Automated high-content imaging to study the response of human cells to novel anti-cancer treatments and identify new therapeutic targets

Italia Anna Asteriti[1,2], Annalisa Verrico[1], Valeria Colicchia[3], Paola Rovella[1], Beate Neumann[4], Pietro Cirigliano[5], Giuseppe Giannini[3], Giulia Guarguaglini[1,2], Patrizia Lavia[1,2]

1. Institute of Molecular Biology and Pathology-CNR National Research Council, c/o Sapienza University of Rome, IT; 2. Nikon Reference Center for Central-Southern Italy at IBPM; 3. Department of Molecular Medicine, Sapienza University, Rome, IT; 4. Advanced Light Microscopy Facility, EMBL, Heidelberg, DE; 5. Nikon Instruments S.p.A., Campi Bisenzio, IT

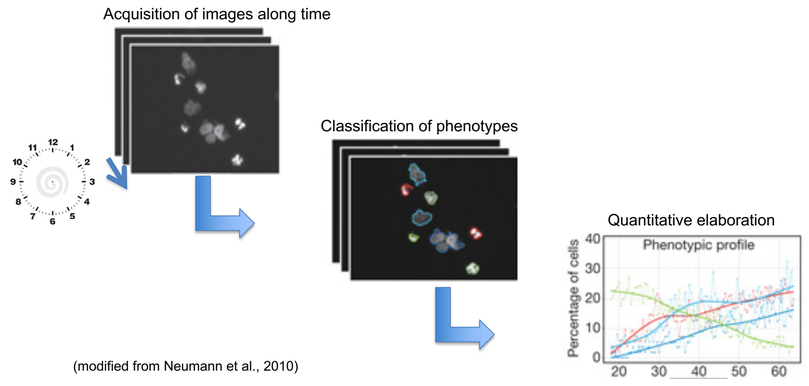
email: patrizia.lavia@uniroma1.it; giulia.guarguaglini@uniroma1.it; lia.asteriti@uniroma1.it

INTRODUCTION

High content imaging of dynamic processes

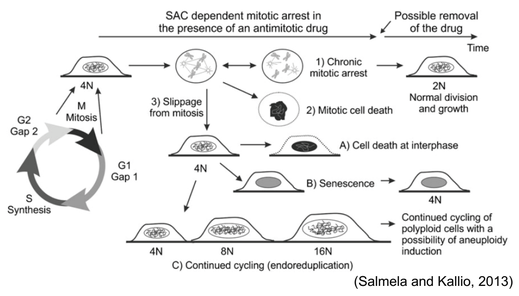
Image-based assays in the biomedical field are rapidly evolving from qualitative to more quantitative approaches ("high content") that provide high resolution spatio-temporal information.

Time-lapse video recording of live cells highlights dynamic information that would remain unnoticed with analyses of fixed samples and that are fundamental to understand complex biological processes (cell division, cell differentation, senescence, intra- and inter-cellular signalling, cellular migration, infection, host/pathogen interactions, response to drug treatments, induction of cell death).



(modified from Neumann et al., 2010)

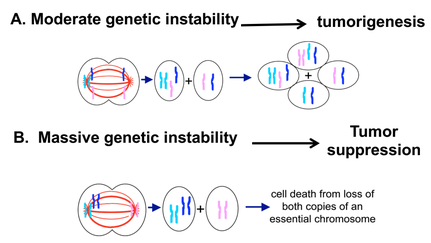
Cell-to-cell variability in response to anti-cancer drugs



(Salmela and Kallio, 2013)

The introduction of single-cell analyses, e.g. in live cell imaging, has provided significant advance in the drug design field, evidencing a cell-to-cell variability that had gone unnoticed in whole cell population studies.

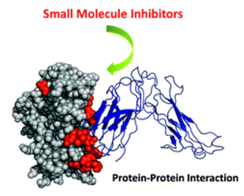
MITOTIC CATASTROPHE, a poorly understood death pathway, is the main form of cell death response to **anti-mitotic** drugs, to eliminate genetically unstable cells.



