EFFECTS OF PERILIPIN I & V ON THE PHENOTYPE OF ZEBRAFISH EMBRYOS

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ABSTRACT

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This study has been done in cooperation with Institute of Biomedical Technology, University of Tampere. Aim of the study was to perilipin proteins on the phenotype of zebrafish by using gene silencing and microscopy.

Protein production is regulated by genes. For each protein there is a gene coding it and by manipulating the gene for example production of the protein can be prevented. Morpholinos are oligonucleotides which can temporarily knock down genes. In this study, two perilipin coding genes were knocked down in zebrafish: perilipin 1 and perilipin 5. Perilipins regulate lipid storage and usage in lipid droplets. Perilipin 1 increases lipid accumulation and storage and perilipin 5 helps body to use lipids as energy.

Because perilipins have an effect on the lipids, lipid staining was used to see the effect of Morpholinos. Nile Red and LD540 dyes were used for staining. According to results, knocking down perilipin 5 increases the lipid content in zebrafish and has an effect on the appearance. Knocking down perilipin 1 produced extremely poorly developed individuals. Lipid content of these fish was slightly smaller than that of wild type control fish. The concentration of Morpholinos had significant effect on results and survival rate. With perilipin 1 too high Morpholino concentration produced dramatically poorly developed embryos. The Morpholino concentration of 175 ng/nl produced the best results. With perilipin 5 the concentration of injection solution needed to be higher, the best results were got with the concentration of 500 ng/nl.

More studies should be done to determine the real amount of lipids, because in this study lipid content was only estimated visually. In future studies control Morpholinos should also be used to eliminate side effects caused by Morpholino itself.

Key words: Zebrafish, perilipin, PAT protein, lipid droplet, Morpholino, gene silencing, gene knock down

TIIVISTELMÄ

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Tämä Pro Gradu-tutkielma on tehty yhteistyössä Tampereen yliopiston Biolääketieteellisen teknologian yksikön kanssa. Tutkimuksen tarkoituksena oli selvittää perilipiinien vaikutusta seeprakalan fenotyyppiin käyttäen tutkimusmenetelminä geenien hiljentämistä ja mikroskopiaa.

Geenit säätelevät proteiinien tuotantoa elimistössä. Jokaista proteiinia koodaa geeni, jonka toimintaan puuttumalla voidaan esimerkiksi estää kyseisen proteiinin muodostuminen. Morfoliinot ovat oligonukleotideja, joiden avulla voidaan tilapäisesti estää geenien toimintaa. Tässä työssä hiljennettiin morfoliinojen avulla kaksi geeniä seeprakaloilta: perilipiini 1 ja perilipiini 5. Perilipiinit ovat lipidien varastoitumista sääteleviä proteiineja. Perilipiini 1 edesauttaa lipidien varastoitumista ja perilipiini 5 puolestaan säätelee rasvojen käyttöä energiaksi.

Koska tutkittavat proteiinit vaikuttavat elimistön rasvoihin, morfoliinojen vaikutusta tutkittiin lipidivärjäyksellä. Värjäyksessä käytettiin Nile Red- ja LD540-lipidivärejä. Tulosten perusteella voitiin todeta, että perilipiini 5 eliminointi lisäsi seeprakalojen rasvapitoisuutta ja muutti selvästi niiden ulkomuotoa. Perilipiini 1 tapauksessa huomattiin, että geenin hiljentäminen tuotti alikehittyneitä, erittäin epämuodostuneita kaloja. Lipidivärjäyksen tuloksena havaittiin, että rasvan määrä oli näillä kaloilla hieman vähäisempi kuin kontrolleina käytetyillä villityypin kaloilla. Morfoliinokonsentraatiolla todettiin olevan vaikutusta lopputulokseen ja kalojen selviytymiseen. Perilipiini 1 tapauksessa parhaat tulokset saatiin 175 ng/nl konsentraatiolla, suuremmat pitoisuudet nostivat alkioiden kuolleisuutta dramaattisesti. Perilipiini 5 tapauksessa sopivin morfoliinokonsentraatio oli huomattavasti korkeampi ja parhaat tulokset saatiin 500 ng/nl konsentraatiolla. Alhaisemmilla pitoisuuksilla vaikutukset eivät näkyneet kovin selkeästi.

