Personalization of cancer treatment is the way to successful treatment of cancer.

More than 100 anti-cancer drugs are used in the clinic today, and new drugs are under development for use tomorrow. The challenge is to match a patient with the right drugs. The clinicians face this challenge with practically every patient, because of the limitations of diagnostics. Information obtained with current diagnostics is still far from predicting securely how the tumor and patient would respond to a treatment.

The recent developments of cancer research have opened for the qualitative improvement of diagnostics. In this document is described Functional Molecular Diagnostics (FMDx) which is for the clinical use.

FMDx tests responsiveness of individual patient's tumors to different drugs by testing responsiveness of the living tumor samples in organ culture (Organ Culture FMDx), testing targets and modulators of the drugs' action (Functional Biochemical Assays), and by unbiased testing of the tumor's proteome profile (Proteomics FMDx).

Personalized Diagnostics of Cancer

This document turns at the first place to clinicians working with patients. However, cancer patients and researchers working with cancer may also find information of interest for them.

Content:

What is FMDx?

FMDx is a set of assays performed on living and processed tumor cells following removal from patients upon surgery or biopsy. These assays measure in a real time how the patients' tumor may respond to different drugs before the patient is offered treatment, and whether the tumor is of an aggressive type. The successfulness of FMDx is based on novel proprietary technologies.

FMDx assays are of 3 types – organ culture-based, functional biochemical assays and proteomics-based. The assays may be used all together or separately, depending on the clinical requirements. All 3 types of assays analyze a tumor with different methods, but for the common goal of finding the best treatment. This increases the confidence of the recommendations for treatment.

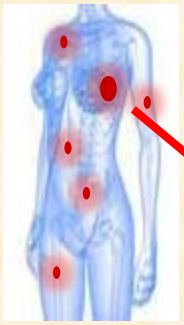
Organ culture FMDx answers the question "Which drug and treatment is the most efficient in killing the tumor?" Tests are performed with samples of tumors obtained upon surgery or collection of biopsy. Cells in the samples are alive and preserve their natural environment of the tumor.

Functional biochemical assays answer the question "Which molecular mechanisms are corrupted in the tumor?" Tests target selected molecular mechanisms, and are performed with processed tumors. Processed means that the biomarkers are extracted from the tumor, and the tumor cells are not alive.

Proteomics FMDx answers the similar question as the Organ Culture FMDx, but with processed tumor biopsies. Proteomics FMDx is unbiased screening for drugs which would be most efficient for treatment of the tumor. Proteomics FMDx may be performed before chemotherapy and adjuvant treatment, or/and after pre-operative treatments, according to decision of the clinician.

What is FMDx?

Schematic presentation of
3 parts of FMDx



***Surgery**
***Biopsy**
***Circulating Tumor Cells**

Living tumor
(collected upon biopsy or surgery)

Processed tumor
(sample is frozen)

Organ culture FMDx

Drug 1

Drug 2

Resistant

Drug 3

Partial Response

Full Response

Selection of the drug which destroys the tumor,
e.g. Drug 3.

Functional Biochemical Assays

Drug X

Modulator

Drug target

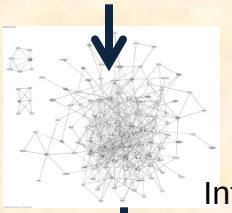
Modulator

Effect:
inhibition
of tumor growth

Measure activity and expression of target, modulators and markers of effect of drugs, to predict efficacy of the drug.

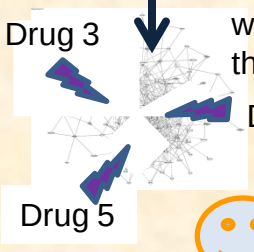
Proteomics FMDx

Proteome profile



Network

Interrogation with drugs which destroy the network



Selection of drugs which destroys the tumor,
e.g. Drugs 3+4+5.

Who may benefit from FMDx?

FMDx is already used for helping clinicians to select the best treatment. These recent successes allow an expansion of application of FMDx to more patients, and new patients are being recruited.

In this folder, there is information for clinicians, at the first place. This information may also be of interest for the cancer researchers, patients and members of the public.

For clinicians, this is the invitation to collaboration. If you want to apply FMDx to your patients, do not hesitate to contact Oranta Cancer Diagnostics (contact information is on the last page).

For scientists, this is the invitation to collaboration on development of novel diagnostics.

For patients and members of the public, this is the information about availability of FMDx and what it can do.

When can FMDx be of help?

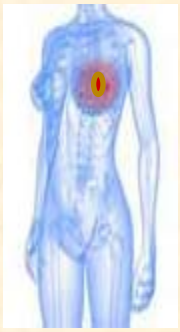
FMDx can be applied to all types of cancer. The most of the benefit FMDx provides for patients with advanced cancers.

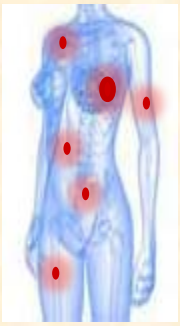
Example of breast cancer

First changes in cells, which lead to uncontrolled growth of the cells Changes may be on the levels of genes, gene transcripts, metabolites and proteins. This is "**pre-malignant lesions**".

Accumulation of the changes leading to **formation of a tumor**. Cells acquire ability to build a colony – a tumor. This is "a **local tumor**".

Accumulation of the changes leading to **spreading of a tumor**. Tumor cells acquire ability to spread in the body and start new tumors in different organs. This is "development of **metastases**".





Survival today:

Up to 80% of lumps are not malignant tumors

Up to 90% of women survive breast cancer if it is detected at the early stage.

Survival is less than 30% for women with metastatic breast cancer

OCD provides diagnostics for individually adapted treatment. We apply:

***Organ culture and CTC FMDx**

***Proteomics FMDx**

***Functional Biochemical Assays**

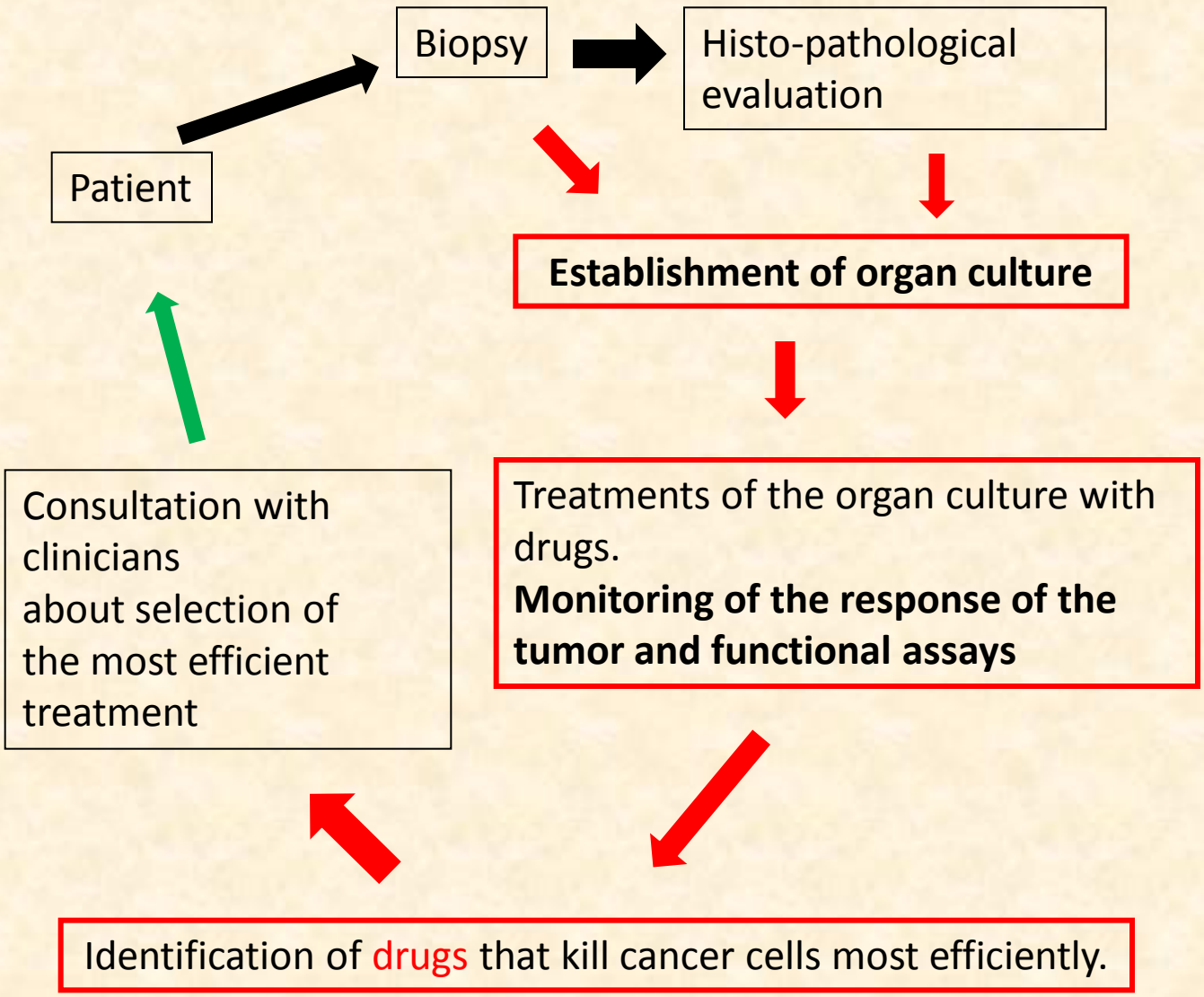
*CTC – Circulating Tumor Cells

Personalized selection of treatment of patients.

workflow for

Organ Culture-based FMDx diagnostics

Activities outlined in black are performed at a hospital, in red – at the OCD.



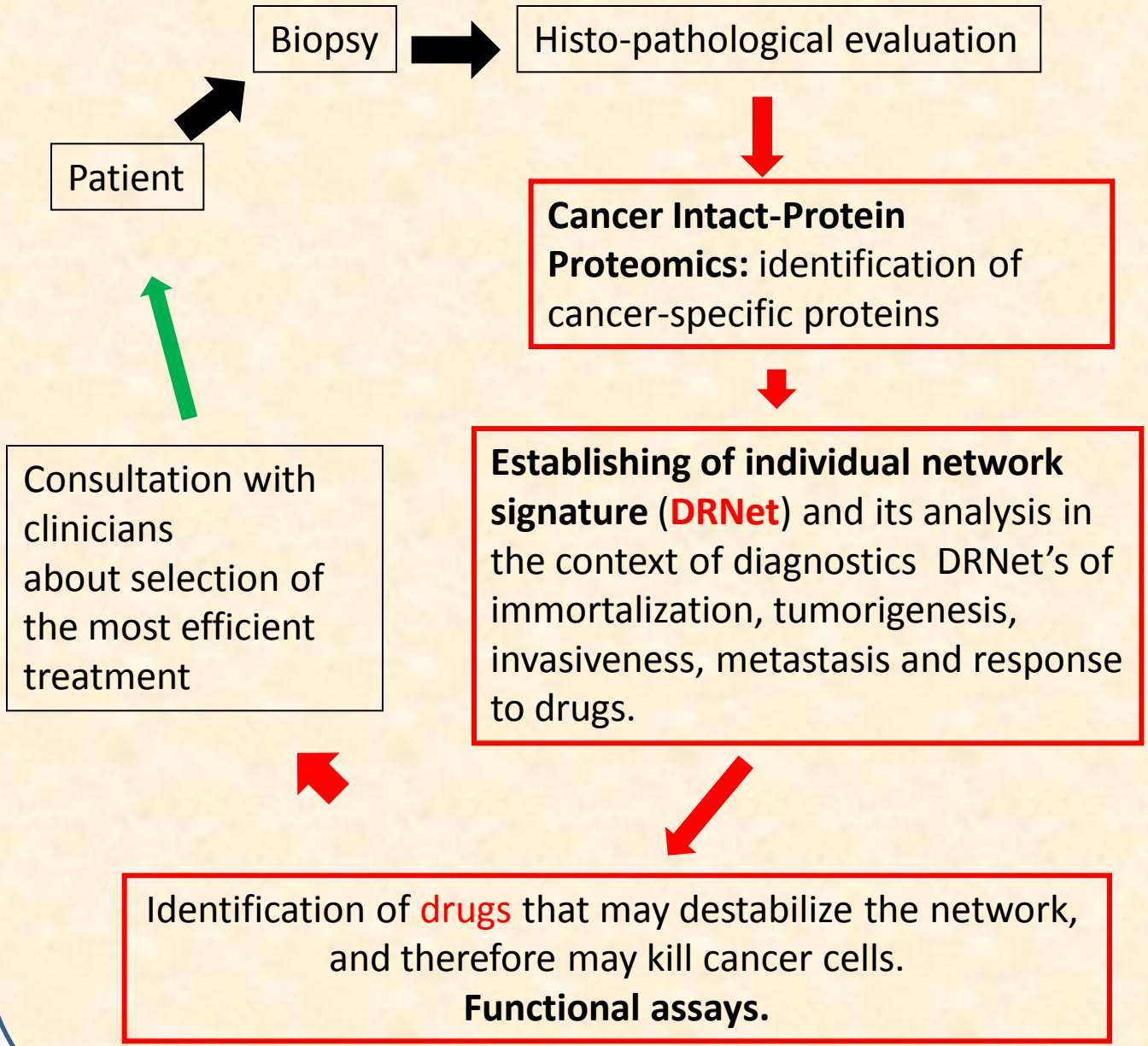
*Organ culture was found to relfect features of a tumor much better as compared to single cells obtained from a tumor. This is partially due to preservation of the tumor structure and native shape of the tumor cells.

