GORDANA VUNJAK-NOVAKOVIC: Good morning everyone, and welcome to the first overview talk that will focus on tissue engineering for regenerative medicine, and the needs and opportunities for functional imaging.

As you know, MATES, with all its constituents, is extremely successful in bringing different communities under the same roof. And some of the previous meetings of this kind that were done on a different topic, such as tissue engineering and developmental biology, for example, actually resulted in some really important and helpful initiatives that changed the field in some way.

So we do have this expectation, as Mark was explaining, that tomorrow, at the closing discussion, we will look for the opportunities for existing methods of functional imaging, the need to develop new methods, and we will also look for the mechanisms that would catalyze interactions between the tissue engineering, regenerative medicine and imaging communities.

This will be an introduction on the topics of tissue engineering, and then Ralph will give an introduction on imaging. My introduction is more conceptual, Ralph's is more technical, and we know that these

introductions will be followed by super-high-quality talks that will help us summarize the meeting tomorrow and make a plan on where to go from here.

Let me start with the rationale for tissue engineering. An obvious question is why are we so interested in tissue engineering? I would like to offer you three reasons. One is that we live longer and better than ever before in human history, and, therefore, our aging population needs, like old cars, a lot of maintenance. This is why the biological spare parts that can reestablish function of our failing tissues or organs are more important than ever.

At the same time, tissue engineering fosters the development of the biological and medical research beyond the direct replacement of tissues.

The logic of tissue engineering can be different from lab to lab, from person to person. I would like to propose an approach in which the cells are the real tissue engineers, and what we do is not to engineer tissues, but rather to provide enabling environments for the cells to do their job.

I think we will all agree that the ultimate goal of tissue engineering is to reestablish function. This

is why we do what we do. And there are two components that are very important, and they are the heart of our conference. One is the control of cell environment, which we achieve by controlling scaffolding materials, bioreactors, in vivo environment. The other component is to have insight, in possible in real time, into the biological processes- and this is where imaging comes into the picture.

The interesting and complex aspect of the whole field comes from the fact that cells do not respond to just one single, dominant factor in their environment. They respond to the entire context of the cytokines, tissue matrix, other cells, physical forces which collectively determine the expression of genes, and, therefore, the fate of the cell that can undergo a number of different processes, from proliferation and differentiation to senescence and death. It is important to keep in mind that this process also goes in the other direction. The cells are secreting cytokines, making and breaking their matrix, affecting other cells, and generating forces.

So this brings us to the situation that actually the old, good petri dish is not always the best solution for cell culture. The cells in a culture plate are attached with one-half of their surface to the

substrate and bathing the other half of their surface in culture medium, a situation very different from the complex three-dimensional environment they experience in vivo.

This is why we do need something more in-vivo like, more biomimetic. And this is how we do it. The tissue engineering is all about designing and using cell-instructive environments that will subject the cells to the milieu of factors they would normally experience in vivo, under normal or pathological conditions.

And the overall concept is very simple. There are just three components, the most important one being the cells themselves. The other component is the scaffold, which is the structural and logistic template for the cells, defining the cell responses, as well as the shape and overall structure of the tissue that we are building. And then we have a bioreactor acting as a substitute body, and providing environmental control and time and space sequences of the molecular factors and physical forces.

From here on, you can go in two different directions. One is the classical tissue-engineering approach to regeneration. So we make tissue grafts or

combine cells with biomaterials or use biomaterials alone. There are major needs and opportunities to repair tissues. And the other direction is a sort of reverse paradigm, when we make pieces of three-dimensional tissues that they're small enough to be studied in high throughput and almost look two dimensional from the practical point of view, but they are three-dimensional from the cell perspective. We build these platforms for studies of development and disease and drug screening. And these platforms can become very complex, if they are designed to include tissue-to-tissue, organ-to-organ or tissue-to-immune-system interactions. We can now build human-in-a-dish systems that can aid to the biological relevance of our studies.

The instruction to the cells also extends to in vivo. This is a schematic exemplifying that you can go towards regeneration by combining cells with scaffolding materials and implanting. You can also take human cells from a variety of tissues and make IPS cells, and then use these cells to study a number of clinical conditions, for example, to study neurological disease and test protective drugs or to study heart disease and look into the drugs that can correct conduction defects.

