Introduction to Developmental Biology

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Lecture-09

Genetic basis (Part 3 of 5)

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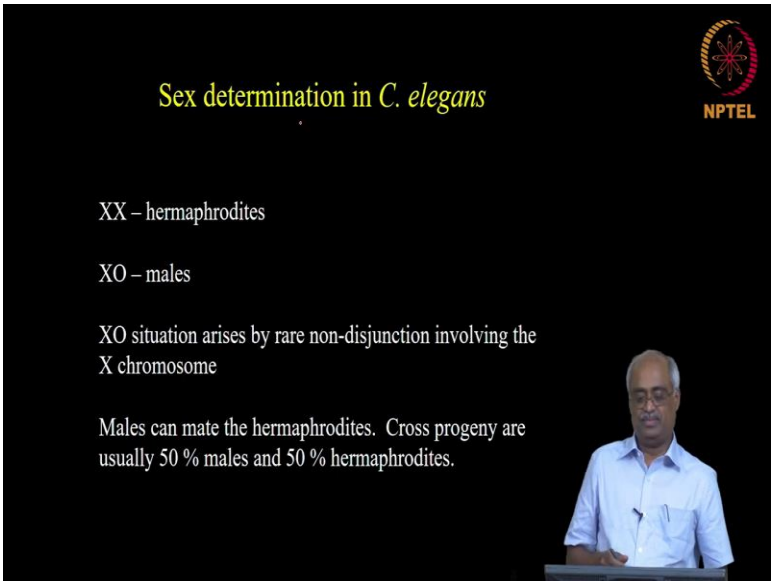

So, in the last class we just began getting familiar with *C. elegans* as a model. We stopped at this slide like sex determination is done by XX means hermaphroditic X0 is male. So, we also saw the advantage of this type of sex determination. Because it is easy to set up a cross as well as a self- cross, self-cross is simply cloning a hermaphrodite. So, one of the big advantages of that is bringing recessive alleles to homozygosity and therefore seeing the phenotype is relatively straightforward. If you pick hermaphrodites from a mating cross and clone them and one-fourth of their progeny will be homozygous so right away you will be able to see the phenotype. So,

that is one of the big advantages of this system. So, let us move on so how to make use of this to discover new genes.

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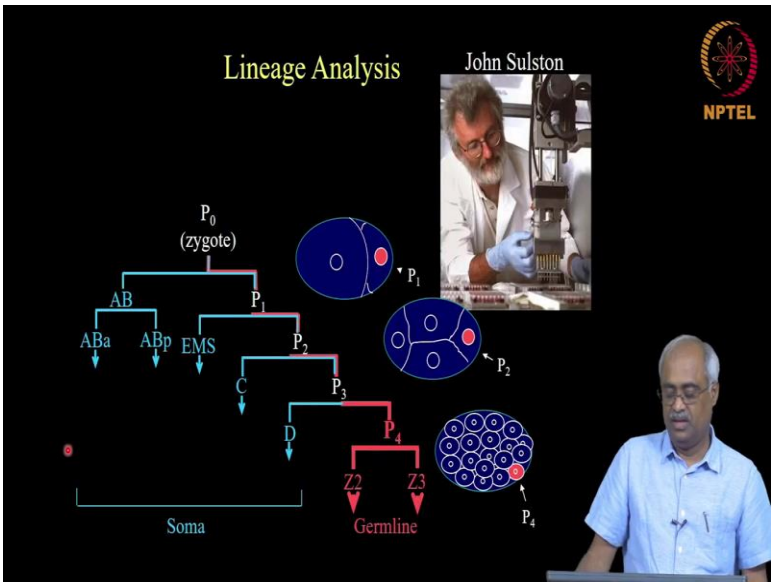

So, we will see one more set of experiments. We have already learned about fate mapping, so in that lineage tracing. In fate mapping we focused on finding the original cell from which an entire set of descendants develop. For example, to identify from which primordial cells a given tissue is derived.

So, here we are going to do a more advanced way of thinking about it, so it will become clearer as we go through. So, this slide shows you the early embryonic lineage of *C. elegans*, it focuses mainly on germline. So, the zygote with one nucleus is called as the P0, it divides into two cells; one cell is called as AB and another cell is P1 and if you look at the horizontal line, the one on the left is little longer from this vertical line compared to the other one, so it is not random it is intentionally drawn that way to indicate that the cell on the left, that is AB, is a larger cell compared to P1, the smaller cell. So therefore, there is an asymmetry. Some of the cytoplasmic components get segregated during this process to the posterior side of P0 and therefore they are inherited only in P1 and not in AB and vice-versa.