Lipidien todellisen määrän selvittäminen vaatii lisätutkimuksia, sillä tässä työssä käytetty menetelmä on hyvin epätarkka ja perustuu visuaaliseen arviointiin. Jatkotutkimuksissa tulee myös käyttää kontrollimorfoliinoa, jotta morfoliinon itsensä aiheuttamat sivuvaikutukset saadaan eliminoitua.

Avainsanat: Seeprakala, perilipiini, PAT-proteiini, lipididropletti, morfoliino, geenien hiljentäminen

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Elina Esko

LIST OF TERMS AND ABBREVIATIONS

Adipocyte	Cell type typical for adipose tissue a.k.a. fat tissue.
Adipophilin, ADRP	Previous name for perilipin 2. PAT protein.
DNA	Deoxyribonucleic acid, contains all genetic information.
dpf	Day(s) post fertilization
ER	Endoplasmic reticulum
Gene	Segment of DNA, responsible for pheno type and inheritable characteristics.
in vivo	In the living organism
Knock down	Temporary method to prevent gene function.
LD540	Lipid dye. Multicolor fluorescence in lipid rich environment.
Lipid droplet, LD	Cell organelle, fat storage in body
Morpholino	Gene silencing agent, oligonuckleotide which can inhibit protein production.
Nile Red	Lipid dye. Fluorescences red in lipid rich environment.
OXPAT	Previous name for perilipin 5. PAT protein.

PAT proteins	Lipid droplet associated proteins, regulate lipid storage and usage in body.
PCR	Polymerase chain reaction. Common laboratory method for DNA amplification.
Perilipins, PLIN1-5	PAT proteins which regulates lipid storage and usage in lipid droplets. Separated by numbers 1-5.
Phenotype	Physical, observable appearance which is resulted by genes and environment.
RNA	Ribonucleic acid, transfers information from DNA to protein forming cell organelles.
S3-12	Previous name for perilipin 4. PAT protein.
TAG	Triacylglyceride
TIP47	Previous name for perilipin 3. PAT protein.

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1 INTRODUCTION

Adipose tissue, and adipocytes in it, is the most important fat storage in the body. Fat storages are needed for energy if food supplies are limited or energy consumption suddenly increases (Beller et al. 2010), but if fat consumption and intake are not in balance some serious problems may occur. Overweight and obesity are increasing problems all over the world and are risk factors for chronic diseases like type II diabetes, cardiovascular diseases and cancer (WHO, http://www.who.int/topics/obesity/en/).

Energy from nutrition will be either used or stored in the body. Excessive energy will be stored as lipids in lipid droplets, mainly in adipocytes. Lipid droplets are dynamic cell organelles with still unclear functions. They were long time almost ignored in research, but nowadays many researchers are interested in their role in the energy metabolism and homeostasis. Especially lipid droplet associated proteins, including perilipins, and the functions of them have evoked interest. (Ahmadian et al. 2010; Beller et al. 2010; Brasaemle et al. 2004; LaFontan and Langin 2009.)

In protein related research modern gene technologies, like gene knockdown, provide good tools for experiments. Genetic information to produce proteins is stored in DNA of chromosomes. Genes are sequences of DNA and all proteins are coded in the genome of the organism. In protein synthesis RNA polymerase 1 synthesizes pre-mRNA from DNA in the cell nucleus. This is called transcription and in this process genetic information of DNA copy to pre-mRNA. In process called splicing, pre-mRNA is processed in nucleus resulting mRNA which moves out of nucleus to the cytosol. In cytosol, mRNA interacts with ribosome and its information is translated to specific amino acid chain. Formed amino acid chain with structure coded in the gene of DNA is the protein. (McArdle et al. 2007, pp. 1007-1010.)