Personalized selection of treatment of patients.

workflow for

Proteomics-based FMDx diagnostics

Activities outlined in black are performed at a hospital, in red – at the OCD.



Example of the Organ-Culture FMDx

Pages 9 to 13 show an example of how a test of sensitivity to anti-cancer drugs is performed with an organ culture of the tumor.
The organ culture was made from a tumor sample obtained upon surgery. Organ culture of this tumor was tested for responsiveness to drugs, including drugs considered by the clinician for treatment of this patient.

Summary of the test shows an example of the recommendation to a clinician. Images of the organ culture are shown in the following slides.

1. Sensitivity to drugs, on the scale from 0 (no cell death) to 5+ (100% cell death)
*Fluorouracil – Cell death is strong, 5+.
*Gemcitabine and Oxaliplatin – Cell death strong (4+), some cells survive.
*Tarceva –Cell death strong but less than with Gemcitabine or Oxaliplatin (3+).
*Melphalan-flufenamide(J1) – Cell death 3+ to 2+.
*SB431542 – Cell death 3+.

Conclusion: The tumor is highly sensitive to Fluorouracil, and full response of the patient is expected. Gemcitabine and Oxaliplatin showed a strong effect, but not complete, partial response of the patient is expected.
Targeting drugs Tarceva, J1 and SB431542 showed inhibitory effect on the tumor cells. These drugs may also be used, but in combinations with other drugs. Tarceva showed impact after 24h and continued after 7 days; J1 had a good impact after 24h, but less after 7days; and SB431542 had weak after 24h and much stronger after 7days.

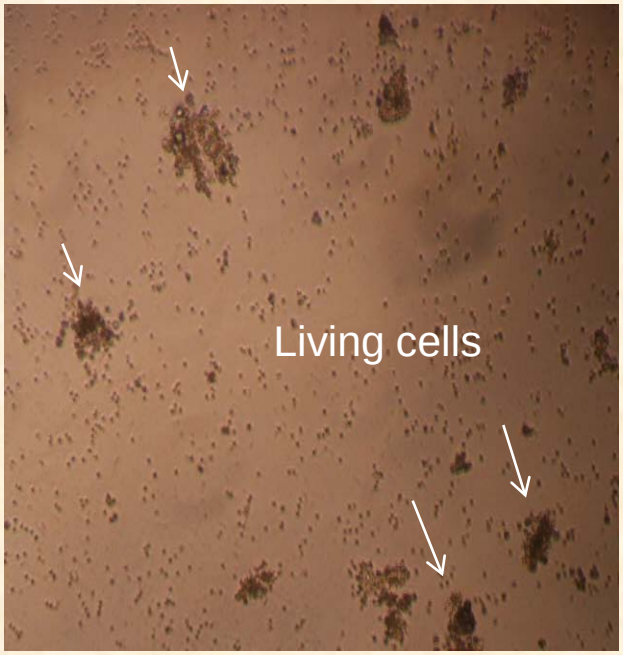
It was possible to establish a primary cell culture for this patient, which is shown on page 14. Morphologically, the cells show mesenchymal phenotype. These primary cells unlikely to reflect true responsiveness of the tumor to drugs, therefore the recommendation on the basis of the Organ Culture FMDx has to be considered.

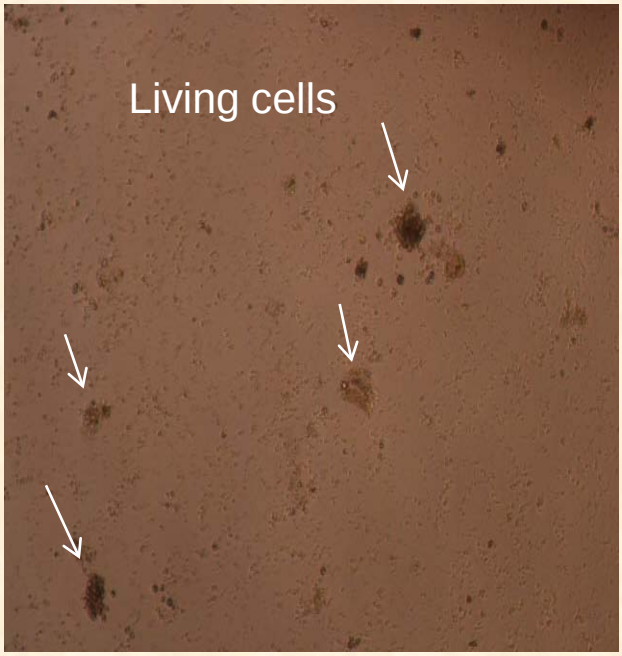
2. Proliferation status by Functional Biochemical Assays
The data indicate that drugs inhibiting classical mitogenic pathway may be of benefit, e.g. MEK, B-Raf, CDK's inhibitors which are currently in clinical trials.

***NOTE:** Decision about treatment has to be taken by clinicians who treat the patient. Described here assays are to help the clinician to take decision.
Patients should always consult with their doctors.

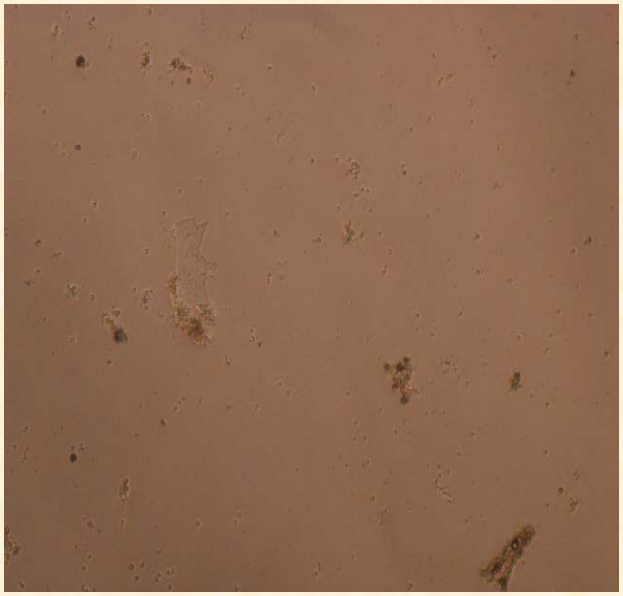
Images of cells of the organ culture after the treatment with drugs

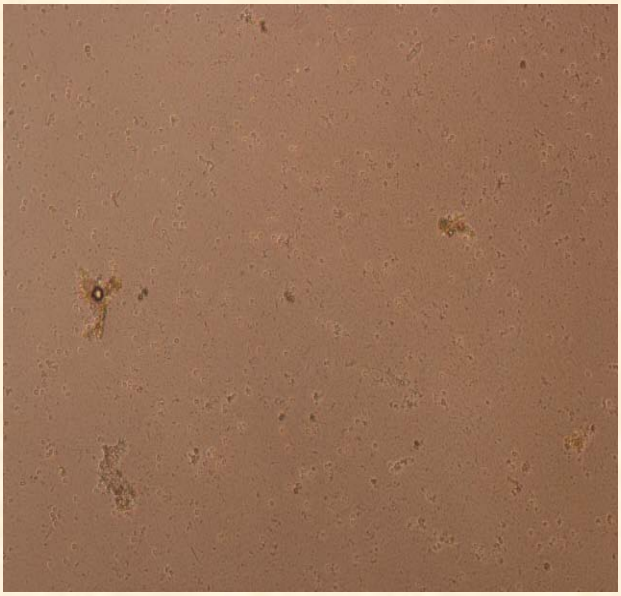
Controls, no treatment





Fluorouracil (no living cells)





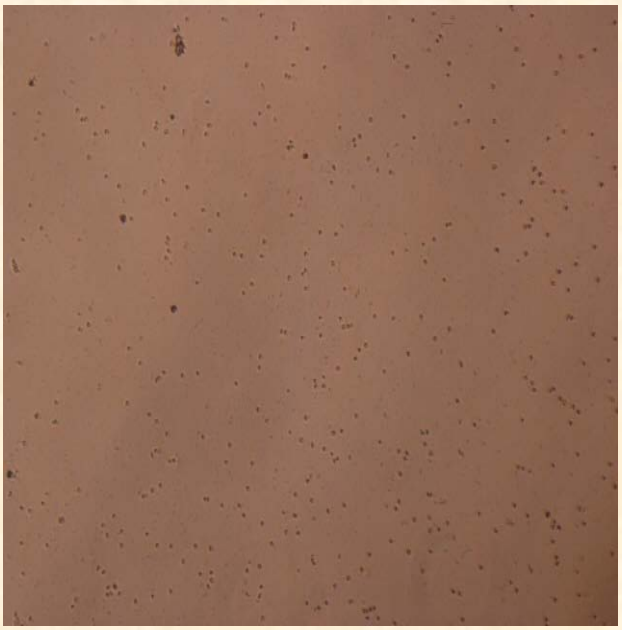
Notes about ORGAN CULTURE-based assays

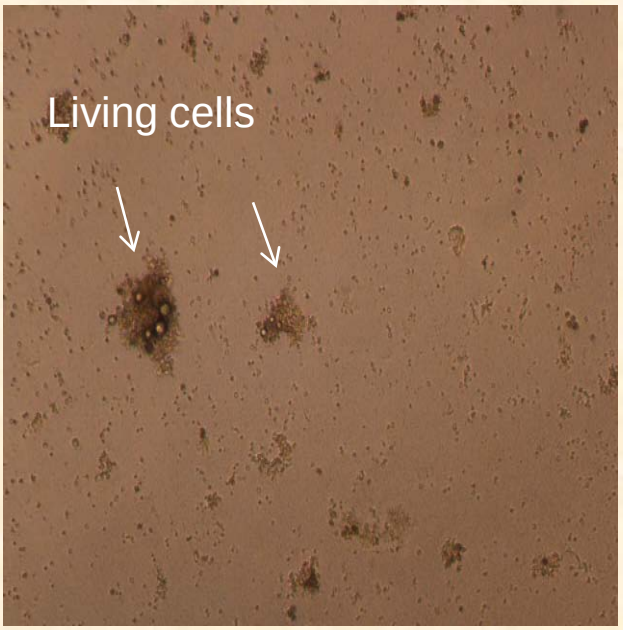
White arrows show living cells forming clusters. Such clusters are formed by cells which are proliferating. Cell clusters observed in conditions when drugs were applied, indicate that the cells are resistant to these drugs.

Two images of the same condition are shown, as examples of different areas.