In the next division AB divides to give ABa and ABp. So, this small letter a indicates anterior daughter of AB, p indicates posterior daughter of AB and this might continue dividing like AB

aaa, AB ppp. Each letter indicates one generation. And P1 divides into two cells one is called EMS another one is called P2. So, these are same nomenclature as you saw in Ascaris embryo that was observed by Theodor Boveri in his experiments. And then P2 divides as C and P3, again P3 divides into D and P4. This is a partial lineage; this is just to familiarize with how this lineage is shown in a diagrammatic way. So, this is called as a lineage tree. So, this kind of lineage tree exist for all the 959 cells that make up the adult *C. elegans* body. So, like Ascaris here again the embryonic cell division is invariant. So, later in the adult stage you might find worms of various sizes and that is due to the changes in the cell size itself. So, the embryonic cleavage pattern is invariant in contrast to our cell divisions which are not invariant.

So this lineage tracing was performed by John Sulston. He followed the early embryonic lineage up to the adult stage. During that he accidentally discovered evidence for apoptosis, he found that some cells born in a certain lineage always died and he reasoned out that the cells must be developmentally programmed to die.

So that is how they obtained the first conclusive evidence that apoptosis exists and that is where then Bob Horvitz figured out the genetic mechanisms, the genes involved and how do they function in executing apoptotic program and that is the reason he shared the Nobel Prize with John Sulston. So, John Sulston got Nobel prize for complete lineage of an organism for the first time. And Sydney Brenner got it for recognizing the importance of this organism.

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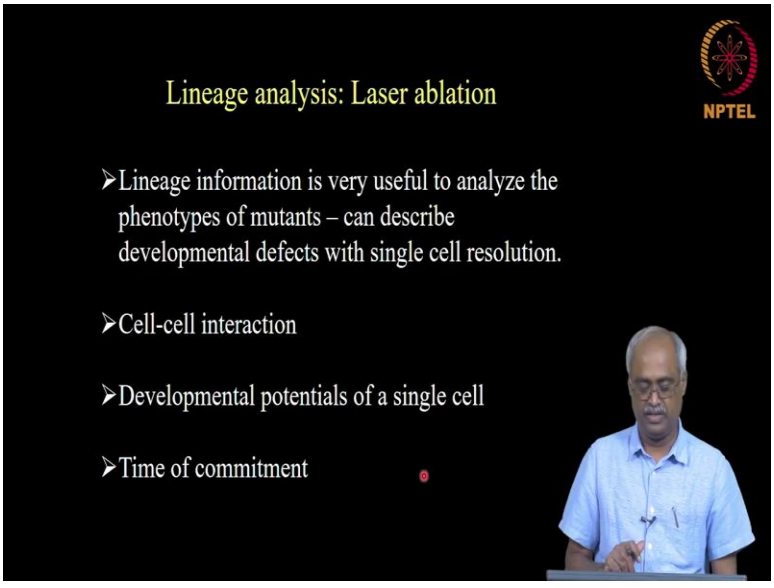

So why are we interested in lineage analysis is coming in the next to next slide. So, if you destroy a specific cell without affecting the other cells during different stages of embryogenesis, and monitor the consequences of the lost cell, you will be able to define a phenotypic defect at a single-cell resolution. It helps us to understand the role of a gene present that cells whose function is required at a given time for the embryo to develop naturally.

Instead of telling in the absence of a gene, the plant will be dwarf; you identify the tissue that does not develop naturally, where the proliferation is less, and you try to understand why is that tissue having fewer cells and go back up to a single cell. This is called as precise phenotyping.

Also, this helps in identifying potential cell-cell interaction. For example, if one cell is destroyed by laser ablation, then what happens to the fate of other neighboring cells? Do they develop autonomously, or were they dependent on the cell that was destroyed? So, you can discover potential cell-cell interactions, and then you can also find out the developmental potentials of a single cell. For example, a given cell might develop into muscle when the normal neighbor is present, that neighbor could have induced the adjacent cell to develop into a muscle cell, and if that cell is absent, then muscle cells are not developed.

Similarly, this helps in identifying the default potential of a cell; for example, if you get rid of the Y chromosome that determines the male development, then that embryo is going to develop into a female. So then you realize the developmental potential in humans is becoming a female, so that is the default state. So, you can find out what is the default state of that cell and when does that cell get committed to a particular developmental path. So, for example, if a neighbor induces a specific cell to become muscle, when does it induce? Like how early should I ablate that? So, these are the things you can discover simply by doing laser ablation.

So what laser ablation is? Laser ablation is when we shoot a laser beam on a specific cell under a microscope mounted on a glass slide. Since the beam size is so small that it can specifically kill the nucleus of a given cell. This technique is widely used in many model systems, and a variation of this called laser capture microscopy where you can specifically pinch off a cell from the tissue. Then you isolate RNA or protein or whatever you want from a single cell and study about it.

Let us see an example of how all these can be learned through lineage tracing you with the help of laser ablation.