Function of different proteins can be studied by affecting the protein synthesis and thus either inhibit protein expression or increase it. This can be done for example by knocking down a gene and examine what happens. Gene knockdown or silencing can be temporary or permanent process and there are several silencing methods available. One practical and economically reasonable silencing technique is to use Morpholino oligo-

nucleotides. (Bill et al. 2009; Brent and Drapeau 2002; Draper et al. 2001; Kim et al. 2010; Summerton 2005 ; Zhu et al. 2007.)

2 LIPID METABOLISM AND LIPID DROPLETS

2.1 Lipid metabolism

White adipose tissue is the major energy storage in mammals. Energy is stored as triacylglyceorols (TAG) in lipid droplets of adipocytes. If energy intake exceeds consumption, the excess energy will be stored in the body. Excessive TAG storage leads to obesity and related diseases, such as type II diabetes, cardiovascular disorders and hypertension. (Ahmadian et al. 2010; Beller et al. 2010; LaFontan and Langin 2009.) Adipose tissue is the main fat reserve in the body, but there is some fat stored also in other types of cells, primarily as cholesterol esters (Brasaemle et al. 2004).

Lipolysis is hydrolytic degradation of TAGs in adipocytes which releases fatty acids and glycerols. Released degradation products will be carried with bloodstream in other organs where they will be used in energy producing processes. Lipolysis is special reaction for white adipose tissue and it is mediated by three lipases: adipose triglyceride lipase, hormone-sensitive lipase and monoacylglycerol lipase. (Ahmadian et al. F01; Lafontan and Langin 2009.) If TAG synthesis and hydrolysis are not balanced, risk of obesity increases (LaFontan and Langin 2009). The processes controlling lipid trafficking in adipocytes are very important in the regulation of energy metabolism (Brasaemle et al. 2004).

2.2 Lipid droplets

Lipid droplets (LD) are metabolically active organelles found inside almost all cells and taking part in cell signaling, intracellular vesicle trafficking and lipid homeostasis. Globular shaped LDs are the most important energy reserves in the body and act as fat storages. In mammals LDs are enriched in adipocytes and in the corresponding cell types in the other animals. (Beller et al. 2010; Bickel et al., 2009; Brasaemle et al. 2004; Digel et

al. 2010; Farese et al. 2009; Guo et al. 2009; Hickenbottom et al. 2004; Londos et al., 1999; Robenek et al. 2005.)

Structure of lipid droplet is shown in the figure 1. LDs have phospholipid and cholesterol monolayer surface and neutral lipid core which consist of triacylglyserols and sterol esters as shown in the figure. LDs structure has been studied by Tauchi-Sato et al. (2002) and they confirmed the monolayer structure of the surface. (Brasaemle et al. 2004; Farese and Walther 2009; Guo et al. 2009; Tauchi-Sato et al. 2002; Thiele et al. 2008.) The size of LDs varies. In adipocytes LDs are larger than in other cells and the diameter can be 50-100 µm. In the other cells, LDs can be as small as <1 µm. (Farese et al. 2009; Hickenbottom et al. 2004.) Numerous proteins, e.g. PAT proteins, with different functions can be found embedded in LDs phospholipid monolayer (Bickel et al. 2009; Brasaemle et al. 2004; Farese et al. 2009).

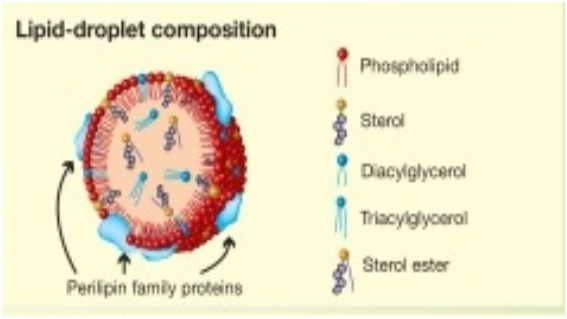

FIGURE 1. Structure of the lipid droplet (Guo et al. 2009).