ANEUPLOIDY can drive or inhibit tumorigenesis: since responses to anti-mitotic drugs are heterogeneous, getting a better understanding is important to predict whether these compounds will have clinical efficacy

(modified from Weaver and Cleaveland, 2007)

Targeting protein-protein interactions in modern drug discovery



Protein-protein interactions (PPIs) offer a rich reservoir of novel and specific drug targets. Significant progress has been made in the discovery and characterization of relevant interactions and in the development of small PPI inhibitors.

(modified from Sheng et al., 2015)

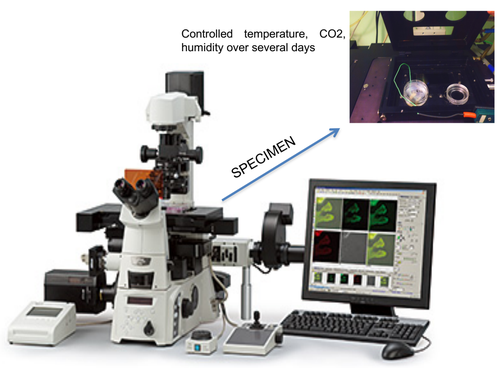
AIMS

- Developing quantitative high-resolution time-lapse imaging methods, to characterize the behaviour of single cells treated with molecules of potential therapeutic value
- Developing the in situ proximity ligation assay (is-PLA) to an automated mode, to identify interactors of cancer-associated proteins

METHODOLOGY

INSTRUMENTAL SETTINGS

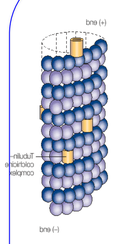
Nikon Eclipse Ti at the Nikon Reference Center at IBPM-CNR



- NIS-Elements HC (High Content Analysis)
- supports for simultaneous analysis of several conditions
- complete motorization
- multidimensional acquisition: xy multipoints, Z-stacks, multichannels

ANTI-CANCER COMPOUNDS

I) ARYLTHIOINDOLE TUBULIN POLYMERIZATION INHIBITORS (ATIs)



Colchicine: very effective binding to tubulin, but toxic

↓

ATI STRUCTURAL DESIGN

1. based on the structure of **colchicine**-binding pocket on MTs
2. capable of **displacing colchicine** from MTs
3. small molecule class (MW < 500)
4. Addition of stabilizing lateral chains that can confer resistance to esterase enzymes

(collaboration with R. Silvestri, Sapienza University)

II) AURORA-A KINASE INHIBITORS

Small chemical inhibitors directed against the ATP-binding site in the catalytic domain (ATP-competitors) of Aurora-A are under evaluation in clinical trials

	Inhibitor commercial name	Clinical trials
Pan-Aurora inhibitors	VX-680/MK-0457 (Vertex/Merck)	Phase II (terminated due to toxicity)
	Tozasertib	Phase II
	PHA-739358 (Pfizer/Nerviano)	
	Danusertib	
	PHA-680632 (Pfizer/Nerviano)	Phase I
	CYC-116 (Cyclacel)	Phase I
	SNS-314 (Sunesis)	Phase I
	R763 (Rigel)	Phase I
	AMG-900 (Amgen)	Phase I
	AT-9283 (Astex)	Phase II
	PF-03814375 (Pfizer)	Phase I
	GSK1070916 (GlaxoSmithKline)	Phase I
Aurora-A inhibitors	MLN8237 (Millennium)	Phase II
	ENMD-2076 (EntreMed)	Phase II
	MK-0457 (Vertex)	Phase II

(D'Assoro et al., 2016)

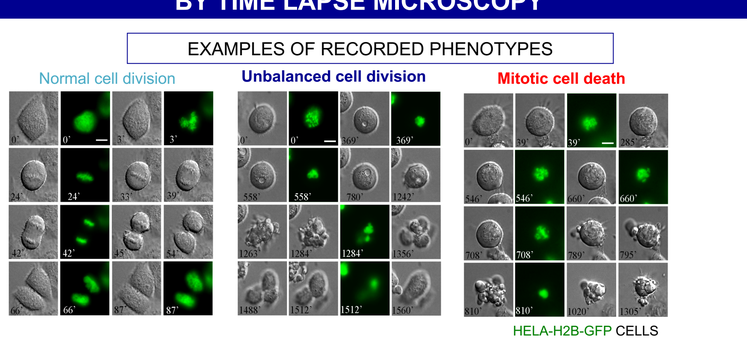
III) PARP INHIBITORS

Small molecule inhibitors of polyADP-ribose polymerase (PARP) are thought to exert their anti-tumor effects by blocking the repair of DNA single strand breaks.