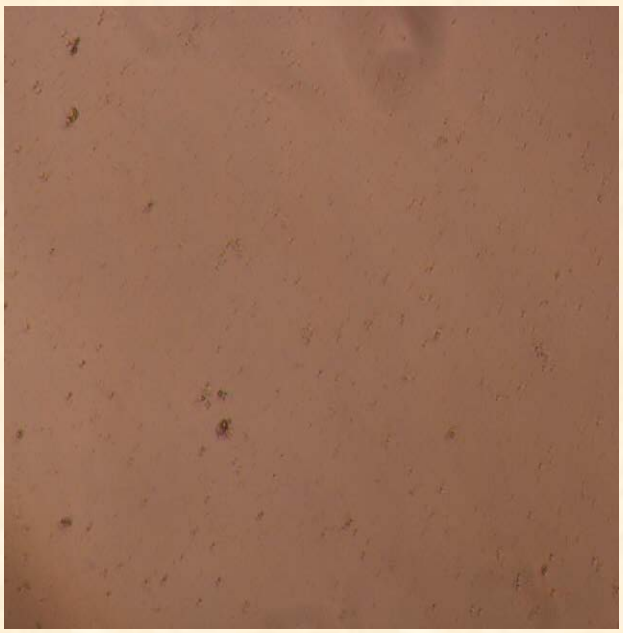
Images of cells of the organ culture after the treatment with drugs

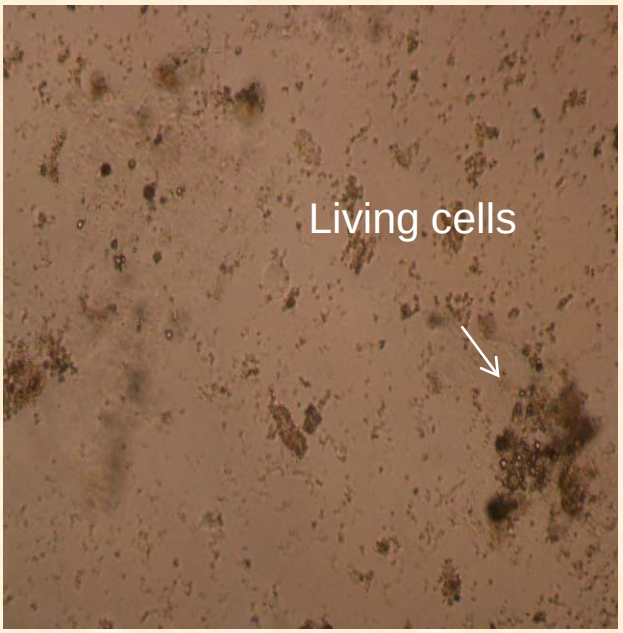
Gemcitabine





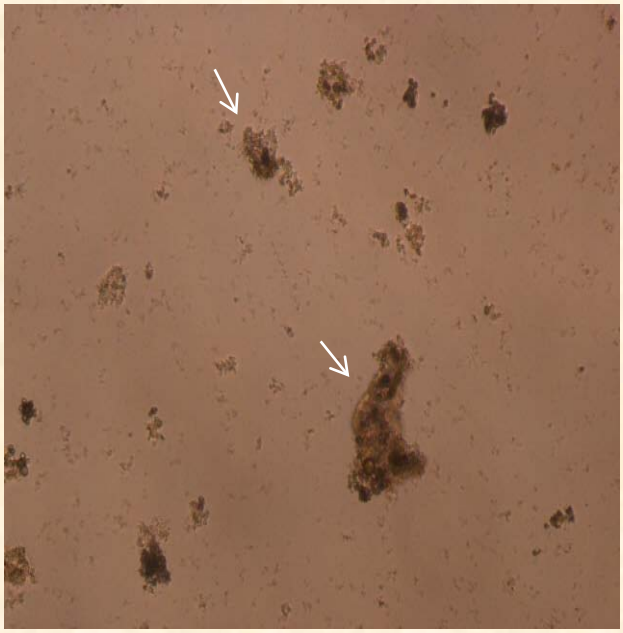
Oxaliplatin

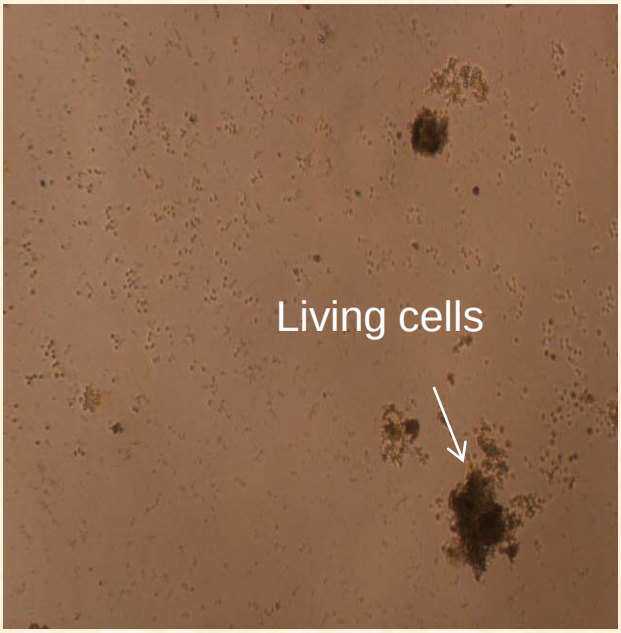




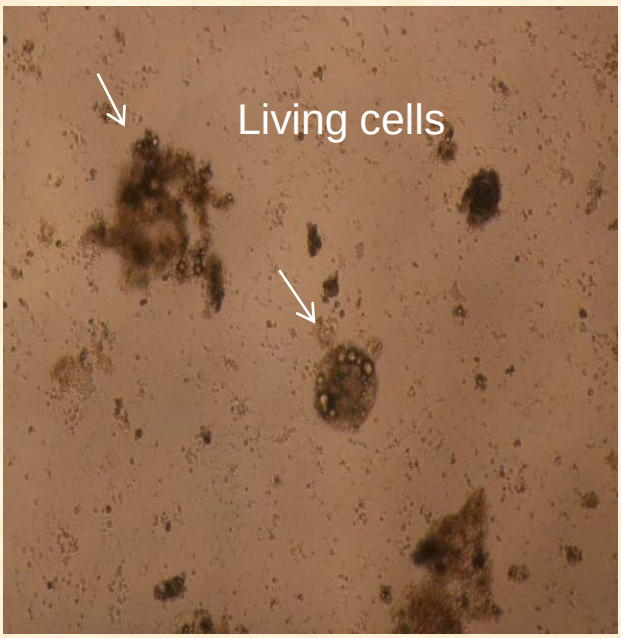
Images of cells of the organ culture after the treatment with drugs

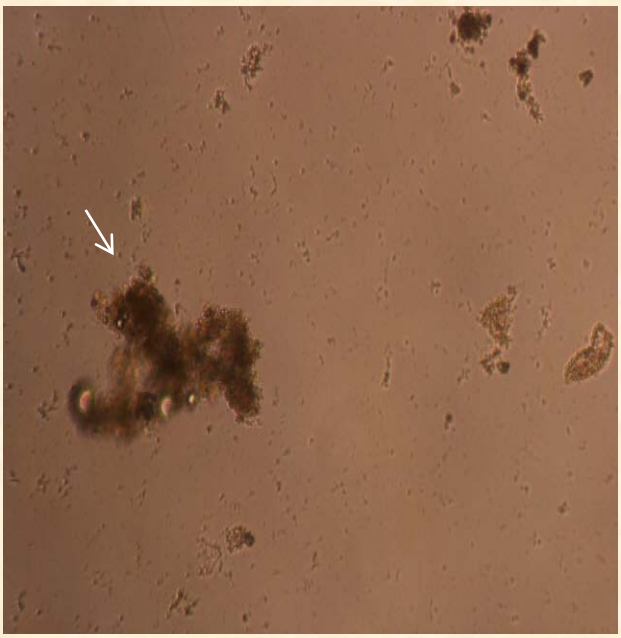
Gefitinib/Tarceva/Iressa (EGFR inhibitor)





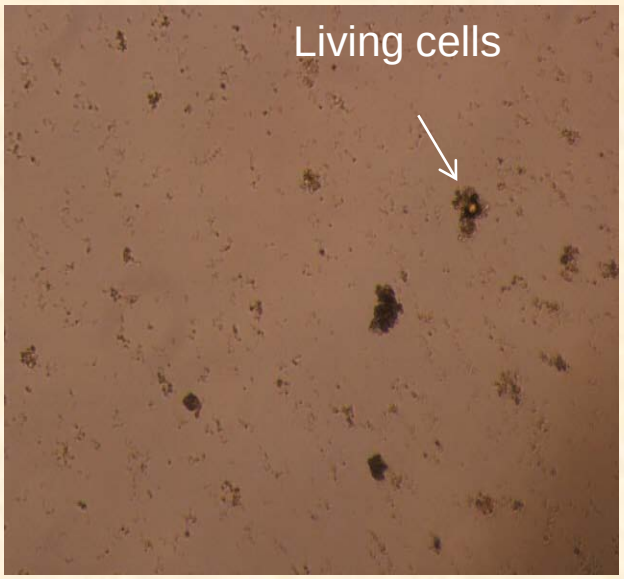
Melphalan-flufenamide (J1)

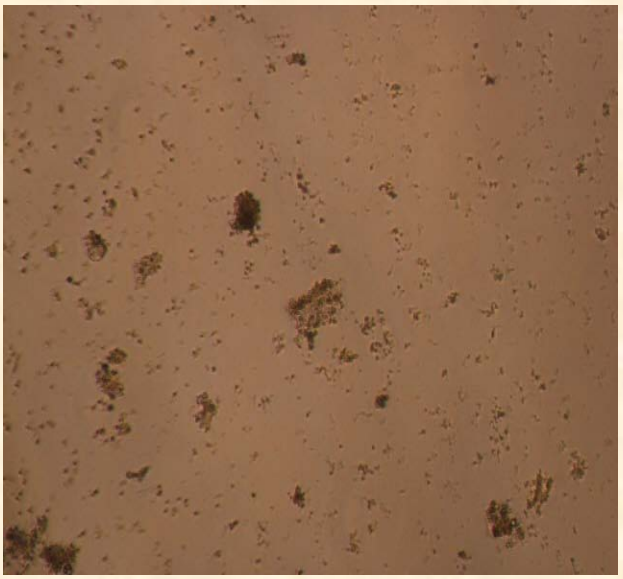




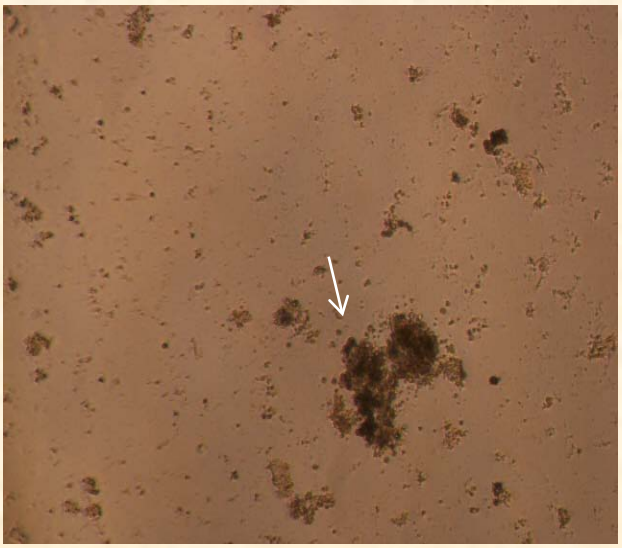
Images of cells of the organ culture after the treatment with drugs

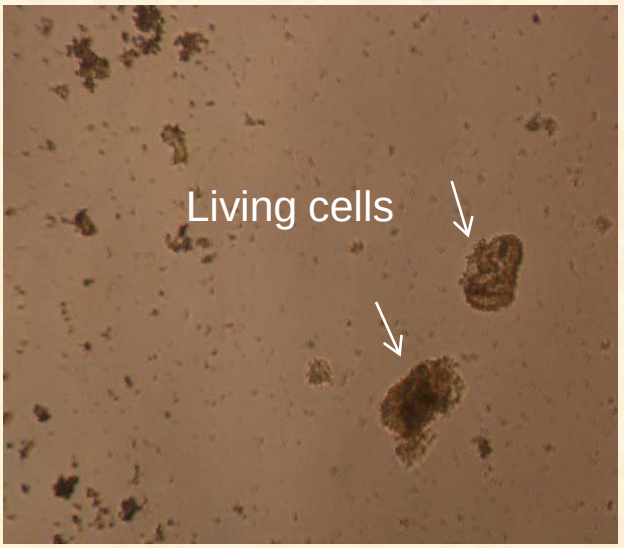
SB431542 (TβRI inhibitor)