Formation of lipid droplets is still unknown. One theory is that LDs form from endoplasmic reticulum (ER), because the ER is the place of triglyceride biosynthesis, but for example Tauchi-Sato et al. (2002) found that LDs have a unique fatty acid composition which differs from ER's and for that reason this theory of LDs formation is uncertain. (Farese et al. 2009; Guo et al. 2009; Tauchi-Sato et al. 2002; Kadereit et al. 2008.) The findings of Tauchi-Sato et al. (2002) motivated Zanghellini et al. (2010) to study formation of lipid droplets and they presented a theoretical thermodynamic model for LD formation from ER. Despite of all contradictory results, the most likely and widely ac-

cepted theory is that LDs form from ER membrane. How this exactly happens is unclear. (Robenek et al. 2005; Kadereit et al. 2008; Walther and Farese 2009.)

Three different possible mechanisms for LD formation from ER membrane are shown in the figure 2. According to the first theory, lipids accumulate within the bilayer of ER which induces budding of a droplet from the cytosolic leaflet of the ER membrane. It is still unclear how the lipid molecules get into the droplet and how the budding happens. The second theory presents other possible mechanism where both leaflets of the ER bilayer form a bicelle with lipids. There are many unsolved problems also in this case. It is unknown how LDs are directed toward the cytosol and not toward ER lumen if the formation happens this way and does this violate the ER membrane because of the hole existing during LD formation. The third theory is called vesicular budding, LDs are formed within bilayer vesicles. Neutral lipids fill the vesicle while it is still part of ER membrane or after budding. In both cases, lipids fill the intermembrane space. Vesicular lumen makes up a little part of the LD or it is obliterated. Remaining lumen as an aqueous environment could be the explanation why some hydrophilic proteins have been found in the otherwise hydrophobic LD core. (Walther and Farese 2009.)

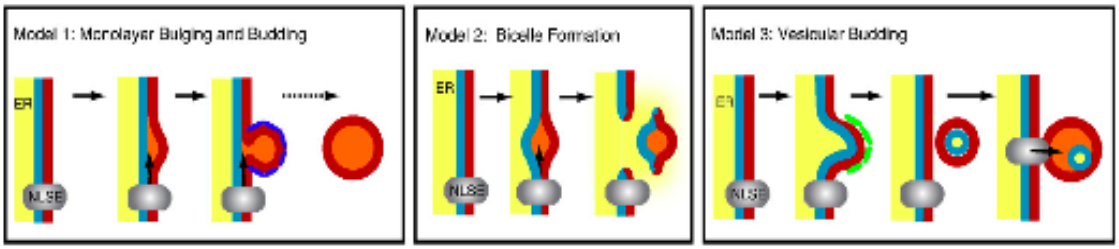

FIGURE 2. Three theories of LD formation: 1) Monolayer bulding and budding. 2) Bicelle formation. 3) Vesicular budding. (Walther and Farese 2009.)

The dynamic functions of LDs are unclear, but something is already known. Lipid droplets can merge each other and form larger droplets. This is called LD fusion. LD may also undergo fission, when one droplet splits and forms smaller droplets as presented in the figure 3. Fission increases surface area of droplets and may require extra phospholipids available for covering new droplets. (Walther and Farese 2009.)

FIGURE 3. Lipid droplet fusion and fission (Modified from Walther and Farese 2009).

Wolins et al. (2005) have studied LD formation and they found out that LDs form peripherally and move toward the center in adipocytes. Mature storage droplets are located in the middle of the cell. Newly formed droplets enlarge when they move centrally. Growing of droplets happens by new TAG synthesis directly in the droplet rather than by fusion with other droplets. Proteins coating LD also change during movement toward centre. Because no significant LD fusion has been found, it is possible that TAGs are synthesized on the droplets. Enzymes required in TAG synthesis were found in lipid droplet enriched fractions from multiple cell types which supports this theory. (Wolins et al. 2005.)