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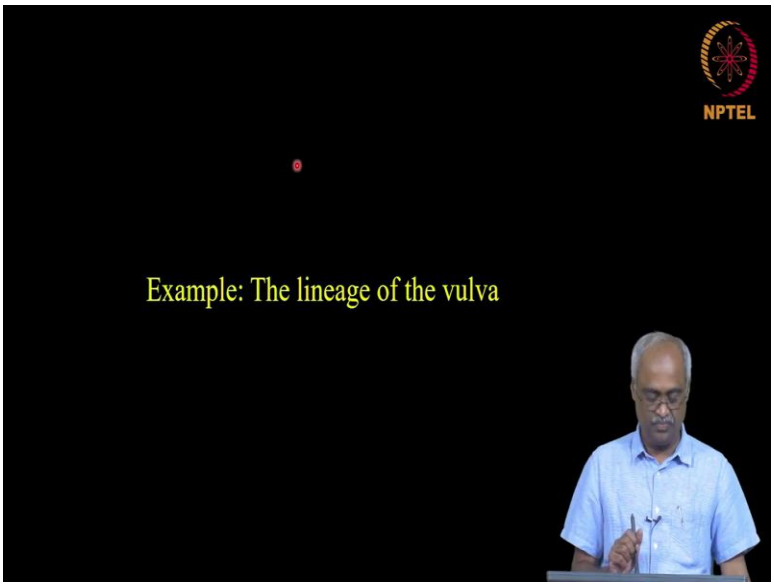

So, we are going to learn some of the genetic concepts in the context of making a particular organ. So, let us say our question is organogenesis, how an organ develops? What are the signaling pathways involved? When does it gets committed? All aspects of organogenesis. So, the organ about which we know the most of any organism is the *C. elegans* vulva. The apparatus through which it lays out the embryos or mates with a male to get sperm. That is the organ in which we first found out that ras is not a proto-oncogene. So, people thought that there are specific genes that are waiting to be activated; therefore, they can cause cancer. Cancer is caused when a normal gene that should be turned off at a certain point is not turned off, or a gene that should not be expressed in a specific context gets expressed. And as a result, they end up stimulating proliferation. So, people realized ras is not an oncogene and is required for normal development. In its absence, development does not take place. Also, another signaling like notch delta has been discovered while solving the problem of how a vulva develops. So, therefore we are going to use vulval development as an example to learn basic genetics.

Let us begin with the vulval lineage itself. Before that, let us familiarize ourselves with the vulva.

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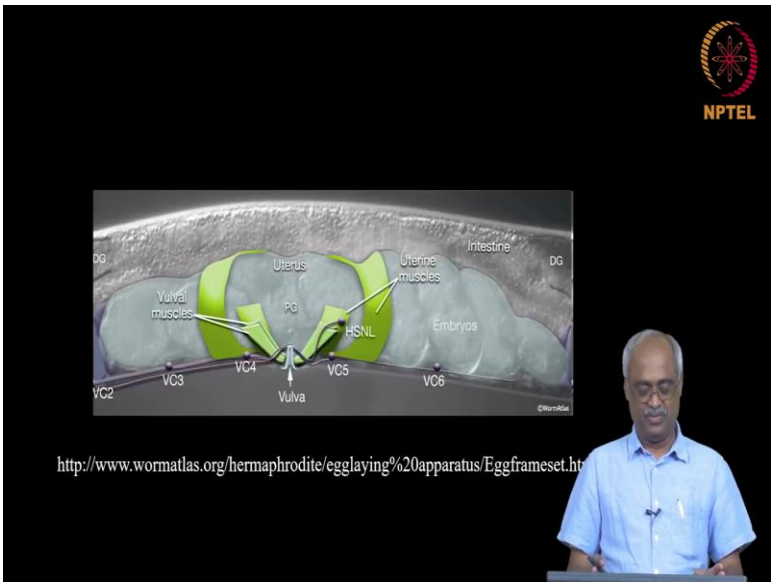

This image shows a section of the worm; yesterday, I showed you the full worm, so this is roughly the middle part of the worm that you see here, and where the arrow is pointed is the pore-like organ through which the embryos come out. So, this is the opening of the uterus. So, you have two gonadal arms that connect to a central uterus where you have embryos. And the green-colored tissue is the muscles that help in the contraction, to push the embryo out. So, this is a pictorial view of the *C. elegans* vulva. Our focus is on only the vulval muscle cells and the vulva itself.