Controls, no treatment





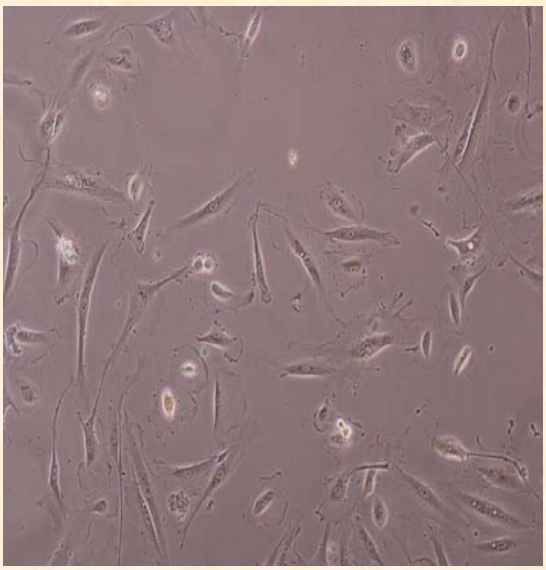
Established cell culture from the tumor

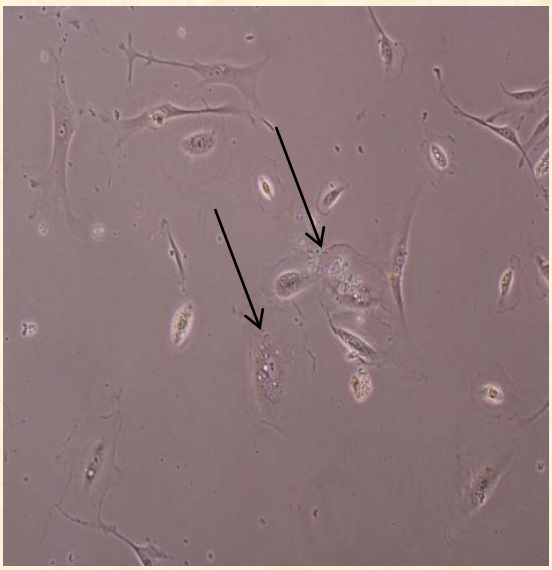
This example is shown to indicate that the Organ Culture FMDx allows also generation of a primary cell culture. However, diagnostic value of the organ culture is higher as compared to the primary culture, due to preservation of the tumor stroma.

Established cell culture from tumors may be used to confirm results obtained with the organ culture. Established cells grow in the 2-dimensional culture on a plastic surface, while the organ culture allows growth of cells in 3-dimensions and in the environment similar to the environment in the tumor. The primary diagnostics is performed with the organ culture which reflects natural environment of the tumor better.

Below are two images of isolated cells obtained from a tumor. Cells which stopped to proliferate and change morphology are indicated by arrows. Appearance of such cells indicate that the tumor cells may be sensitive to drugs inhibiting the cell cycle.

Thus, the cells are highly proliferative and may be sensitive to anti-proliferative drugs.





Functional Biochemical Assay of molecular markers

For application to a patient, the assays are selected in consultation with clinicians treating this patient.

Types of biomarkers

* Proteins (expression and activity)
* Transcripts of genes, mRNA (expression and mutations)
* Genes (genomic mutations)
* Tumor antigens

Regulatory processes which are monitored by FMDx

* Tyrosine kinase receptor signaling (EGF, FGF, VEGF, IGF, PDGF)
* Serine/threonine receptor signaling (TGFβ, BMP)
* G-protein coupled receptors signaling
* Intracellular kinases signaling (kinases involved in regulation of cell proliferation, cell death/apoptosis, DNA repair, invasiveness, cell-cell interactions, cell-substrate interaction)
* Proteases (MMPs)
* Ubiquitintransferases (E3 ligases)
* Oncogenes and tumor suppressors, expression and activity.
* Check-points regulators, expression and activity.

Activities which are monitored by FMDx

(assays which measure activity)

* Phosphorylation/kinase activity and specificity
* Ubiqutylation
* PARylation
* Acetylation
* O-Glycosylation

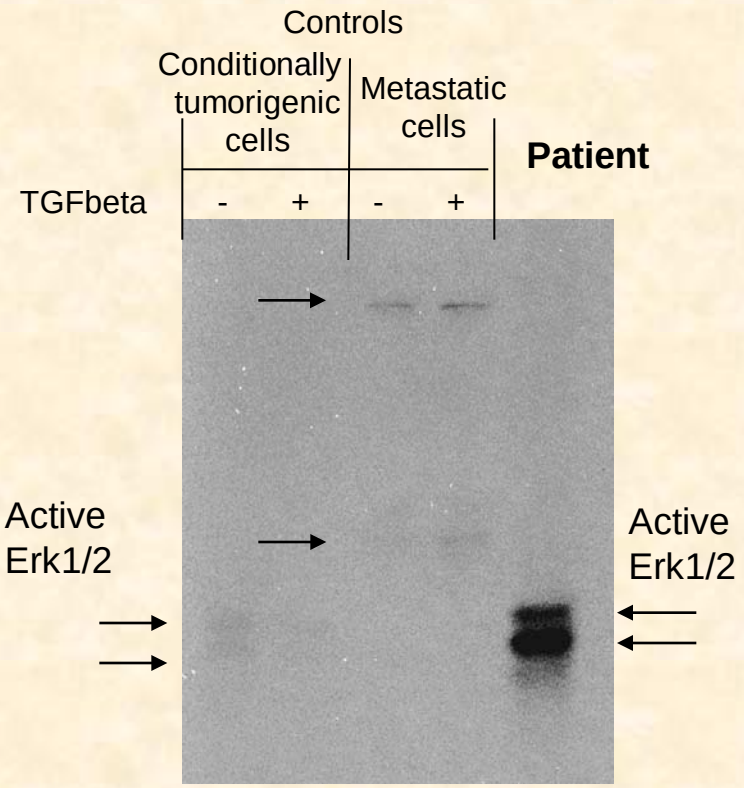
Another category of the assays is the **companion diagnostics of drugs**. The companion diagnostics assays are designed to evaluate efficacy of a specific drug for a given patient.

Functional Assay of molecular markers

example of testing the mitogenicity marker Erk1/2 as a functional biochemical assay

Erk1/2 activity is high in highly proliferating cells. Erk1/2 activity reflects status of the classical mitogenic pathway by Ras-Raf-Erk to pRb/CDK/ Cyclins.

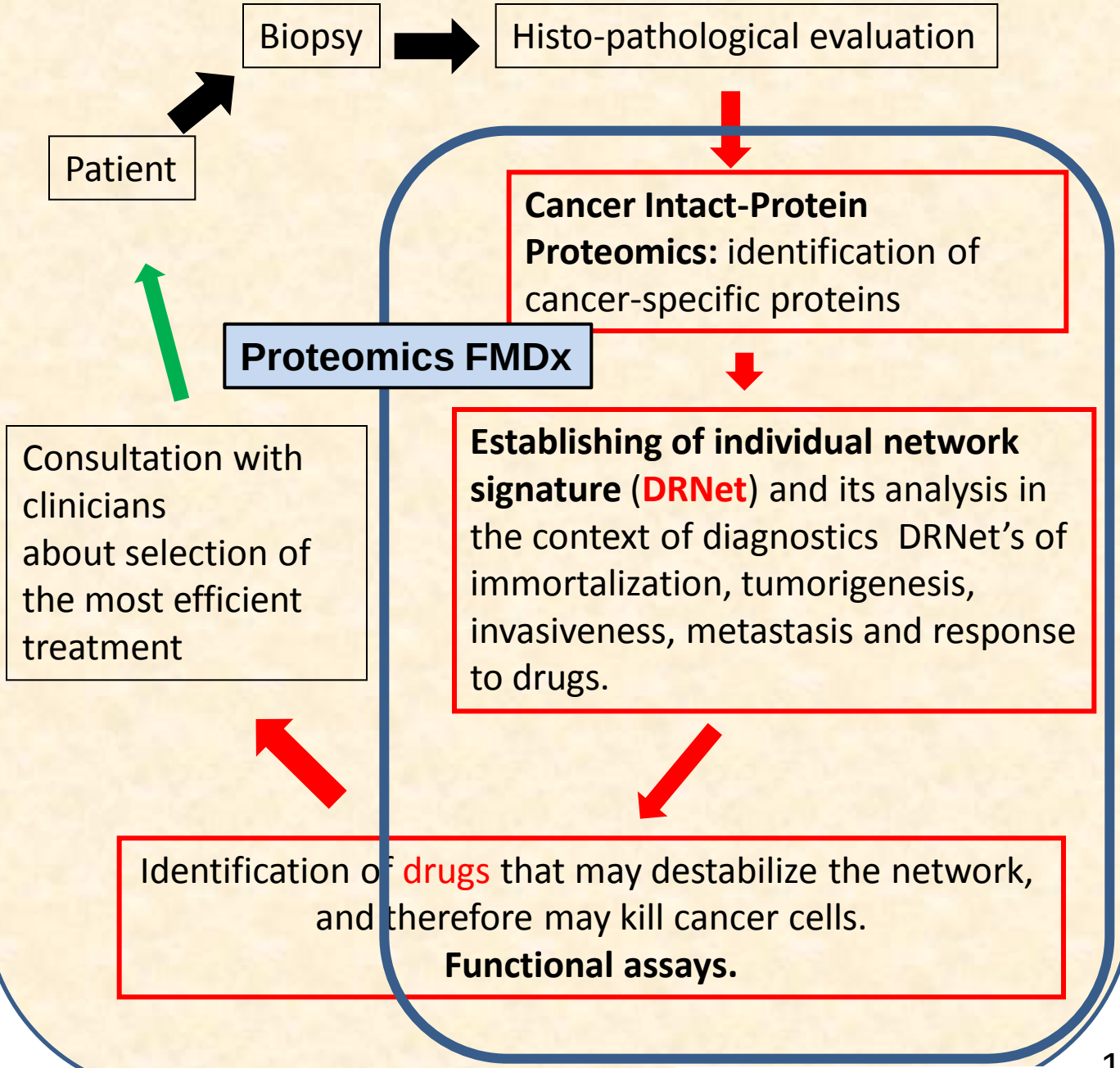
The patient has a strong Erk1/2 activation, which indicates a strong proliferative activity. Note that the patient does not have activation of Erk-like proteins specific to highly metastatic cells.
The data indicate that drugs inhibiting mitogenic pathway may be of benefit, e.g. MEK, B-Raf or CDK's inhibitors. These drugs are already in clinical use, and can be used for the patient treatment, together with 5FU and possibly with Tarceva.



*Other markers reflecting status of signaling mechanisms were also tested (not shown).

Proteomics FMDx

This page shows the workflow of Proteomics FMDx. The dark-blue frame marks proteomics and systems biology activities.
On pages 18-20 are shown examples of unbiased search for individually-tailored drugs for two patients.
On pages 21-23 is shown an example of how decision about use of a specific drug was made for four patients.



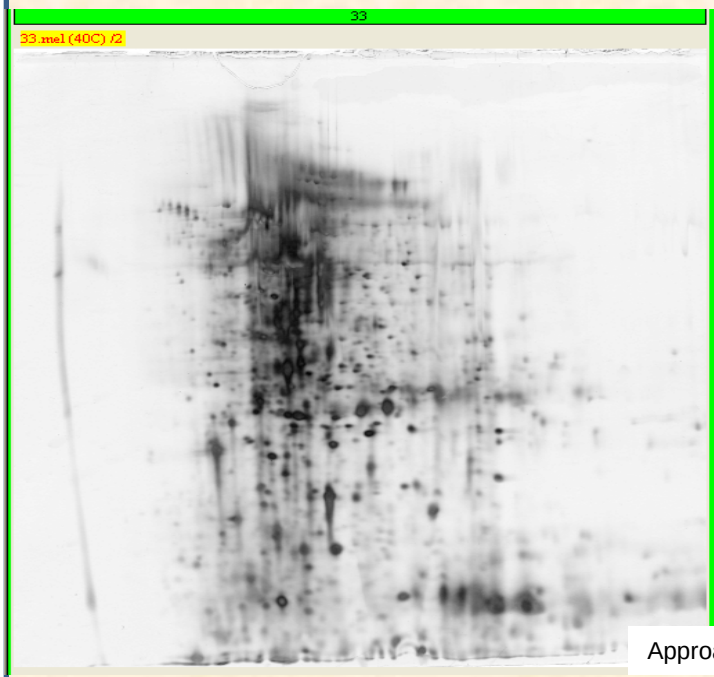
Proteomics FMDx

Examples of separation of the proteins from a tumor and a normal tissue of the patient A.