3 PAT PROTEINS

PAT family proteins are lipid droplet associated proteins and in mammals. This family includes 5 members: perilipin, adipophilin (a.k.a. adipose differentiation-related protein ADRP), OXPAT (a.k.a. MLDP/PAT-1/LSDP5), tail-interacting protein of 47 kDa (TIP47) and S3-12. The name PAT comes from initials of Perilipin, ADRP and TIP47. These proteins have ability to bind LDs and regulate access to the core of droplets. They have also structural similarities and PAT domain is found in all of them, except S3-12 which has other similarities in the structure. PAT proteins are highly polar molecules. Perilipin and adipophilin are only expressed in environment containing lipids, basicly in cells with neutral lipid pools and they are bounded to lipid droplets. TIP47, S3-12 and OXPAT are stable also in environment lacking lipids and able to bind to new droplets when TAGs are synthesized. Perilipin and adipophilin regulate energy storages responding to energy demands. TIP47, S3-12 and OXPAT help to store excess fat from nutrition in lipid droplets. (Bickel et al. 2009; Robenek et al. 2005; Wolins et al. 2003, 2006a.)

PAT proteins have been considered as surface proteins of LDs, but Robenek et al. (2005) have shown by freeze-fracture immunocytochemistry methods that they actually have access to the core of LDs. How these polar proteins get in the hydrophobic TAG containing core is unknown, probably because there are no suitable research methods available. (Robenek et al. 2005) In the study of Wolins et al. (2005) was shown that PAT proteins were recruited on the surface of the nascent droplet from preexisting protein pools. These findings were made with adipocytes and Wolins et al. (2005) suggest that conclusions from data which has been obtained by studying different cell type or organism should be made bearing in mind that there might be differences between cell types and organisms.

PAT protein nomenclature is complicated there are various names for the same protein. To avoid confusion, the nomenclature of PAT proteins has been updated recently. A new, unified nomenclature for mammalian PAT proteins is shown in the table 1. (Kimmel et al. 2010.)

TABLE 1. Unified nomenclature of mammalian PAT-proteins. (modified from Kimmel et al. 2010)

Approved human symbol	Approved name	Previous aliases
PLIN1	Perilipin 1	Perilipin, PERI, PLIN
PLIN2	Perilipin 2	ADRP, ADFP, adipophilin
PLIN3	Perilipin 3	TIP47, PP17, M6PRBP1
PLIN4	Perilipin 4	S3-12
PLIN5	Perilipin 5	PAT1, LSDP5, OXPAT, MLDP

3.1 Perilipin 1

Perilipin 1 (PLIN1) is the most important PAT protein in adipocytes and steroidogenic cells. Four variants of perilipin 1 have been found and they are separated with letters a,b,c,d. (Bickel et al., 2009; Brasaemle et al., 2000; Kimmel et al. 2010; Robenek et al. 2005.) Perilipin 1 acts as a regulator of lipolysis. PLIN1 increases TAG storage by inhibiting lipolysis. It forms a barrier on lipid droplet surface which shields TAGs from action of lipase enzymes. In the study of Brasaemle et al. (2000) cells lacking PLIN1 degrade synthesized TAGs rapidly, so it proves that perilipin 1 acts by inhibiting degradation of TAGs. (Brasaemle et al., 2000) According the studies of Wolins et al. (2005, 2006a) PLIN1 coats especially mature storage droplets in adipocytes and it is not present in striated muscles in mice. These results are consistent with results of Brasaemle et al. (2000).

Xu et al. (2006) studied the degradation of perilipin 1. They found out, that in cell culture lacking fatty acids, which are important for TAG synthesis and LD formation, PLIN1 degraded through an ubiquitination-proteasome pathway. They suggest that stored lipids stabilize PLIN1 and inhibit degradation.

