell culture media. The cells were analysed on DNA strand breaks by automated FADU. DNA strand breaks increase with increasing the MMS concentration.

Mode of action

Involvement of XPA and or XPC in Base Excision Repair

Accumulation of DNA damage may lead to cancer and ageing, therefore repair mechanisms are necessary for maintaining genomic stability. Base excision repair (BER) and nucleotide excision repair (NER) are important pathways involved in the removal of non-bulky and bulky damaged nucleotides respectively. XPC and XPA are crucial proteins involved in NER but several recent studies provide information about the involvement of XPC in BER (Hazra *et al.*, 2007). The putative role of XPC in the removal of oxidative damage is supported by in vitro data, in which $Xpc^{-/-}$ mouse embryonic fibroblasts exhibit a severe decrease in survival when cultured at 20% oxygen compared with 3% oxygen pressure (Melis et al., 2008).

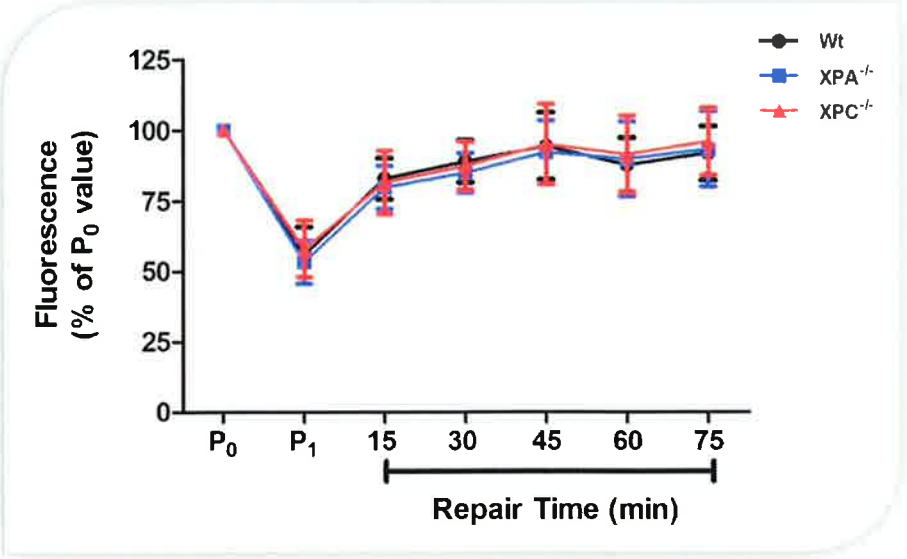
DNA repair after x-ray in XPA and XPC deficient MEF measured by FADU assay did not differ after 75 minutes incubation time at 37°C

It was possible to show that Wt, XPA-/- and XPC-/- MEFs all possess the same DNA repair kinetics of X-ray induced DNA damages (Figure below). Afterwards, fluorescence values rose rapidly, due to an ongoing BER, and finally, after 75 min, reached a value of about 94%. This increase in fluorescence was just interrupted once: a nick in fluorescence after 60 minutes of incubation could be observed. It should be mentioned that this nick in fluorescence was already recognized in earlier studies and several cell types. The biological significance of this nick remains to be solved. One possible explanation was that this nick displays the NER, processing DNA damages later than the BER. In this study it was possible to neglect this hypothesis, since the nick could be observed in cells deficient in nucleotide excision repair as well.

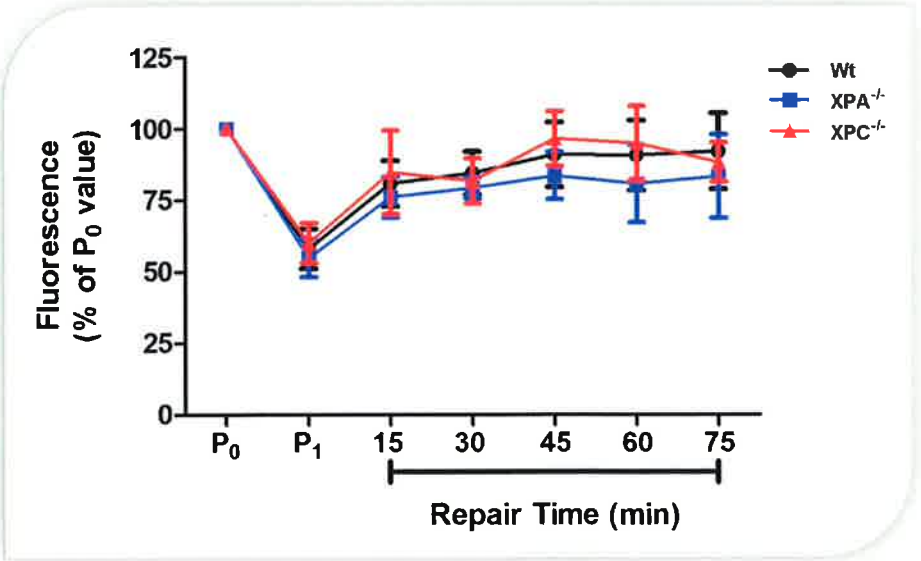
Oxygen culture conditions

The analysis of the DNA repair kinetics of MEFs (Wt, XPA-/-, XPC-/-) cultured at 3% oxygen was performed with the FADU assay and the means of the results of 30 independent experiments are displayed in Figure 13. Data were normalized to the P0 values, representing the endogenous DNA damage. Wt, XPA-/- and XPC-/- MEFs showed similar DNA repair kinetics after irradiation with 7.5 Gy.

Culturing the primary MEFs at 21% oxygen posed a challenge. The cells presented a much more vague and complex behavior than MEFs cultured in 3% oxygen. Cell growth, survival rates as well as the outcome of the FADU assay after DNA damage induction were subject to strong fluctuation.



DNA repair kinetics of MEFs cultured at 3% oxygen. Cells of all three genotypes (Wt, XPA-/-, XPC-/-) repaired the X-ray (7.5 Gy) induced DNA damages (P1 values) with the same dynamics (Ri values). Each data point is expressed as mean relative to the corresponding P0 value + SD (n = 30each). Black: Wt; Blue: XPA-/-, Red: XPC-/-.



DNA repair kinetics of MEFs cultured at 21% oxygen. MEFs of all three genotypes (Wt, XPA-/-, XPC-/-) repaired the X-ray (7.5 Gy) induced DNA damages (P1 values) showing similar kinetics without significant differences. Each data point is expressed as mean relative to the P0 values + SD (Wt n = 8. XPA-/- and XPC-/- n = 6). Black: Wt; Blue: XPA-/-, Red: XPC-/-.

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