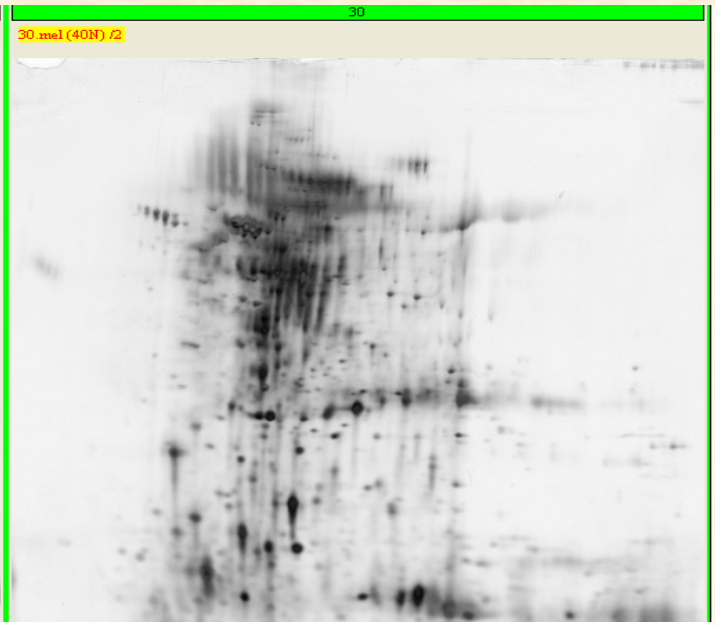
Case A, IDC; 2D gels for this case are shown

Similar proteomics work was performed for the patient B (not shown)

Cancer



"Normal" –(peripheral)



Approach: Histo-pathology, 2D-GE, MALDI-TOF MS, IP, IB, TMA IHC.

Notes about PROTEOMICS-based assays

This page shows an example of separation of proteins from a tumor and from a normal tissue of a patient.

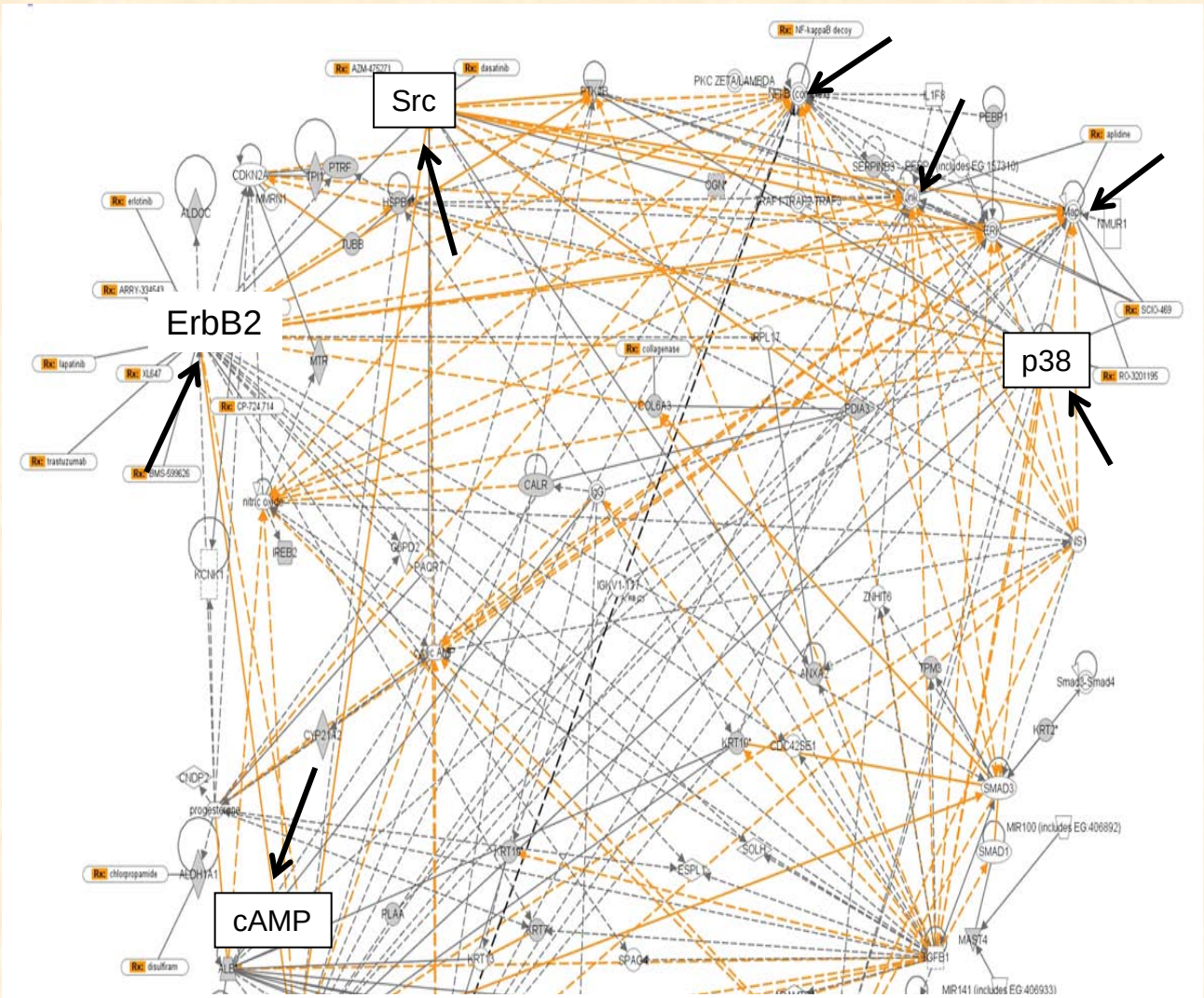
The pages 19 and 20 show examples of networks built to extract information about drugs which may block growth of tumors. The networks were built using proteins identified by proteomics as cancer-related in the patients' tumors. The networks represent relations between the identified proteins, dominating molecular regulatory processes in the tumors, and indicate which drugs may stop proliferation and induce death of the tumor cells. The examples show diagnostics of two patients. Note that the patients have different profiles of drugs.

Validation of Functional Molecular Diagnostics-Proteomics is done by functional assays. The combination of Proteomics FMDx with Functional Assays gives higher confidence to suggestion of treatment, as compared to a single assay.

*Images of 2D gels are from Zakharchenko et al., 2011.

Patient A: cancer-related signature and drugs which act on the proteins

Note differences in the networks for patients A (this page) and B (next page). This indicates individual differences between the patients' tumors.



Suggestion of drugs for this patient
(only few selected drugs are mentioned)

*lapatinib, trastazumab; disulfiram; ruboxistaurin; SCIO-469;

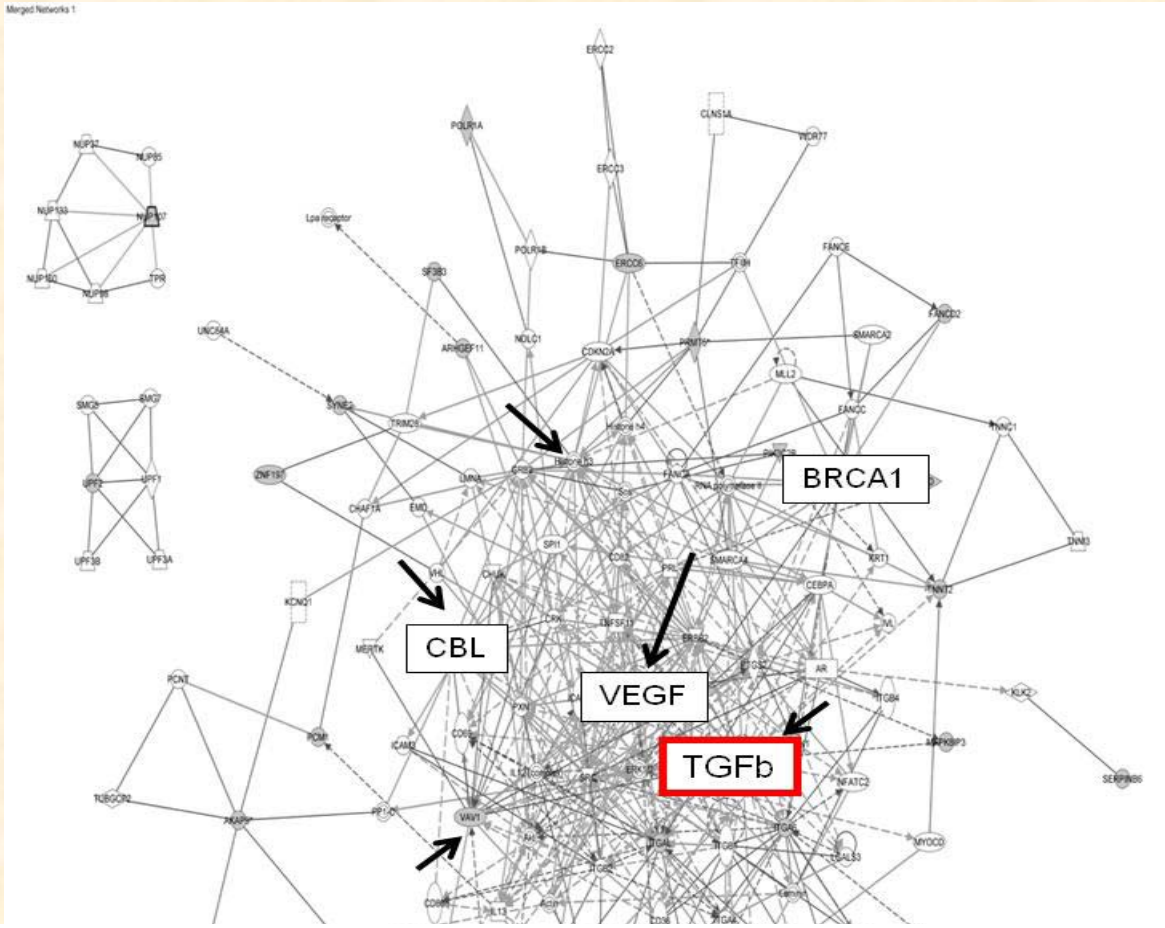
No TGFβ activities, no need of the inhibitors.

**One of the Network representation is shown; we use different complementary SysBio tools*.

*Zakharchenko et al., 2011

Patient B: cancer-related signature and drugs which act on the proteins

Note differences in the networks for patients A (previous page) and B (this page). This indicates individual differences between the patients' tumors**.**



Suggestion of drugs for this patient
(only few selected drugs are mentioned)

*enzastaurin, bevacizumab, erlotinib, pegaptanib, ibuprofen, ustekinumab, diclofenac, trastuzumab,

Over-activation of the TGFβ signaling.

The TGFβ signaling inhibitor may be efficient.

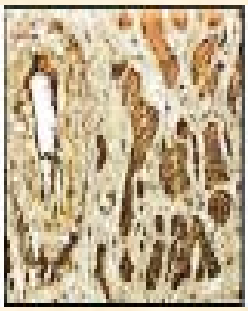
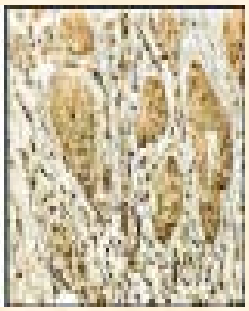
**One of the Network representation is shown; we use different complementary SysBio tools*.

*unpublished data 2012

Validation with functional assays strengthens recommendations to clinicians about selection of treatment.

This is an example of how decision about use of a specific drug was taken.