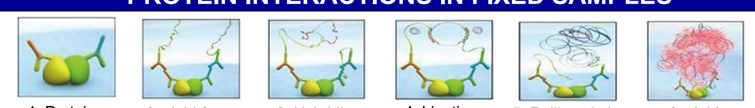
Agent	Tumor types	Company	Most advanced development stage
Olaparib (Lynparza)	Breast, endometrial, gastric, glioblastoma, head and neck, lung, ovarian, pancreatic, prostate, sarcomas	AstraZeneca	• FDA-approved in ovarian cancer • Phase III in breast cancer, pancreatic, ovarian, gastric
Rucaparib	Breast, ovarian, pancreatic	Clovis Oncology	Phase III in endometrioid epithelial ovarian, primary peritoneal or fallopian tube cancer
Niraparib (MK4827)	Breast, Ewing sacroma, ovarian	Tesaro	Phase III in ovarian, breast cancer
Veliparib (ABT-888)	Breast, cervical, colorectal, glioblastoma, head and neck, lung, leukemias, multiple myeloma, non-Hodgkin lymphoma, ovarian, pancreatic, prostate	AbbVie	Phase III in breast cancer, non-small cell lung cancer, glioblastoma
Talazoparib (BMN-673)	Breast, endometrial, leukemias, ovarian, solid tumors	BioMarin Pharmaceutical	Phase III in breast cancer
BGB-290	Solid tumors	BeiGene	Phase I

(Source: NIH Clinical Trials registry. www.ClinicalTrials.gov.)

RECORDING INDIVIDUAL FATES OF LIVE CELLS BY TIME LAPSE MICROSCOPY



IN SITU PROXIMITY LIGATION ASSAYS (PLA) TO STUDY PROTEIN INTERACTIONS IN FIXED SAMPLES

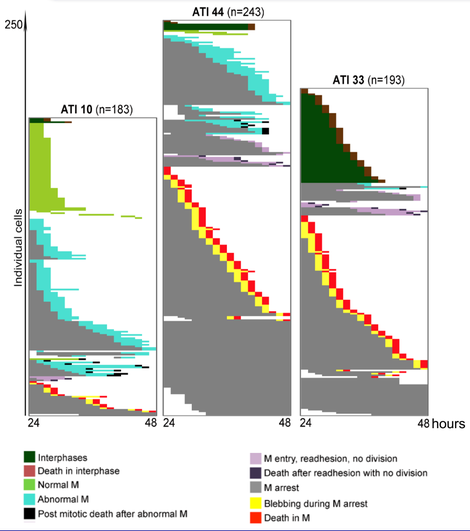


PLA signals reveal amplification of antibody-bound oligonucleotides: hence, they are only detected when the two proteins of interest interact or are very close.

RESULTS

1. Quantitative time-lapse imaging methods in human cancer cells to depict specific responses to small molecules

1.1. SAR (structure-activity relationship)-based drug design: single cell time-lapse recording to visualize the heterogeneous response to novel inhibitors of the mitotic spindle