I use two sets of examples that are from our lab, to illustrate what are some of the directions of tissue engineering and to explain some of the imaging needs.

An approach you can take to regenerating the terminally failed heart function - for example after myocardial infarction, is to engineer a cardiac patch. This is a set of old data that generated all this promise and potential for cardiac-tissue engineering to build a functional patch starting from the associated cells and the scaffolding material.

We learned along the road that cells have an amazing capability to assemble themselves into functional tissue structures if you give them just a few essential cues that they need, so you don’t need to do too much. And this ability of the cells to use some of the cues you give them and do the rest by themselves is the basis for many of the successes of tissue engineering.

We also learned that physical signaling is extremely important, as the implementation of electrical and mechanical signals made the whole difference. The cells that were subjected to biophysical stimulation would have much higher beating amplitudes, better conduction of the signals and yield much better tissue

structures than the cells that were not stimulated during the culture. And you can see in some of these examples that the ultrastructure and the expression of markers in engineered tissue could be made very close to what you see in the native tissue.

You can also take a different approach and use a biomaterial as a delivery platform for the cells. What we have done here was to take the native tissue matrix, which is probably an ideal scaffold for the cells because it has the right composition and architecture and right mechanical properties. And then we loaded this matrix with the cells, immunoselected adult stem cells in this case, that were suspended in a native occurring hydrogel fibrin, and this hybrid construct consisting of cells in scaffold was then implanted onto the infarct bed.

One imaging modality that was used in these and similar studies very extensively is ultrasound. Ultrasound can help you determine what is the pumping capacity of the heart, by determining the standard physiological factors and comparing different groups.

What you see here is the standard comparison between the normal heart and untreated infarcts, serving as controls, with the implantation of cells alone and

implantation of engineered patch. We can see that the engineered cardiac patch is the closest to the normal healthy heart muscle.

When looking into what is underlying these processes, what we normally do is to take histologies that you see here. You can see the implant, the infarct and a line between them for the implantation of the acellular scaffold, cell-seeded scaffold and scaffold with the cells that were conditioned during culture. one can see clearly that the cell conditioning made a major difference in terms of the number and strength of the blood vessels that were formed in the infarct. And we can also see something very interesting - that the stem cells are migrating from the implant into the infarct bed, where they are most needed.

It would be really helpful to monitor these processes in real time because the usual scenario, as you know, is that you sacrifice animals, take these samples at timed intervals, and wait for several weeks until your histology is done. Equally important, samples taken at different time points are actually from different animals, reducing the consistency of collected data and increasing data scattering.

Based on the results of this study, we looked into cardio-protective capabilities of these cells, and to make this possible implemented a real-time imaging modality. Apoptose was studied as a measure of survival of cardiomyocytes using an in-vitro model in which the cardio construct was overlaid with a patch with stem cells in a hybrid scaffold. The cardiac construct was cultured under hypoxic conditions to mimic the infarct situation, and the cell patch was the same as the one we previously implanted in mice.

And we could see that the conditioned cells had capability to reduce quite substantially the apoptosis incardiomyocytes both immediately and over seven days of culture under hypoxic conditions as compared to the cardiac constructs cultured alone without the patch.

We investigated the same cardioprotection method in live animals, and it was interesting to see that the implantation of cell patch onto the heart resulted in an initial apoptotic situation that was resolved over time. The reduced apoptosis correlated with the decrease in the size of the infarct over the time of the study and with the uptake of the tracer as compared to the group with no patch in which apoptosis remained pretty much the same.The imaging data

correlated very well with the classical histological (Caspase) assay for cell apoptosis.

For cardiac patch, we are always trying to control the cell environment and to obtain as many functional readouts as we can. To this end, we are developing a variety of bioreactor systems. One of the most used ones in our lab, and now disseminated to many other labs, is what we call a “plug-and-play” bioreactor, because it can combine any set of signals and controlling factors that you want to implement, such as electrical stimulation, stretch, or time-change of molecular factors in the culture medium. So this is a pretty flexible system that allows us to both control and monitor the processes in our cardiac patch.

Using this system, we can compare, for example, the effects of electrical stimulation on the expression of cardiac markers that are associated with the function of sodium and potassium channels. We tried to correlate these readouts with the calcium signals that we measure using calcium florescent dyes to evaluate communication between the cells.