Four patients' tumors were tested by Proteomics FMDx, by Functional Biochemical assays (IHC is shown here) and by network analysis to answer the question from a clinician "Will TβR-I inhibitor be of help for the patients?"

	Case1	Case 6	Case 45	Case 47
Activity of TGFβ *measured by proteomics*	**Weak** **+**	**Considerable** **+++**	**Weak** **+**	**Considerable** **+++**
IHC pSmad2, *reflects TβR-I activity*	 **+**	 **+++**	 **+++**	 **++**
Shall the drug be used? *Network analysis suggestion of combination of drugs*	**No**	**Yes**	***+Inhibitor +Tmx +Iressa/ Herceptin***	***+Inhibitor +Inflammatory/ +Immune modulation drugs***

*Zakharchenko et al., 2011

Proteomics FMDx

Conclusion for the example of diagnostics to decide about use of the inhibitor of TβR-I kinase, described on the page 21:

Patient #1 will not benefit from SB431542.

Patient #6 will benefit from SB431542.

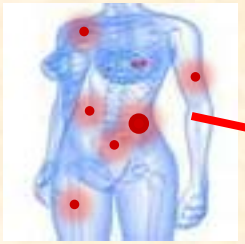
Patient #45 will benefit from SB431542 only if at the same time are applied tamoxifen and Iressa, Lapatinib or Herceptin.

Patient #47 will benefit from SB431542 only if at the same time are applied immunomodulators.

FMDx, Part 2

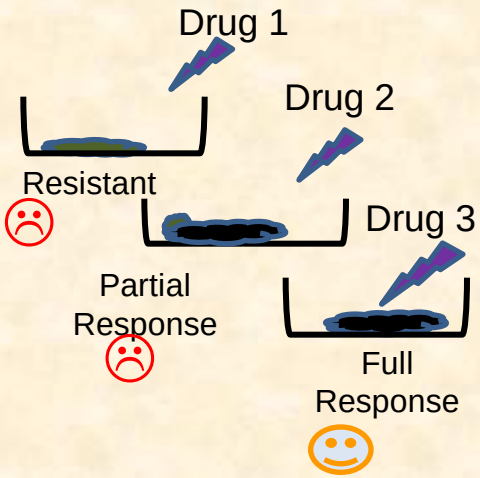
FMDx

Examples of diagnostic with blood obtained from a patient.



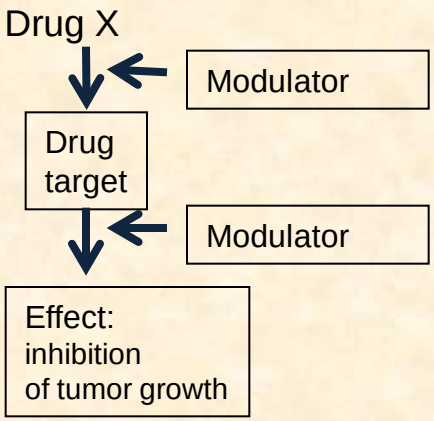
Blood for:
***Vaccine**
***Circulating Tumor Cells**

CTCs prepared for testing with drugs



Selection of the drug which destroys the tumor, e.g. Drug 3.

Functional Biochemical assays with CTCs



Measure activity and expression of target, modulators and markers of effect of drugs, to predict efficacy of the drug.

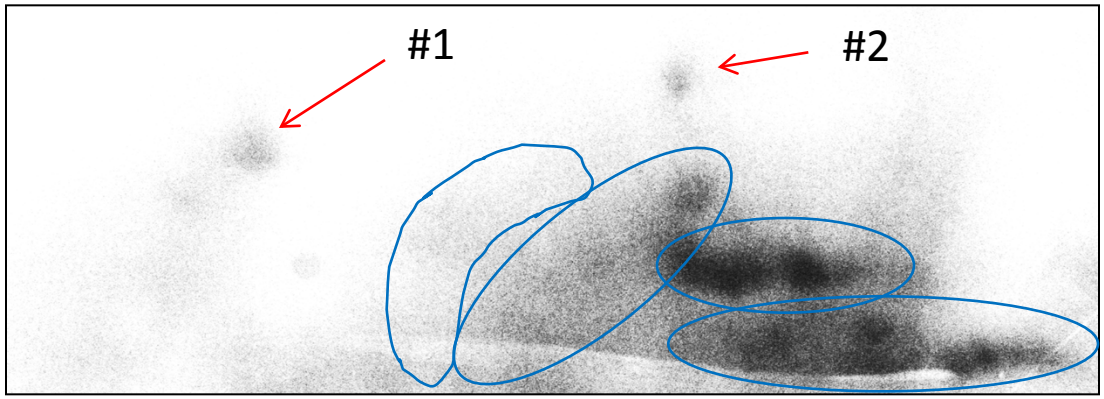
Proteomics
FMDx

Detection of tumor antigens
Production of vaccine

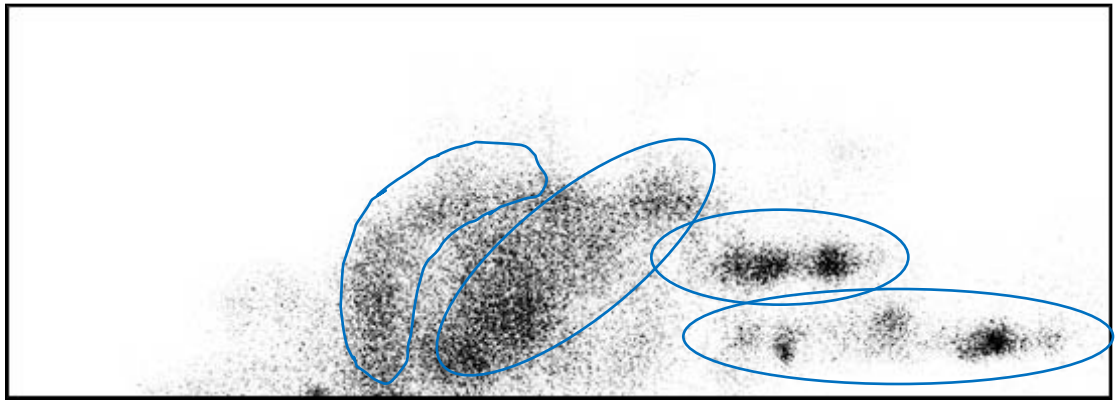
Drug screening and diagnostics by proteomics are done with CTCs

Proteomics FMDx for development of individualized vaccine

Detection of antigens



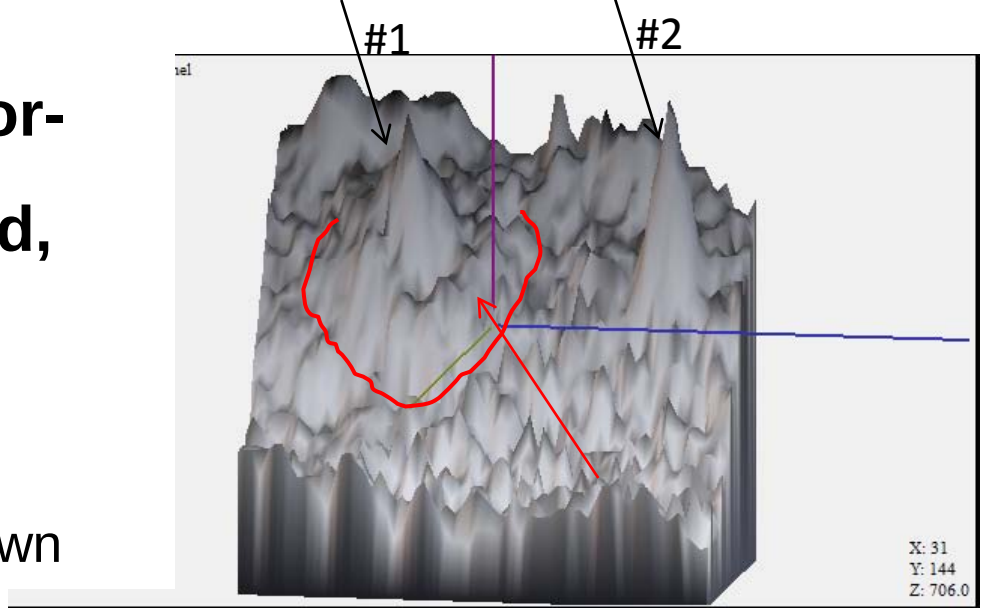
Cancer



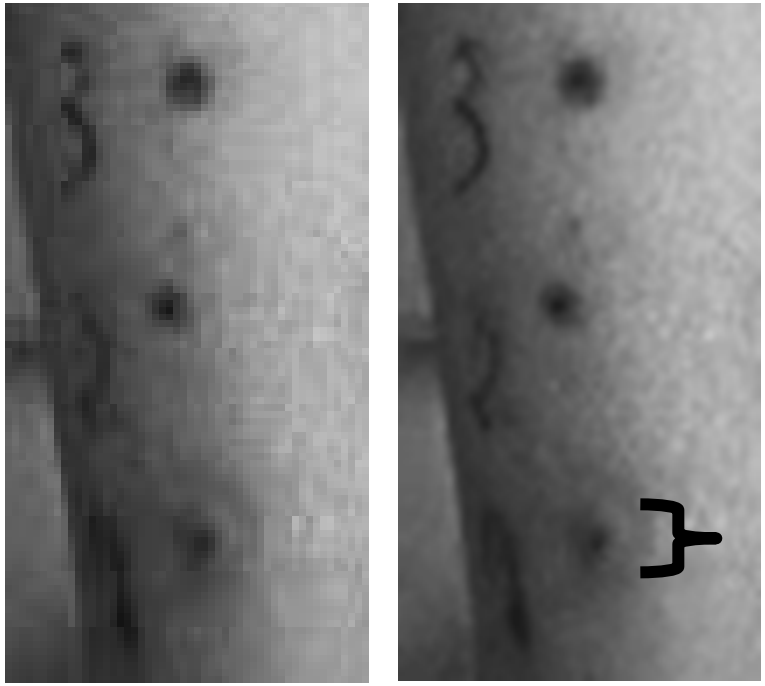
Healthy

Red arrows indicate protein-antigens specific for the given patient, so-called individual tumor antigens.
Blue areas indicate antigens which are common with a healthy person.
Individual tumor antigens (specific only for the patinet) are used for development of personalized vaccine.
Technology used: ZP-tech for intact proteins. An image of the last stage by 2D-GE is shown.

Identified immunogenic tumor-related antigens were purified, and prepared as a vaccine.



The prick test with the vaccine is shown



Red line indicates elevated area (swelling)
#1 and #2 indicate areas of pricking.

Time after pricking is 28 h.

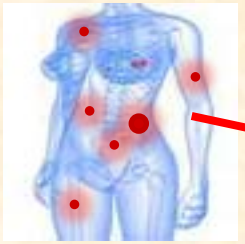
Positive reaction for the #1 (swelling).

Confidential

FMDx, Part 2

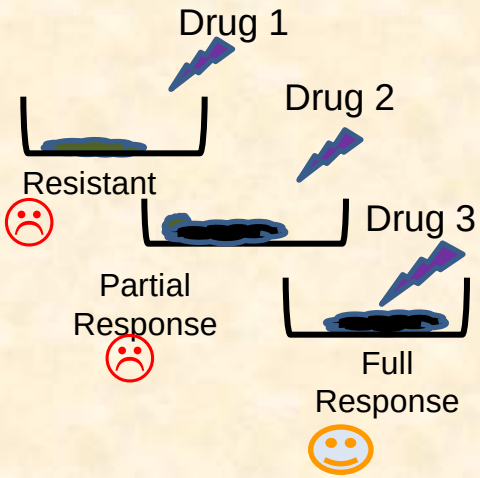
FMDx

Examples of diagnostic with blood obtained from a patient.



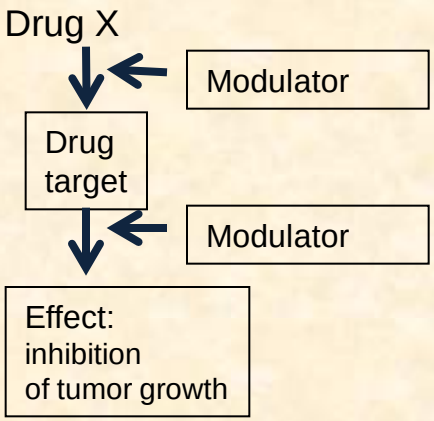
Blood for:
***Vaccine**
***Circulating Tumor Cells**

CTCs prepared for testing with drugs



Selection of the drug which destroys the tumor, e.g. Drug 3.