Mottagui-Tabar et al. (2003) have studied perilipin 1 functions in humans by measuring perilipin 1 content and rate of lipolysis in subcutaneous fat cells. Subjects were obese and non-obese women. They found that in humans low perilipin 1 content in adipocytes is connected with obesity and higher rate of lipolysis.

Tansey et al. (2001) noticed that PLIN1 elimination produced a lean phenotype in mice. As a summary of these studies, it seems that perilipin 1 inhibits lipolysis and increases fat storage. Results of Mottagui-Tabar et al. (2003) differ from others, but it should be considered that experiments with different organisms can't necessarily be compared. (Brasaemle et al. 2000; Tansey et al. 2001; Wolins et al. 2005, 2006a; Xu et al., 2006.)

3.2 Perilipin 2

Perilipin 2 or ADRP is found in all mammalian cell types also in skeletal muscle. It is expressed all over the LD, also in the core. (Minnaard et al. 2009; Robenek et al. 2005.) Xu et al. (2005) have studied degradation of Perilipin 2 and they found that it is degraded through the post-translational proteasome pathway. The studies of Masuda et al. (2006) also support this degradation theory. PLIN2 competes with PLIN1 in adipocytes. There are evidences that PLIN2 is present in young, differentiating adipocytes and during development of the cells expression of perilipin 1 increases and PLIN2 is degraded by proteosomes. Experiments were made with Chinese hamster ovary fibroblastic cells. (Xu et al., 2005)

Findings of Bonggi et al. (2009) suggest that PLIN2 may act as a stabilizator of stored TAG in lipid droplets. These experiments have been made with mice and they noticed that after high-fat diet PLIN2 levels are higher. They also found that after long, chronic high-fat diet protein level was higher than after acute diet. In the study of Dalen et al. (2004) PLIN2 level was increased in obese rats compared with lean rats.

Imai et al. (2007) used antisense oligonucleotides and knocked out PLIN2. They found out that amount of free circulating TAGs decreased when level of PLIN2 is reduced in obese mice. In this study PLIN2 expression was reduced in the liver, but not in the muscle or adipose tissue. Fat content was reduced, but there were no effects on food intake or body weight. (Imai et al. 2007.)

Masuda et al. (2006) found out in their studies that expression of PLIN2 is associated with accumulation of triglyserols in cells and PLIN2 levels decreased when triglyserols were consumed. In lipid storing cells PLIN2 prevents the reduction of triglyserols (Masuda et al. 2006). As a conclusion, PLIN2 seems to stabilize stored fat in adipocytes

and dietary conditions might have effect on protein expression. PLIN2 level also seems to be high in obesity and prolonged high-fat diet. (Bonggi et al. 2009; Dalen et al. 2004; Imai et al. 2007; Masuda et al. 2006.)

3.3 Perilipin 3

Perilipin 3, or TIP47, is expressed in many cell types (Ohsaki et al. 2006). Ohsaki et al. (2006) have shown that PLIN3 expression in LDs increases when PLIN2 expression is reduced by knocking it out. Overall expression of PLIN3 didn't increase, thus presence of PLIN2 is restricting factor of PLIN3 in lipid droplets. PLIN3 function in LDs is unclear. It has been shown that knocking out PLIN3 did not influence significantly LDs lipid ester storage or integrity. PLIN3 may have a role in intracellular vesicular trafficking (Ohsaki et al. 2006), but this has not been confirmed yet.

Bonggi et al. (2009) made some experiments with mice and used different dietary challenges. They found that PLIN3 may take part in the synthesis of lipid droplets in early stages of dietary fat absorption. In the experiments mice were under high-fat diet and they found out that PLIN3 level was higher after acute diet than after chronic dietary challenge.