We also look into the propagation of electric signals that we measure using microelectrodes, to determine the direction and amplitude of the conduction

velocity. Finally, we measure the development of mechanicalforce, as the ultimate readouts for conatruct functionality. We used the plug-and-play bioreactor also to look into the possibility ofelectrical conditioning of the immature human cardiomyocytes derived from embryonic or IPS cells.

Most of the heart development in the human body occurs in the presence of electrical signals, and the heart is in fac, the first functional organ in the human body, and it starts to beat only three weeks into gestation. Therefore, for most of the development, heart cells are subjected to electric signals.

What you normally see when you derive human cardiomyocytes by staged molecular induction from stem cells of one or the other kind are these relatively non-uniform spontaneous beating profiles, where each small construct beats at its own amplitude and frequency. After only seven days of stimulation of these constructs with, in this case, the frequency of 2Hz and using signals that are similar to those in the heart, y a significant amount of synchronization can be achieved. We are now looking into how to use this feature to make better cardiac constructs.

Another area of great interest are the interactions of the engineered patch or implanted cells with the host environment, which is everything but neutral. In the best case, you have a relatively healthy organism with a cardiac infarct, and then the patch with its cells and matrix is placed over the infarct to drive the repair. In more complex situations and actually in most cases in real life, you have a patient that doesn’t only have heart disease but also has diabetes or some other systemic disease. In this case, we would see a milieu with cascades of macrophages that start from neutrophils which turn into monocytes and then they polarize, first into M1s and then into M2s.

The last and latest in the field is that there is a continuum of transients between M1 macrophages that are considered to be inflammatory and M2 macrophages that are considered to be pro-healing, and it is still not entirely clear how much they can change their phenotype going from one to another.

We are trying to look into these interactions, and because the in-vivo situation is so unbelievably complex, we are trying to use the in vitro platforms we developed to study the interactions between cardiomyocytes (at differet levels of maturity) with macrophages (at different stages of polarization). So

we look into the effects of diffusing factors, cytokines created by microphages, how they affect cardiomyocytes, how cardiomyocytes affect secretion of cytokines by macrophages, and how all these cells affect each other in direct contac, within a practically unlimited spectrum of possibilities. The question that we are trying to address is how this interaction is changing with the maturation stage of human cardiomyocytes because you can, in principle, implant them at a more or less mature stage.

The studies *in vitro* are correlated with the experiments in-vivo, and this is where labeling of the cells, to distinguishing the macrophages from the cardiac cells, for example by using a regular mouse and a GFP mouse, is most helpful. In parallel, many useful reporter cell lines are being developed for in-vitro studies.

These were just a few examples from the cardiac-tissue engineering area and how imaging can help us to get a better insight into what’s happening at various hierarchical scales.

I would like to give you a similar set of examples from another area and in a slightly different context. This is the area of burn-tissue engineering where I

will emphasize translational studies that are being done to come closer to the clinical application of some of the tissue-engineering modalities that are being investigated.

Basically, the experimental space is in this case defined by two different scaffolding materials. We're using the native-bone as we used the native-heart matrix as a scaffolding material and a learning tool about how to design a synthetic bone scaffold. And then we use a number of protein mineral scaffolds, such as mineralized silk fibroin scaffolds that we obtain in cooperation with David Kaplan from Boston, who will speak here tomorrow.

The osteogenic cells - mesenchymal stem cells derived from bone marrow or adipose tissue aspirates or embryonic-like stem cells - are seeded into the scaffolds and cultured in bioreactors. Ideally, the scaffold needs to have the architecture and mechanical stiffness that are very close to those in the native-bone, and the bioreactor, among other things, needs to have perfusion, that is an interstitial flow through the cultured scaffold because the diffusional penetration depth of oxygen in bone tissue is less than a 100 micrometers. To grow a sizeable piece of

bone, you absolutely need to have contact of the medium with all cells within the scaffold.

Perfusion is just one of the parameters that we have studied. We also looked into how the amount of mineral in the silk scaffold that we have at the beginning is affecting the formation of bone. What you see here is a microcomputer tomography image of the scaffold that contains no mineral, and several different initial concentrations of mineral.