Functional Biochemical assays with CTCs



Measure activity and expression of target, modulators and markers of effect of drugs, to predict efficacy of the drug.

Proteomics
FMDx

Detection of tumor antigens
Production of vaccine

Drug screening and diagnostics by proteomics are done with CTCs

This reference scale is for comparison only.

Reference scale of aggressiveness as defined by CTCs detection and growth

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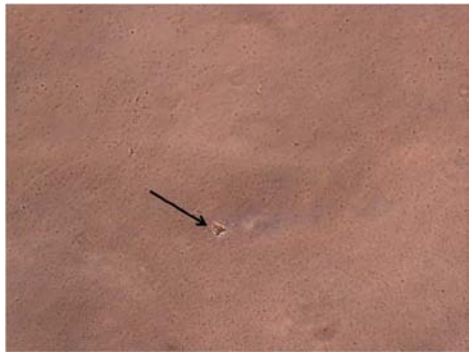
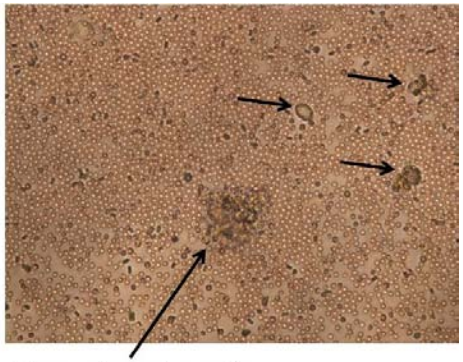
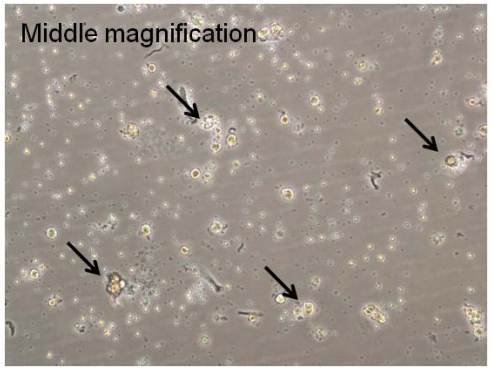
Non-aggressive

Aggressive

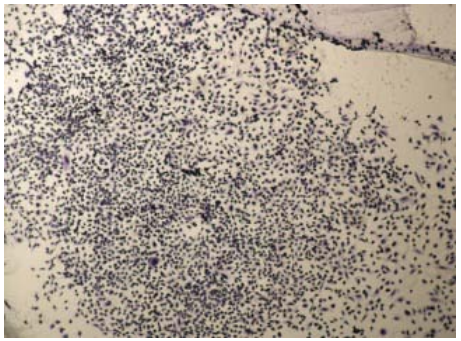


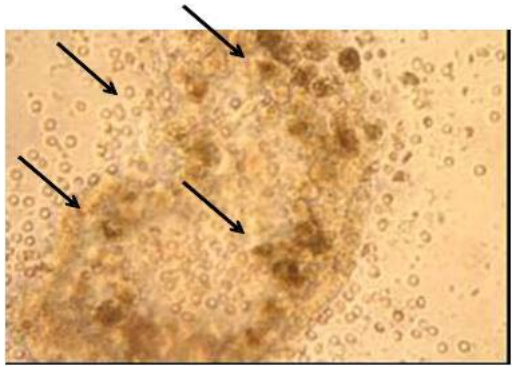
High magnification is used to show single CTC which does not grow.





High and middle magnifications are used to show CTCs with average growth rate.





Low (top) and high (low image) magnification is used to show how fast growing CTCs look and how they form large clusters.

Test of sensitivity to drugs with CTCs. Case 0301TK

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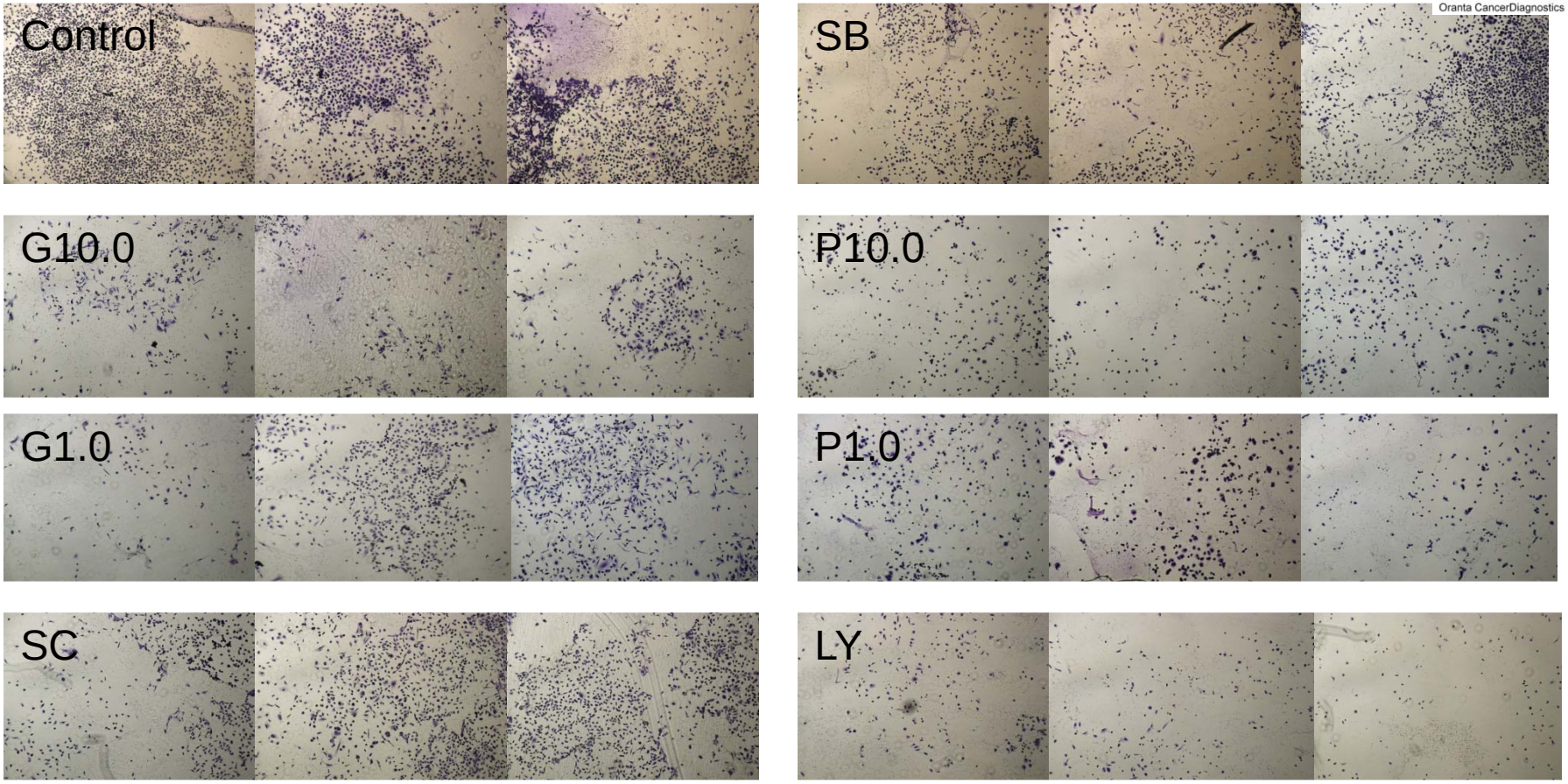


Images of cells which were not treated (Control), or treated with Gemcitabine (G10.0 and G1.0 at two concentrations), Paclitaxel (P10.0 and P1.0, at two concentrations, TbRI inhibitor (SB), PKC inhibitor (SC), and PI3K inhibitor (LY).

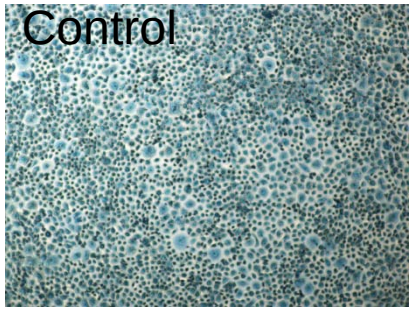
Three images of 3 repeats are shown. Note the cells death as: **LY >> P10.0 > P1.0 > G10.0>SB=G1.0=SC.**

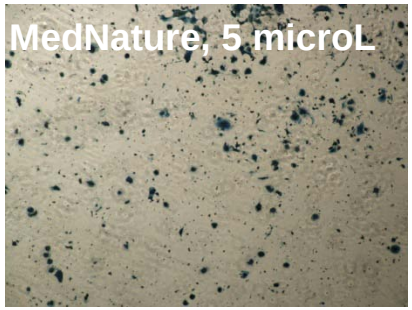
Conclusion: 1) Drugs acting on PI3K/AKT/mTOR may be the first choice to use. There are more than 15 anti-cancer drugs acting on PI3K/AKT/mTOR. 2) Paclitaxel and Gemcitabine may also be used, but unlikely to have a full effect.

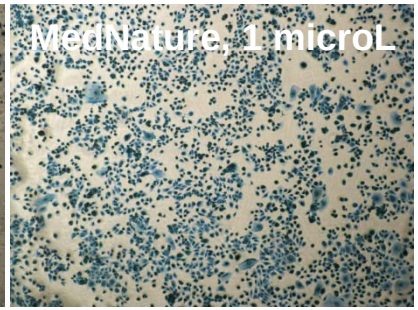
3) Drugs acting on TGFbeta and PKC will have no effect.

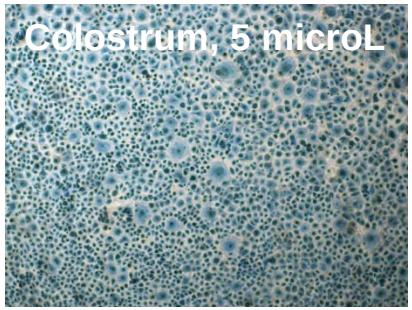
Test of natural products

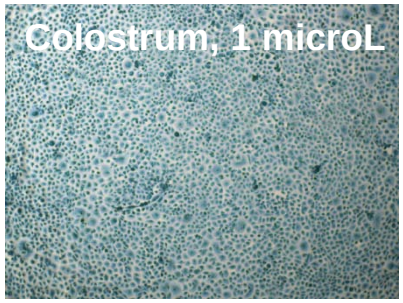
MedNature – good effect; Salvestrol - some effect at high dose; Colostrum and FlorEssence – no effect

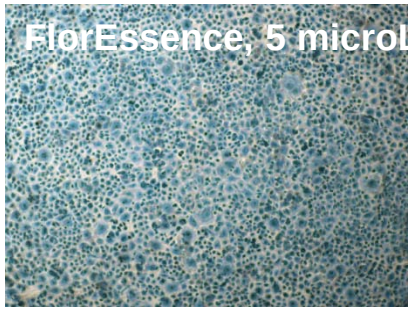


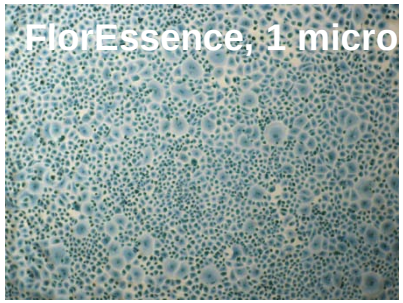


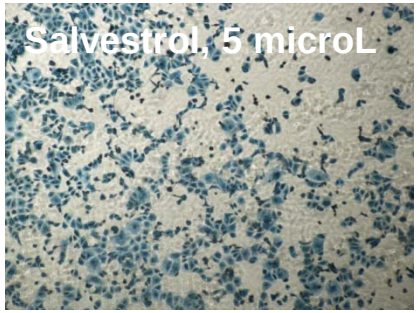












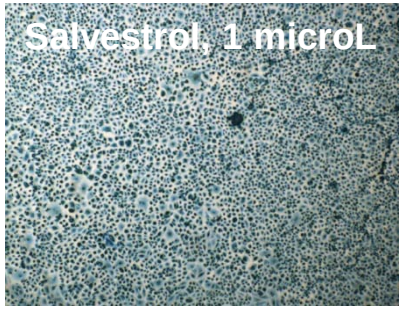
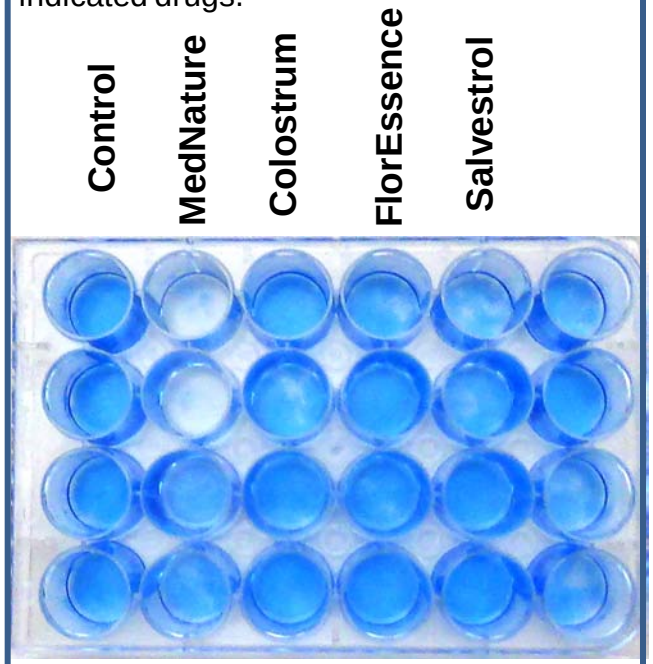


Image of a plate with tumor cells (blue color), non-treated (control) or treated with indicated drugs.