Wolins et al. (2006a) studied how lipid droplet coated with different PAT proteins are divided in adipocytes when incubated in long chain fatty acid containing media. In this case, PLIN 1, 2 and 3 were stained and results are shown in the figure 4. It can be seen, that PLIN1 coats the largest central LDs and PLIN3 the smallest, peripheral droplets. PLIN2 coats the intermediate sized droplets.

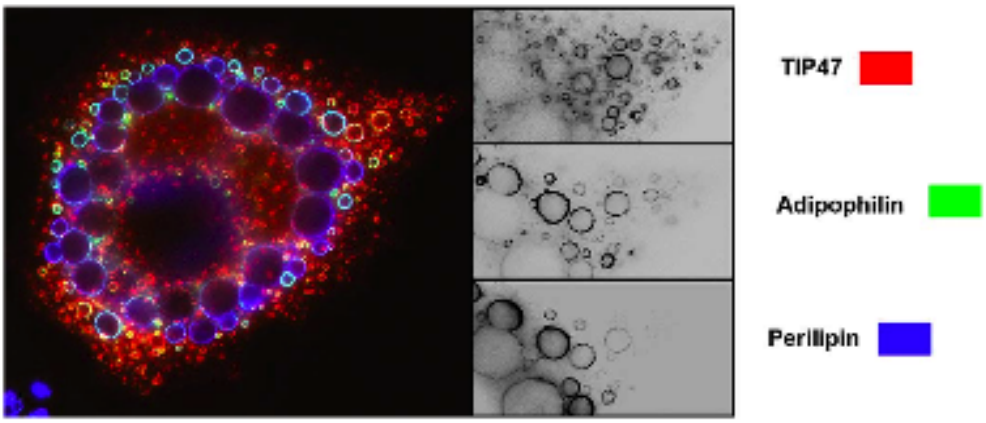

FIGURE 4. Lipid droplet packaging in adipocyte incubated in long chain fatty acid containing media. Perilipin (PLIN1) coats the largest droplets, Adipohilin (PLIN2) the intermediate sized and TIP47 (PLIN3) the smallest ones. (Wolins et al. 2006a)

Perilipin 3 has not only LD related functions but also the other important roles. Hocsak et al. (2010) have found that PLIN3 protects cells from oxidative stress and it has been shown that PLIN3 is overexpressed in some tumors. As a summary, PLIN3 and PLIN2 seems to be competing and dietary challenges may have an effect on PLIN3 expression. The role of PLIN3 in LDs is still unclear. (Bonggi et al. 2009; Ohsaki et al. 2006.)

3.4 Perilipin 4

Wolins et al. (2003) have studied perilipin 4, or S3-12, and they found out that by promoting TAG synthesis in adipocytes lipid droplet coated with PLIN4 were formed separately from PLIN1 coated droplets. PLIN4 coated droplets were smaller than PLIN1 coated ones. But when cell culture medium was lipid-poor, PLIN4 coated droplets did not exist. (Wolins et al. 2003)

Perilipin 4 is not present in brown adipose tissue, but in white adipose tissue it has been found. It promotes TAG packaging in LDs for storage. (Wolins et al. 2006a) Dalen et al. (2004) reported that PLIN4 level is reduced in obese rats compared with lean rats. Results of Wolins et al. (2006a) and Dalen et al. (2004) have contradiction and this protein is the less studied PAT protein.

3.5 Perilipin 5

Perilipin 5, formerly called OXPAT is the last PAT-family protein found. It has been characterized by Wolins et al. in 2006. PLIN5 is hydrophilic and slightly acidic molecule and consists of 463 amino acids. It's molecular weight is predicted to be 51,4 kDa. It takes part in oxidative processes and it is highly expressed in the tissues with high fatty acid oxidative capacity. In the study of Wolins et al. (2006b) PLIN5 was expressed in mouse brown adipose tissue, liver, heart and soleus. In other, less oxidative tissues like white adipose tissue and more glycolytic skeletal muscles the expression was lower. PLIN5 increases fatty acid uptake which supports mitochondrial fatty acid oxidation. Oxidative catabolism related enzyme levels are increased by PLIN5. They noticed also that in tissues where PLIN5 expression is high PLIN4 expression was low, although these two proteins are found in same tissues. PLIN5 regulates TAG packaging in LDs for hydrolysis and oxidation. (Wolins et al. 2006b.) PLIN5 has been found in skeletal and cardiac muscle mitochondria which also support the theory of PLIN5 taking part on oxidative processes (Bosma et al. 2011).