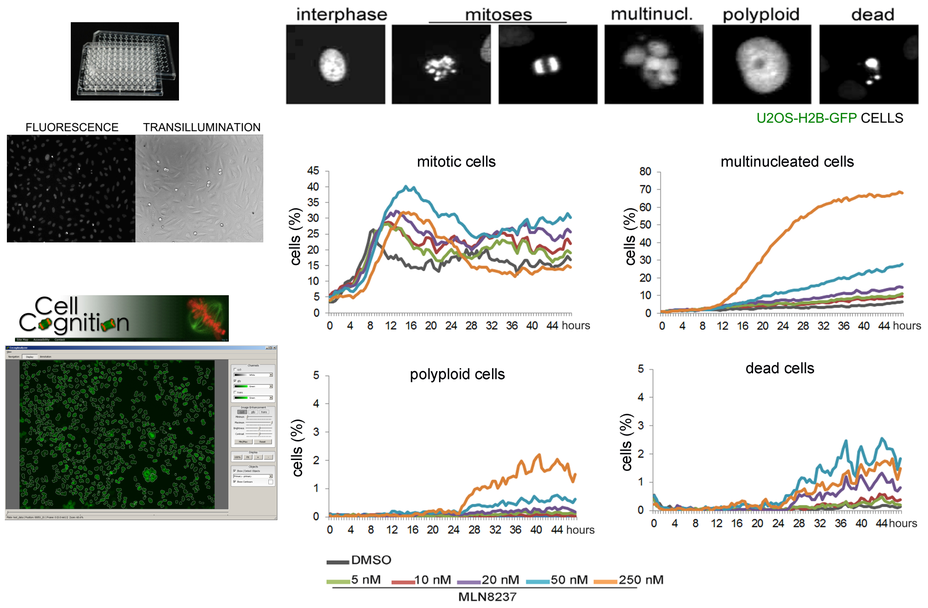


Different cell fates were visualized in cell cultures (HeLa cells) treated with 3 ATI compounds.
Each horizontal bar in the graph represents the fate of a single cell, videorecorded from 24 to 48 h after treatment:
- Small structural modifications may significantly influence the cellular response to the treatment, and, consequently, efficient induction of cell death;
-In all conditions, cell cultures show heterogeneous response to the treatment;

Therefore video-recording is essential to evaluate the frequency of "escaper" cells in response to new anti-cancer treatments.

(Di Cesare et al., 2017)

1.2. A high-throughput video-recording approach and automated analysis to follow the fate of MLN8237-treated cells over time

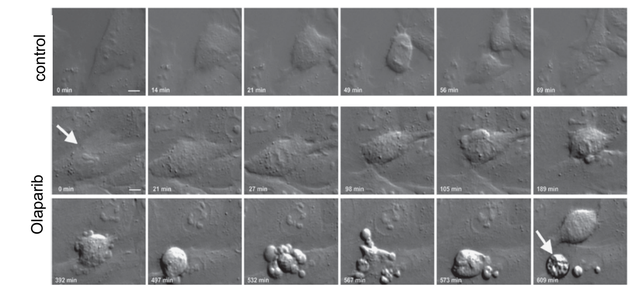


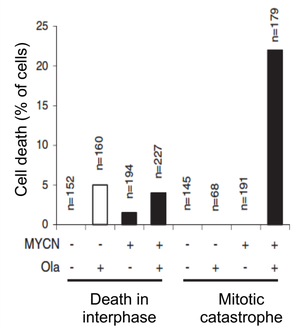
Proliferation of MLN8237-treated osteosarcoma cell cultures is slowed down in a dose-dependent manner, without significant induction of cell death neither from mitosis or the following interphase within 44 hours from the treatment, and paralleled by an accumulation of multinucleated cells.

EURO-BIOIMAGING EMBL Advanced Light Microscopy Facility

(Asteriti et al., 2014)

1.3. Inhibitors of PARP enzymes induce mitotic catastrophe in neuroblastoma cell lines in a MYCN-dependent manner





Time lapse video-recording of neuroblastoma cells treated with PARPi Olaparib showed an unexpected cell death pathway: cells enter in mitosis also in presence of DNA damage and die through mitotic catastrophe.
This fate is specific for neuroblastoma cells with MYCN amplification (a main driver of aggressiveness in neuroblastoma).