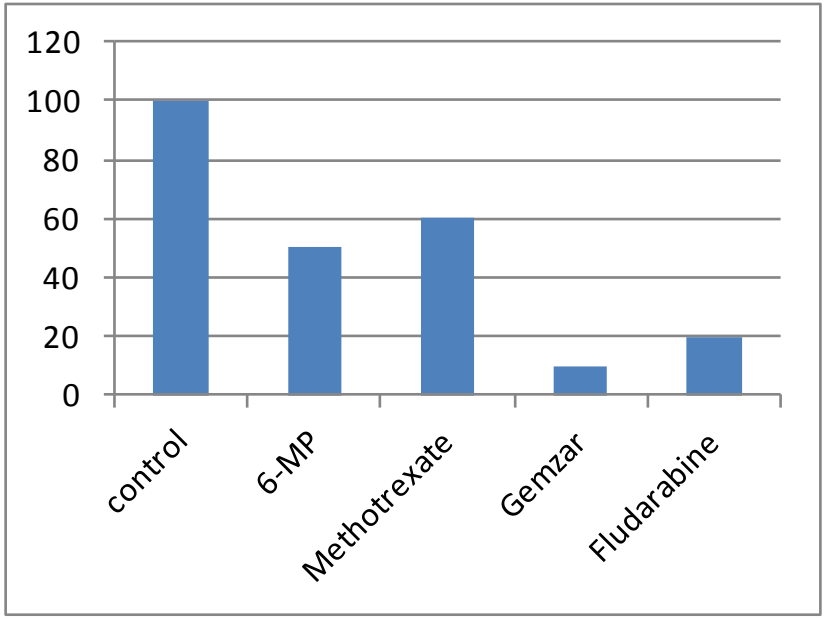


Images of cells are shown. Treatments are indicated. Cells are stained in blue.

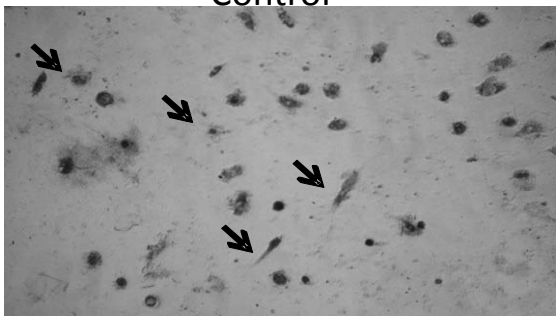
Oranta CancerDiagnostics

Test of sensitivity to drugs with CTCs.

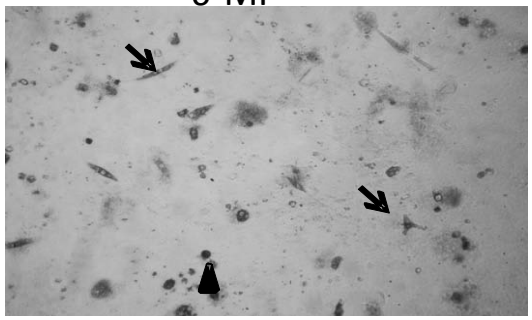
Case 010301



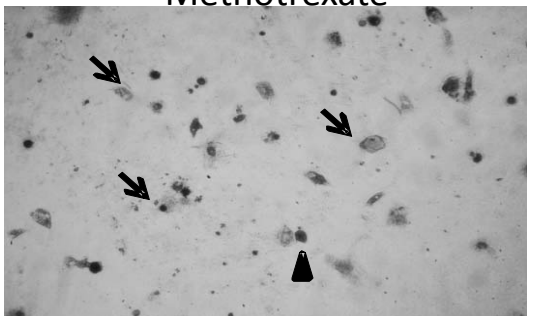
Control



6-MP



Methotrexate

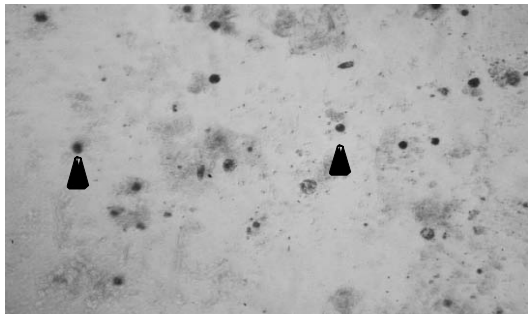


PCD_010303 2013.12.29

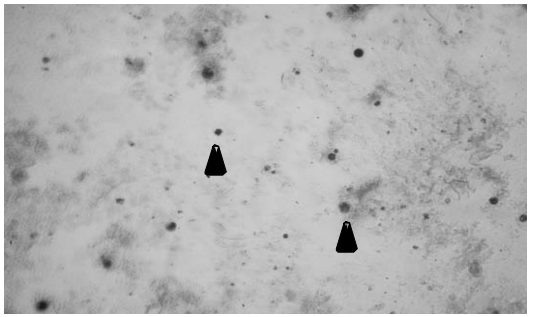
- living cells

- dead cells

Gemcitabine



Fludarabine



-

Combined treatments *(example, case #008).*

G- gemcitabine; P – paclitaxel, control – no treatment . 20 – 1000 mg/m2; 2 - 100 mg/m2, 0.2 – 10 mg/m2.

Comments:

Gemcitabine showed inhibitory effect at both concentrations

Paclitaxel – inhibited, but at lower concentration the effect is weaker

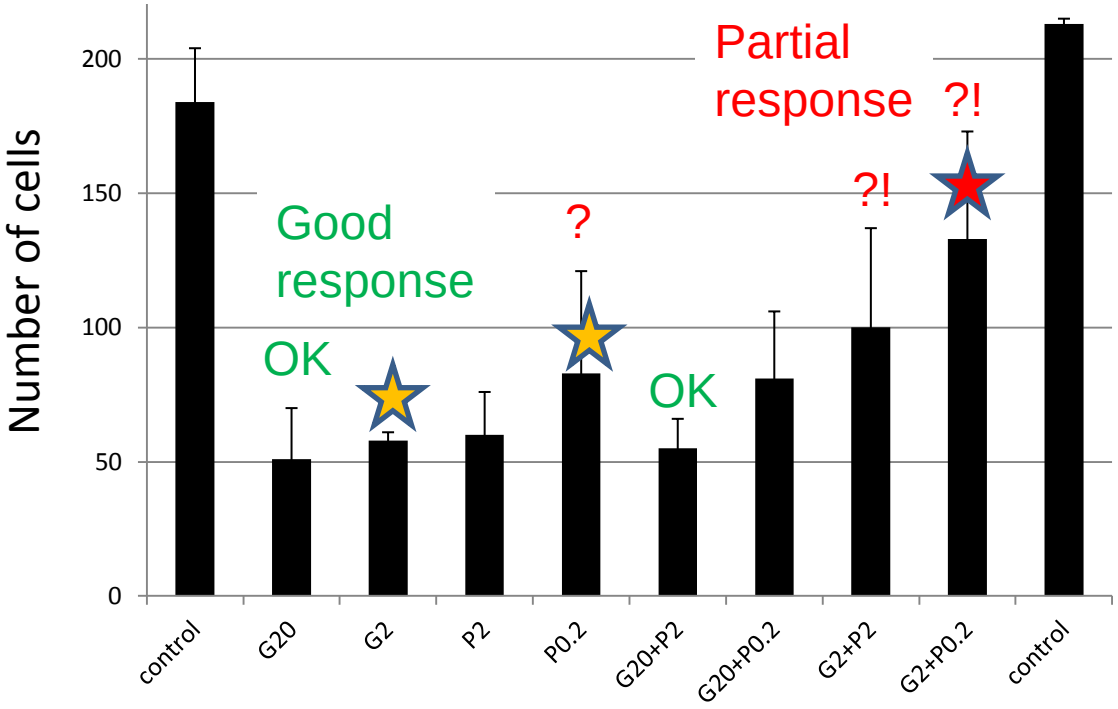
Double treatment - weaker effect at both low concentrations.

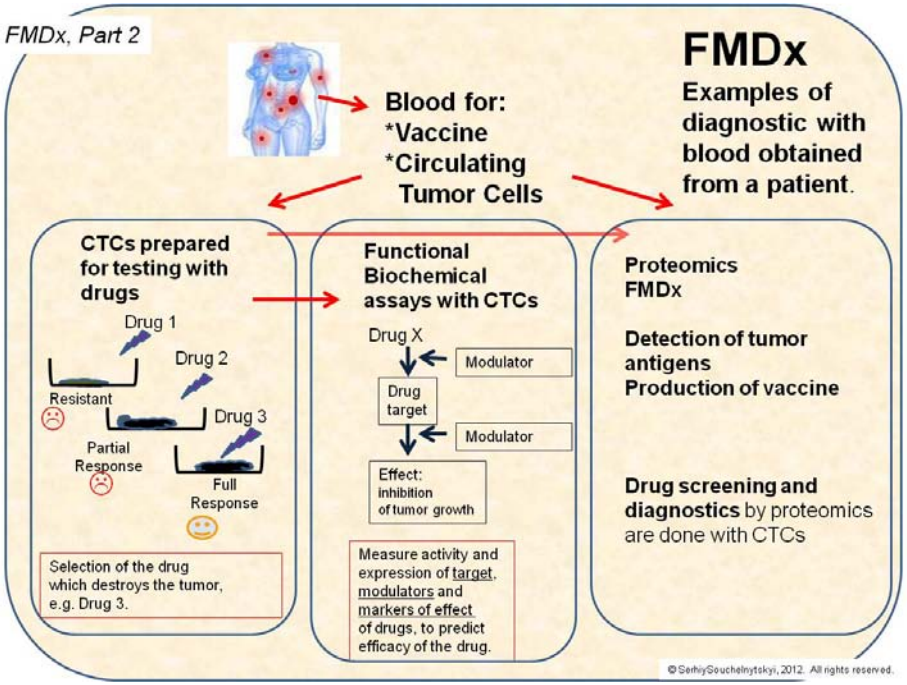
Conclusion about efficiency of the drugs:

gemcitabine (20, 2) > paclitaxel (2) > paclitaxel (0.2) > double treatment (2.0+0.2).

In other words: **use Gemcitabine at 20.0, nab-paclitaxel has to be at 2.0.**

Do not use both at lower doses.





FMDx with CTCs and plasma shows a possibility of individualization of treatment by collecting a blood sample from a patient.

FMDx from point of view of a patient

Functional Molecular Diagnostic for personalization of cancer treatment.

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