As we see from these microcomputed tomographies, both after five and after ten weeks of cultivation, the more mineral was in the scaffold in the beginning, the better bone development there was at the end. Also, the bone grown using scaffolds rich in mineral had markedly better mechanical properties compared to the bone grown using the scaffold without mineral. A more complex question is what is the basis for these effects, because mineral is serving two different purposes. One is that mineral is osteogenic by itself, and all bone-tissue engineering studies that we do are done without any growth factors. In particular, we do not use BMPs, mineral itself is sufficient to promote bone formation.

The other effect of the mineral is that the scaffold becomes stronger, closer to bone-like stiffness, and, as we know that cells are sensing their scaffold, this itself can promote bone formation.

Microcomputed imaging is one of the modalities that we can do longitudinally, I'll show you later how exactly we do this. A huge advantage of this is that we can look into the same sample over and over and follow bone formation over time. And then if you see something interesting happening you can still harvest your sample and do additional end-point analytics.

This is the same engineered bone in animals, in the cranial repair and femur repair models. And what we saw in these studies is that engineered bone, bone that was already preformed to some extent, had much higher capability to heal the defect than either cells on scaffold, scaffold alone or untreated defect.

We move from there to cultivation of bone that can have clinical utility. So it is encouraging to heal a mouse skull, but size matters, and these studies cannot be extrapolated to clinical scale.

We focused on one of the most complex bone repair tasks - regeneration of anatomically-shaped bones in

the head and face. And the rationale was that this region is where the regeneration and precision that we can achieve -- regeneration matters the most, because face is what makes us different from each other, and these are the features we really want to re-establish in case of trauma, surgery or any other reason that requires repair.

So the approach is relatively straightforward. Our first graft was the TMJ, temporomandibular joint, the end portion of your lower jaw, and the only loaded joint in our head. We would use these digitized images to make a scaffold in the exact shape of the defect being repaired, and we can do it with 10 micrometer precision. And we would use the same images to make a matching bioreactor chamber, which is enclosing the scaffold and preventing fluid to flow anywhere else except through the scaffold. We would then load the cells, and put the system under perfusion, for 3-5 weeks. This is a photo of one of the bone grafts that were grown this way.

A big portion of the project was the development of the bioreactor with an anatomically-shaped chamber. You can see the bone here, this yellowish part, and this inner chamber will obviously be different from one graft to another.

This is a microcomputed tomography image of the resulting bone in which you see the mineralized matrix. You don't see the cells or anything else. Here is one of the bone-protein stains, osteopontin, but you see pretty much the same situation with other stains. The light color is the scaffold, and the darker color is the matrix staining for osteopontin.

Under certain conditions, we were able to achieve vascularization. For bone, vasculature comes first, and then it serves as a template for bone development. Clearly, this is a very complicated situation. And,if we can image the mineralized matrix, which we can, and if we could image vascular flow in the bone, we would be able to optimize the conditions for bone formation.

This is a large-animal study that we just finished about a month ago that is testing the exact same thing concept I described. You start from images. You make grafts. This is the cheekbone or zygoma and this is a TMJ. You take cells from the pig in the autologous fashion. We used mesenchymal stem cells from lipoaspirates. Then you culture the grafts, and implant them back into the pig for six months.

To do this study, we decided to redesign our bioreactor to fit into the μCT, which makes a world of difference, as it gives us ability to image in real time. This is now a more elegant and elongated bioreactor. Without disconnecting anything, with the tubing long enough to reach, one can do imaging and continue the culture. This approach enables longitudinal images of each of the individual grafts.

For this particular project mathematical modeling was absolutely necessary to define the flow paths through these large and anisotropic scaffolds and to achieve uniform perfusion of fluid through these large grafts that were fully viable and had physiologic cell density, and this was only possible with profusion.

What you see here is the pig jaw, the part that we were replacing. The reddish graft is the one that was explanted from the pig and the white graft is the one that we made based on images. So you see that they are very close to each other. And by comparing the other set of the explant and engineered graft, you can see the great diversity of shapes that you also see in human. We don’t all have the same TMJ, so off-the-shelf approach is not exactly feasible. For each pig, we would make a separate customized graft and use the cells from this pig for implantation.

The most interesting interim result is that the resorption was much faster than bone formation for acellular scaffolds, and that the midpoint -- a three-month data point – showed an empty area that was filled with fibrous mass. There was no bone, perhaps because there was not enough power to build bone at the rate of scaffold degradation. In contrast, the implantation of engineer graft remodeled really nicely and we are now looking into final six-month data.