So, to get a good view of all parts of the worm in 3D in different sections can be found on the website called wormatlas.org. Both cartoon and scanning electron micrographs are available on this website

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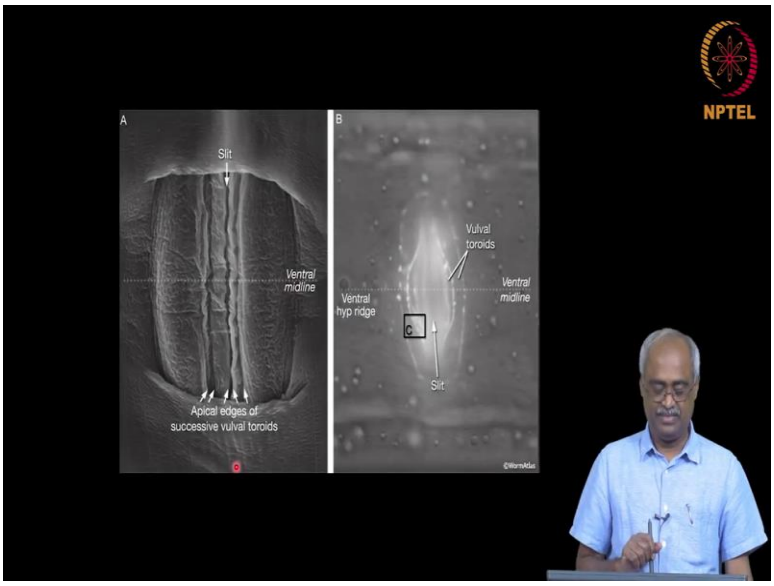

So the first image is an SEM image of the pore. This shows the opening through which the embryo comes, and many structures are seen here. And the second image shows the GFP that is expressed only in this particular tissue in the pore.

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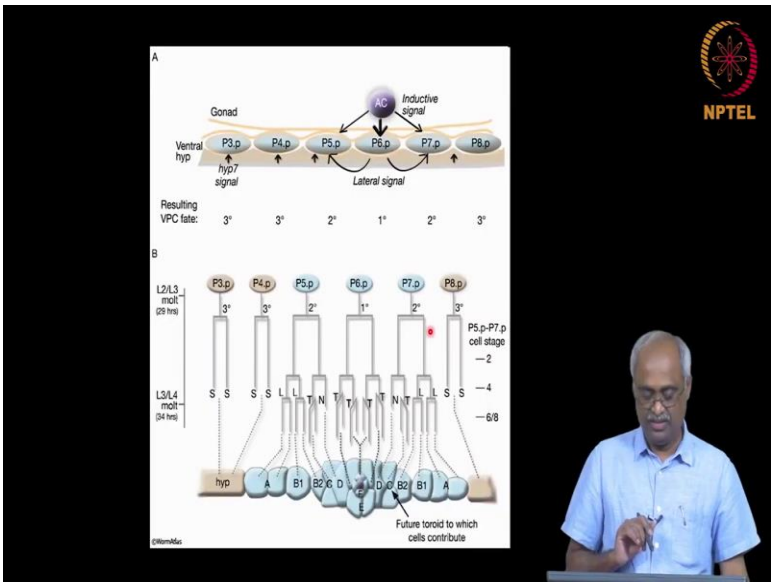

So this is the lineage diagram for vulva, so in the very early larva stage, you have six cells. These are called P3, P4, P6, P7, P8. So, these six cells are the ones that are eventually going to make the entire vulva, and they go through this lineage shown here. P6.p acquires primary fate, P5.p and P7.p acquires secondary fate, and other cells acquire tertiary fate.

Cell during the early larval stage, get these three different fates and based on those fates, then they end up following this lineage. So, for example, a cell with the tertiary fate where the phenotype is still not visible is not going to provide these cells to make the vulva; only the primary fate cell can do that.

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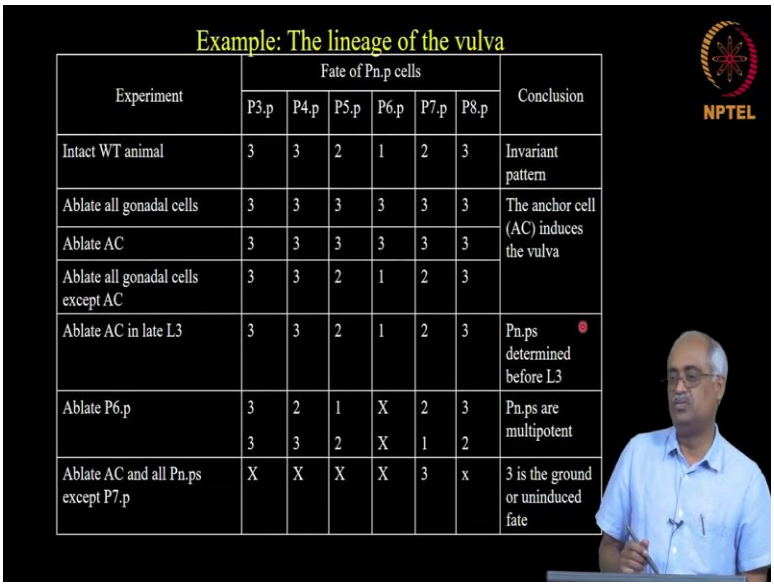