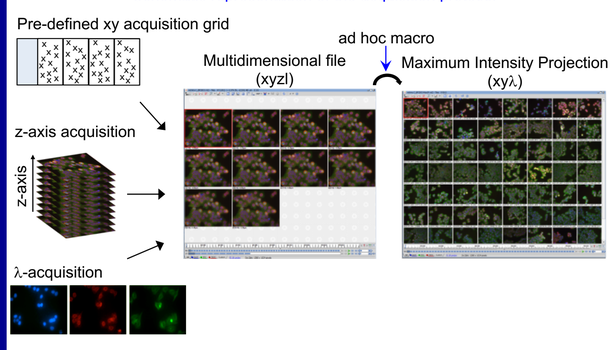
(Colicchia et al., 2017)

2. PLA automation to validate protein interactions identified by proteomic approaches

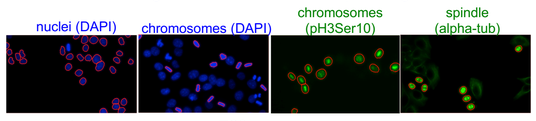
Workflow

Acquisition	- Complete image series (multiple fields)
↓ Segmentation	- Automated generation of segmentation masks
↓ Measurements and analysis	- Simultaneous intensity measurement within selections

Schematic representation of the acquisition protocol



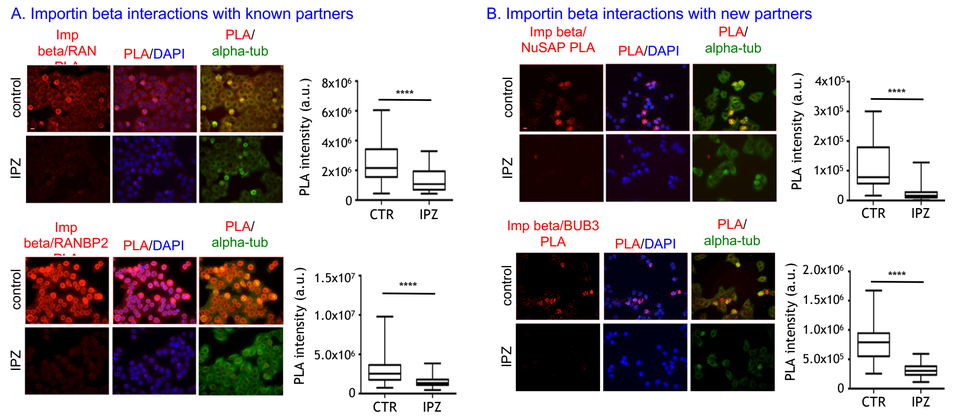
Segmentation: mitotic vs. interphase figures



For all mitotic marks, over 70% (non-synchronized cultures) and 80% (mitoses-enriched cultures) objects were recognized, with >75% or >90% confidence, respectively

Validation of Importin beta interactors by automated PLA detection

Importin beta is the major vector for protein import in interphase nuclei and a RAN GTPase effector. After nuclear envelope breakdown, when nuclear transport ceases, importin beta regulates several steps of mitosis. It is overexpressed in many cancer types characterized by high genetic instability.



Our workflow for automated detection and analysis of PLA products yields rapid and accurate information on i) validation and ii) localization of Importin beta interactors selected in proteome-wide screening. Here, all interactions are specific and are abolished by importazole (IPZ), a specific Importin beta inhibitor (*Soderholm 2011).*

(Di Francesco et al., under revision)

CONCLUSIONS

Our studies highlight the power of imaging approaches in drug development and screening protocols:

- We highlighted important cell fate differences depending on small modifications of drug structures, which may influence the outcome of the treatment
- We depicted the complexity of the cellular response to anti-mitotic drugs of potential therapeutic value, evidencing stochastic effects that may lead to aneuploidy, a potentially pro-tumorigenic condition
- We identified an unexpected form of cell death that may be specific for the response of highly aggressive MYCN-amplified pediatric tumors to PARP inhibitors.
- We have developed an isPLA based automated protocol as a novel, rapid and reliable tool to validate protein interactions emerging from proteome-wide screenings.

We acknowledge support from CNR-InterOmics Flagship Project, grant IBISA; AIRC; Nikon

InterOmics CNR — ASSOCIAZIONE ITALIANA PER LA RICERCA SUL CANCRO AIRC — Nikon