So let me now just go super quickly through the third part, which covers microscale platforms. So what you see here is bioreactor which is the size of a microscope slide with lots of individual culture chambers and you can put cells in a scaffolding material into any of these chambers. And by flow of fluid through these side channels, you can establish a gradient of a molecular factor across the culture space from one well to the other.

And you can also establish multiple gradients so you can have one factor being high on one end and the other factor being high on the other end, such as superimposed promoter and inhibitor.

In this case, these are some human IPS cells undergoing cardiac differentiation, and we were looking into the effects of Wnt and the cells had GFP label on Wnt promoter. So if you see them expressing GFP then the β-cathenin pathway is being activated. And you can see difference from well to well to well in the intensity of GFP. We did a lot of dye studies to figure out the exact levels of the factors in the individual wells.

This is how the system looks - you can change the number of wells, you can change the depth. There is a lot of freedom how you can build it. You can also divide it into segments depending what your experimental scenario is. You can change gradients by simply changing the width of the channel, and have a gradient tha is very, very steep or gradient that's very, very high. So it's actually easy to change these factors.

In this study, we are trying to look into some of the factors defining the development of primitive streak preceding the cardiac development. So we are looking into the Wnt, activin and BMP – factors that are driving this process in vivo by a combination of gradients, and we establishing these gradients in the platform and looking into the cell responses. The

effects of these factors are summarized here. This system that allows you to study different combinations of the factors affecting the same cell-culture space, and also to look into the polarization of the cells in response to gradients.

In another experiment, we looked into the pluripotency vs differentiation of cells that have a Nanog promoter attached, again in a gradient space of LIF, Dox and retinoic acid. We used one of the very nice imaging options that is compatible with this small platform – Fluidigm that allowed us to study just a few cells at a time. We studied groups of 5-10 cells that are small enough to do mapping of the gene expression across the cell-culture space.

So let me now just use this few minutes that I have left to discuss some of the needs and some of the challenges that we are facing.

In summary, I would like to restate that our job is really to design the environment so the cells can do engineering. I would like to argue that biomimetic or in-vivo-like environments are really critical to get realistic responses of the cells, in fundamental biology research as well as in clinical studies.

Scaffolds and bioreactors, alone or in combinations, can be used to “instruct” the cells to make functional tissue constructs or to undergo disease processes.

Interdisciplinary work and young talent are, I believe, the main drivers of advances in the field. As much has been achieved, many more questions remain, and these are some of the needs that I believe we are facing. We need imaging that is capable of capturing phenomena at all levels of scale - from molecules to organs. Biological processes are dynamic, so we also need to capture the changes in space and time.

It would be ideal if we could use real-time imaging and see things as they happen, by longitudinal imaging, which is revisiting the same sample over and over, which gives us consistency, saves on the number of samples and increases the quality of the outputs.

In many cases, we just need to visualize cells and extracellular matrix, but we need to do it over long periods of time. We are struggling with the longevity of our labels – in an animal model, the label works great on day one, two and three, but after nine days, it’s all gone.

We look into matrix composition. For example, collagens change, and looking into features of the matrix using imaging modalities would be extremely helpful, as well as getting insights into architecture of the matrix and its isotropy. Most tissues are anisotropic, both structurally and mechanically.

Functional readouts are most important, from the expression of genes that are activating certain pathways to physiological responses such as signal propagation, generation of force, or establishment of vascular flow.

This is my list of the main challenges at this point. So what are the things that we are trying to approach in tissue engineering to come to the next level?

We still don’t have an ideal source of human cells. Studies that can help us derive and characterize repair cells with respect to the application we are targeting are still ongoing.

Function, how to achieve and how to evaluate function, a requirement that changes from tissue to tissue and patient to patient.

Storage and transport of living tissues. The bioreactor that we built for bone was also built as a storage and transport device, and we were flying across the country living bone samples while they were being perfused. Sharing tissues and samples between the labs through the development of such technologies is very important, I believe.

Vascularization is a universal problem of all tissue engineering, with the blood flow of the host.

Building complex tissues consisting of more than one compartment, more than one cell type, and having functional interfaces between the tissues is a big challenge.

Anoter challenge is how to correlate various types of data - molecular and functional, cellular and organ-level. How do we integrate this? Correlations and modeling that are biologically sound and based on an understanding of the system are really important.