Experiment	Fate of Pn.p cells						Conclusion
	P3.p	P4.p	P5.p	P6.p	P7.p	P8.p	
Intact WT animal	3	3	2	1	2	3	Invariant pattern
Ablate all gonadal cells	3	3	3	3	3	3	The anchor cell (AC) induces the vulva
Ablate AC	3	3	3	3	3	3	
Ablate all gonadal cells except AC	3	3	2	1	2	3	
Ablate AC in late L3	3	3	2	1	2	3	Pn.ps determined before L3
Ablate P6.p	3 3	2 3	1 2	X X	2 1	3 2	Pn.ps are multipotent
Ablate AC and all Pn.ps except P7.p	X	X	X	X	3	x	3 is the ground or uninduced fate

So, this is the summary of all the experiments. Now let us do a simple experiment. We know that these six cells are the precursors of Pn.p's. Now in an intact animal, if you follow the lineage of any one of the six cells, then you will end up finding P3.p is always giving rise to tertiary fate, and P6.p always gives primary fate. There is no difference in these cells between larvas; even if you observe multiple times, therefore this lineage follows the invariant pattern. Now, if you ablate the gonadal cells, then all these six cells will have only the tertiary fate and will not have these primary and secondary fates. Now, if you leave the gonadal cells intact and if you ablate the anchor cell, then you get the same effect, none of this Pn.p's will acquire the secondary or primary fate. Then you do one more experiment where you ablate all gonadal cells leaving the anchor cell, then you end up finding it is like wild-type.

So, these three put together tell you that it is the anchor cell that induces these cells to acquire their fate, primarily the primary and secondary fate for these six cells. So, this is how you work out to a single cell; the anchor cell is the one that induces the primary and secondary fate. This is also an evidence for cell-cell interaction.

So now if you want to find out what are the different things each one of these six cells can make. If you ablate P6.p and now its neighboring cells P5.p or P7.p, one of them randomly acquires primary fate. So, that tells these cells have developmental potential to become primary as well as secondary. So, Pn.p's are multipotent. So now if you get rid of anchor cell and all the Pn.p's and see what happens when there is no induction or the default state a default state when the induction is not there and neighbors are not there what happens to a given cell, so it acquires the tertiary fate. So, this tells you the tertiary fate is the default or the ground state but given the induction they can become primary or secondary. So, essentially any one of these six cells can acquire any one of the three fates depending on the context.

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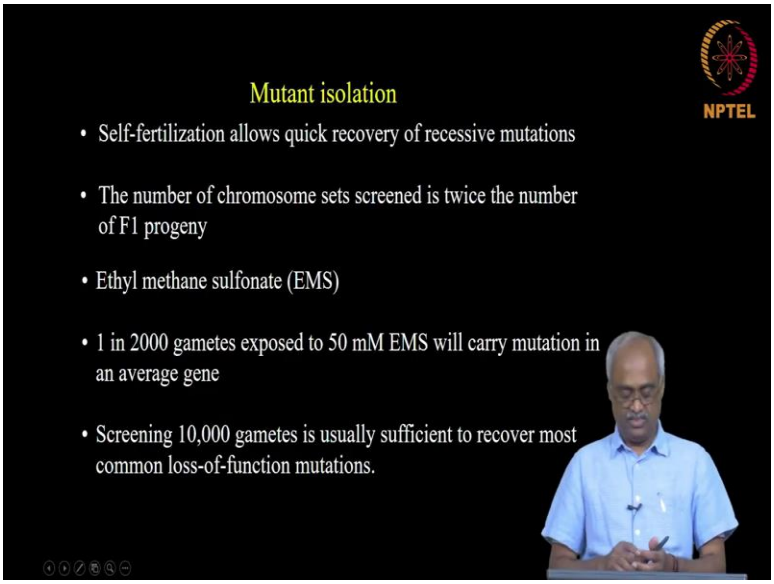

So that is an example of how lineage analysis is useful. Next is we are going to learn about a genetic screen, its importance and how it is done. So, here our goal is understanding how vulva is formed. So, before beginning, I want to mention a fundamental thing. If you look at the DNA sequence, the order in which the four nitrogenous bases occur holds biological information. But chemically, they are either purine or pyrimidine; there is no uniqueness in terms of chemistry for any given sequence. So, as a result, anything that is going to disrupt DNA chemically is not going to be sequence-specific. So, we need to remember this; for example, when Morgan's group used x-ray to mutagenesis, they cannot target the chromosome 1 left side first 100 base pairs. So, therefore, when you are going to mutagenize using any mutagen, you are going to disrupt DNA

randomly. So, depending on the strength of the mutagenesis- the concentration of the mutagen, and exposure time of the mutagen on a given organism, one will be able to mutate in such a way that only a small part of DNA is damaged and not the whole of it. For example, if you are using chemicals like ethyl methane Sulfonate, EMS. If you take more EMS and treat the worms for a long time, they are going to be dead because there you are going to make large-scale disruption on the DNA. So, optimize the concentration such that you will probably create one lesion on the DNA somewhere in one chromosome or the other, not multiple mutations.

So, the effect of mutagen can be followed by the phenotype, for example, whether it is going to make wrinkled seeds or round seeds. So that is always the readout here. So, therefore here what will be our readout? Whether the worm can lay embryo or not after treating with the mutagen. So, we already saw that self-fertilization allows quick recovery of recessive mutations. And in the slide, the advantages of this self-fertilization are listed—the first two bullets are advantages of using *C. elegans* as a model. The second one tells you the number of chromosomes sets screened is twice the number of F1 progeny. So, that means, if you are going to take a few 10,000 of hermaphrodites, the young adult, and soak it in a solution containing EMS, you are likely to have mutations taking place in sperm or oocytes.

So therefore, after mutagenesis treatment with the mutagen, when I am going to clone the worms, I am going to look at the progeny that has effects on oocyte or sperm; it could be either one, but you will be able to screen for both. Suppose you compare this with an organism where you have males and females separately. Suppose I am going to take the female and mutagenize. And in the progeny, I am only having mutations coming through one gamete if there are no mutations in the other one. So, that is the advantage here, so if I am going to screen 10,000 F1, what it means is I am screening 20,000 chromosome sets because sperm and egg come from the same individual. And usually, it is enough if you screen about 5,000 worms like 10,000 gametes to find the loss of function in any given gene.

Given its genome size and the fact that you can screen twice the number of chromosomes sets per individual organism, usually screening about 5000 worms is enough to get a loss of function like lethal or sterile those kinds of phenotypes.

So usually 50 mM EMS is used, and they typically soak it about for a few hours no more than that, then they wash off the EMS and then put on plates allowing them to feed on E.coli, and then you collect embryos from them.

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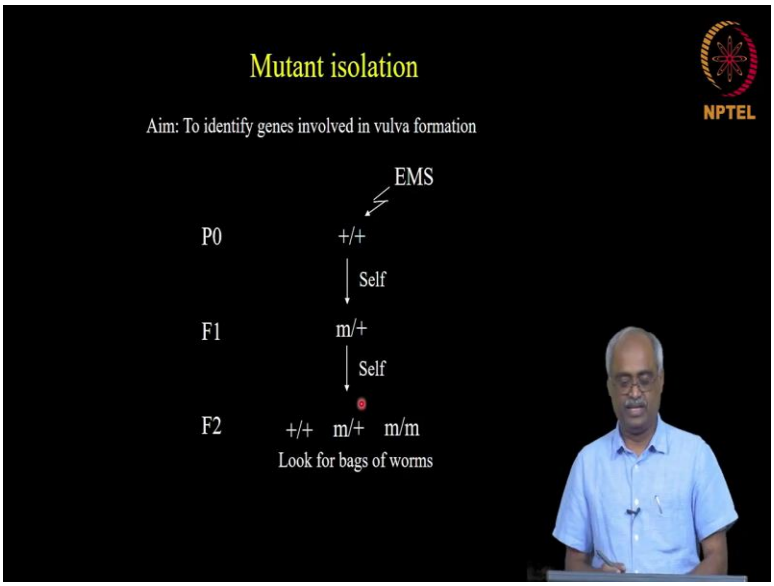

So now, we will see a very complex genetics screen. In the slide, this is an actual screen. So you take the worms that are wild-type for a given gene involved in vulval development. Since these worms have a functional vulva, we will assume that the gene is wild-type +/+; it is diploid. Then after you treat with the EMS and then you allow it to self, meaning hermaphrodites are going to give their progeny using their sperm and egg. Now let us say your gene of interest is mutated in one of the two allelic chromosomes, and we call that as m. And in the other one, the same locus getting mutated is low. So, therefore we write it as m/+. Now, if you clone the F1 worms, in F2, you will get 25% of the worms not laying any embryos. Here they are going to be bag of embryos; they cannot lay embryos, so the worm gets filled with embryos. Since the required factors for the development of the embryo is present within the eggshell, the embryos develop and hatch as larvae inside the worm uterus itself. When you are going to have a worm, filled with worms inside, even 1 in 10,000, that worm will stand out.

So that is another important aspect of how to design a screen. The phenotype that you are going to look for should be very obvious compared to the background wild-type. So, then it is very easy to score or identify even a very rare mutagenic event if your screen, the readout that you are

going to plan is such that you can readily observe it even amid 1000s of wild-type progeny, that is an important thing in genetics screen.

So here even among 10,000 F1's, we can identify the plate with a bag of worms, and then you pick that. So, that was the result of the screen.