And I would like to argue that medicine is moving towards a personalized approach. What’s good for one person is not necessarily good for another, and we do need to take into account the host environment.

And I would like to argue that we also need imaging modalities that not only monitor but also can intervene. They were great studies, for example, in the area of drug delivery where ultrasound or another source of energy would be used to release a factor from the matrix.

Then, how much function we need immediately following implantation? This will vary from tissue to tissue and there is no standard established. Do we need anisotropy or shall we leave it to the body to finish the job? I believe that integration and remodeling are really important. So how much function is enough? How much tissue formation do we need to achieve in vitro before going into the body? Can we induce regeneration in the body and to which extent? And what kind of a real-time imaging we can count on?

Animal models. They are really complex, and the size matters – for many tissues, you cannot study human on a mouse model, a human-like physiology is needed Do we study phenomena in autologous models which we in most cases do in large animals? Then you face the problems of the lack of markers.

And, finally, the host environment. We do need dynamic imaging of cells and their function following implantation if we are going to pursue our ultimate goal, which is to harness inflammatory response to work for us.

And let me just summarize quickly. We have three large areas of impact with tissue engineering work -- regenerative medicine, fundamental biological research and study of disease. And in each of these areas are some grand challenges that we are now facing.

With the personalized approach to regenerative medicine, the imaging modalities that are of greatest interest are those capable of tracking of the cells, tracking of the matrix, scaffold degradation, vascularization and function on the tissue level.

For advanced biological research, we are building cell “niches”, in most cases in a high throughput fashion and in a dynamic way – “perturb and observe” experiments are among the most interesting ones. In this case, imaging modalities with ability for tracking multiple cell types and measuring function at the molecular, cellular and tissue levels are most important.

And, finally, for the human tissue platforms developed for studying disease and screening drugs in the context of the whole body physiology, imaging modalities for measuring viability, metabolism, electrophysiological and mechanical function are most important.

Thank you very much, and I will be happy to answer any questions. [Applause].

MALE SPEAKER: If you have questions, please step up to the microphone. I have one. I guess I should step up to the microphone, too.

Gordana, on the plug-and-play platform that you talked about you had an image where you had troponin plus and minus electrostimulation. Could you flip back to that? I don't know how long that will take, but I noticed there was a really striking difference in this for images. And, clearly, anyone who's trained and knows what to look for sees that there's something there.

So my question is was there any data or was there any image analysis done on this? I mean, it seems to me that you might learn about spatial patterning. So -- Yes.

GORDANA VUNJAK-NOVAKOVIC: Yes, we have done a lot. I can show you or others offline.

We have also done extensive gene arrays, there is a lot of data. Basically, this study showed us, like many other studies, that the development of cell and tissue features goes in parallel. For example, the conditions that gave us more troponin also gave us better conduction behavior. And you can see that the two stains are superimposed and significantly stronger in the group that was electrically stimulated.

The parallel development of these features rationalizes, to some extent, the selection of one dominant marker to follow, because if we studied troponin only in this group, we would still be able to select the conditions of interest on line and then do additional endpoint analytics to capture detailed functional properties.

MALE SPEAKER: So what role did the image analysis in these sorts of images play?

GORDANA VUNJAK-NOVAKOVIC: In this study we mostly relied on endpoint processing. We would love to see these changes online, but we cannot at this point.

Imaging played only a role in post processing of the data and looking into the levels and distributions of these markers.

FEMALE SPEAKER: Very nice, Gordana. So I was struck by the comment that the imaging should not only provide monitoring, but means for intervention. And I think this is very true. I mean, I very much agree with it. So my question is with your bioreactors that you're developing is there capability for the feedback, like if you are seeing something that's happening with the tissue and you want to change it midstream is it possible to change the environment, go back?

GORDANA VUNJAK-NOVAKOVIC: We can change lots of things, and we are not seriously limited by technology at this point, but rather our understanding what exactly to do. There is a lot of freedom on how to build a bioreactor - you can make it ultrasound or MRI compatible. I would be interested to see which modalities may be available at this point to intervene and to change the course of the tissue development. It is really between the tissue culture and the imaging where interesting things may happen, and we should try to take an active role in using imaging beyond "just" monitoring of the phenomena