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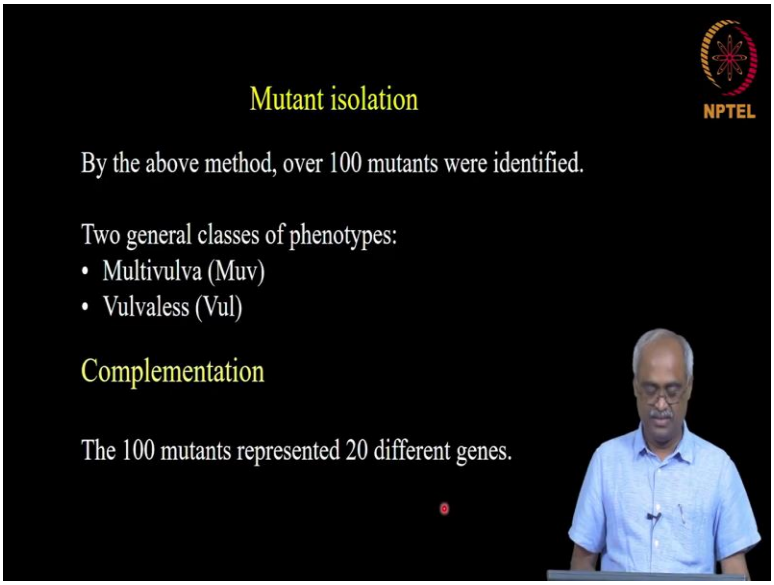

So, they got about 100 mutants in the initial screen where there was a problem in laying embryos. And when they decided to look closely at it, they found some of them had multiple vulval primordium as if it is trying to make more than one vulva. And in some of them, there is no vulva; they could already classify the phenotypes into two. Possibly you isolated multiple loss of function alleles for a given gene. So these 100 mutant alleles, like you have a hundred groups of worms where there is a problem with making vulvas, this does not mean you have identified 100 genes involved in it. Some of them maybe 10 alleles for one gene. And maybe in another gene, you got only one allele, so it is all possible.

So, to identify the genes that got mutagenized, complementation can be done.

So, if you take one of these 100 and then mate it with another group and if the mutation is on the same gene, then they will be homozygous for the loss of function of that particular gene. So, they will be vulvaless, but one is a mutation in gene **a** required for vulval development; another one is

a mutation in gene **b** required for vulval development. If you have **am/a+** and **bm/b+,** then you have a wild-type copy for both of them, and that is called trans-hetero. This is complementation. So, if they complement, then they are two different genes. If they do not, then they are on the same gene, so, therefore, each mutant having a phenotype is called an allele because whatever be that locus, there is a wild-type allele, and you got a new mutant allele. So after doing complementation, some alleles will group into one group; another one will group into another group, so you call it complementation groups. So each complementation group is essentially mutations for a given gene or alleles of one gene. So, this is how these scientists came down to 20 different genes. So, one mutagenesis screen and then complementation has found 20 genes in vulva development.

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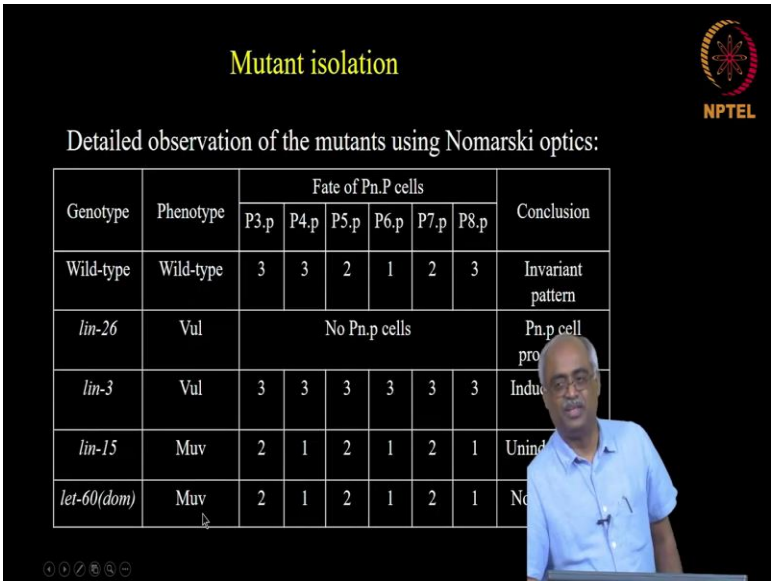

So, now let us go further, 20 different genes involved in vulval development. Still, then we are working not on a difficult organism, so here we know invariant lineage, laser ablation, and we thought that we can define phenotype to single-celled resolution. So, let us do that, can I now find out in each one of the 20 where exactly is the problem to start with during the embryonic or larval development?

So the detailed observation of mutants is essential. So, in the worms that do not have a vulva, we must identify where precisely the problem happens during larval development.

I would like you to familiarize yourself with Nomarski optics. So, when you have a thin transparent specimen and allow light to go through, which we call as bright field, many internal structures would not be readily visible. So, you will not be able to visualize some of the internal structures and you may not get a three-dimensional perspective to the image when you do that sort of a microscopy. So, a person name Nomarski came up with a method, so this uses several lenses and optical components in a microscope such that the differences in the refractive indices of the different cellular components.

Let us say you know the nucleoplasm has a different refractive index compared to the cytoplasm or the nuclear membrane or mitochondria and so on. So, by exploiting the differences in the refractive indices he managed to create a three-dimensional view of an object and that is what we call as differential interference contrast microscopy, DIC, or Normarski, in honor of the person who came up with this method. And that is the image you were seeing when I first showed you worm, that is a DIC image.

So all those images were DIC images. So here it is just a regular light microscopy with no staining. Still, by exploiting the differences in the refractive index you get subcellular detail as well as a three-dimensional perspective of the object. So, using that, you can examine what happens to these six cells. Since we already know that the wild-type has an invariant pattern, then one of those 20, *lin-26*, has a vulvaless phenotype, there we find no Pn.p's, none of the 6 were present, which tells you the wild-type function of *lin-26* is required for Pn.p production. So you discovered a gene that is necessary for the Pn.p formation to start with. Then if you look another one, lin-3; two different complementation groups again, the phenotype is the same, vulvaless, but when you look at where, when exactly the problem started, then you find the Pn.p's do not seem to get the acquired fate. So, they all have the default fate. So conclusively, that shows lin-3 is required for the induced fate.

In no other biological experiment, in one experiment, you come to a conclusion. This is the power of genetic methods. So, this is how the genes are discovered; after that, you could do whatever you want; you can crystallize and solve the structure, find out what happens in the active site or whether it binds to a ligand, etc.

But to get to the molecule, someone has to identify the molecule required for a particular function, and that usually starts with genetics. So, that is the power of genetics here. One experiment and then you come to the conclusion that a gene is required for a specific activity. Then in *lin-15*, you find all of Pn.p's getting induced to default state indicating that *lin-15* is required for that uninduced fate, which we thought as the default state.

Then sometimes, you get dominant alleles. So, in this screen, how would we have identified this? Now in this screen, in F1, you will have the phenotype, the *let-60* dominant allele gives all induced fate but we do not conclude here. Because we do not know what normally *let-60* is doing during vulval development. So when it is a dominant allele, it is not easy to conclude at this stage.

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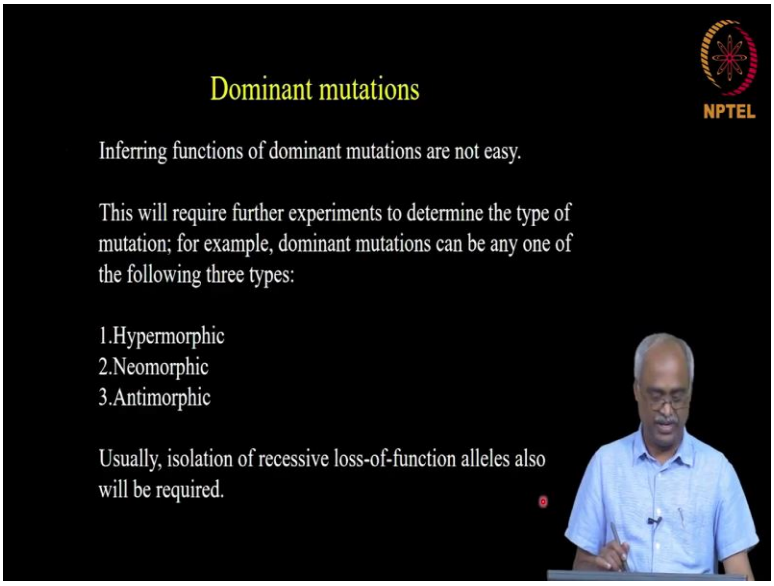

So, the next slide tells you why inferring functions of dominant mutations are not easy. This will require further experiments because the dominant mutation is from any one of these three types listed in the slide. It may be hypermorphic, where multiple doses of a gene product are produced than the normal.

It could be neomorphic meaning it may be a new function. So for many of these neuro degenerations phenotypes, the cause is actually a new function of the protein. Like for example if you take a protein like superoxide dismutase, a mutant version of it aggregates; that aggregation

is a neomorph; it is a new phenotype. The dominant mutation could also be anti morphic, an opposing function compared to what the gene does typically. It usually might suppress some gene but the mutant version is not; instead, it is promoting, so that is anti morphic. So, the dominant allele could be any one of these three and unless otherwise you identify which one of these three you cannot immediately conclude from the observed phenotype.

So usually to settle clearly on such things as what is the wild-type function of that gene, you need to get a recessive loss of functional alleles as well. So, we will stop here. So, in the next class, we are going to continue which chromosome does the mutation is present and what protein it encodes. So, we will try to focus on in the next class then we will move on to, how do we handle dominant alleles.