PLIN5 expression in obesity has been studied. Results of the studies of Wolins et al. (2006b) suggest that in obesity, PLIN5 expression in subcutaneous adipose tissue is decreased. They also found that low PLIN5 level with insulin resistance primarily depends on the linkage between obesity and insulin resistance. PLIN5 can be seen as a marker of positive adaptation to increased availability of fatty acids in the healthy and lean individuals. PLIN5 seems to be relevant for maintaining normal flexible metabolic fuel selection and in obesity this normal adaptation is lost because PLIN5 expression decreases. (Wolins et al. 2006b)

Bosma et al. (2011) found that PLIN5 expression correlates with muscle's oxidative properties. Study was done by taking biopsies from vastus lateralis muscle of healthy male subjects. They also studied if PLIN5 overexpression has effect on mitochondrial density, but they didn't find any effect. However overexpression leads to higher fatty acid oxidation rates.

4 METHODS TO RESEARCH PAT PROTEIN FUNCTIONS IN VIVO

4.1 Gene knockdown

Protein synthesis is regulated by genes, and there is a gene specific for every protein. Protein functions in the body can be studied by manipulating the gene coding it. One gene manipulation method is gene silencing or knockdown. It means that the specific gene is temporarily or permanently inactivated by silencing agent. There are various silencing agents available, but one of the most widely used is Morpholino. Morpholinos are suitable for zebrafish *in vivo* experiments (Brent and Drapeau 2002; Kim et al. 2010; Zhu et al. 2007) and they are relatively easy to use and economically reasonable technique. (Bill et al. 2009; Draper et al. 2001; Summerton 2005) Morpholinos are used with zebrafish by injecting them into the center of the yolk. To ensure ubiquitous spreading, injection must be done in early development stage. Bill et al. (2009) recommend injections during 1 to 8 cell stage and Brent and Drapeau (2002) before 16-cell stage.

Morpholinos, or morpholino antisensing oligonucleotides, were innovated by James Summerton in 1980's. Morpholino oligomers consist of morpholine ring, phosphorodiamidate group and nucleic bases (adenine, guanine, cytosine and thymine). Structure of oligomer is shown in the figure 5. Bases are different in each Morpholino and they are chosen to match the target RNA. (Gene Tools LLC. USA; Bill et al. 2009.)

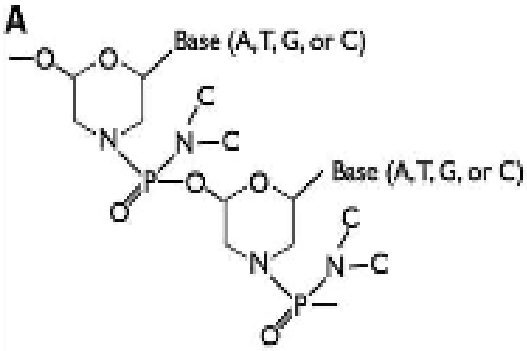

FIGURE 5. The structure of Morpholino oligomer. The molecule consists of morpholino ring, phosphorodiamidate group and nucleic bases which are different in each morpholino. (Picture from Bill et al. 2009.)

When the bases of Morpholino are chosen to match with bases of RNA sequence coding the target protein, Morpholino can inhibit protein formation or function. There are two possible ways how Morpholino can act: steric blocking of translation and splicing. These mechanisms are shown in the figure 6. In steric blocking of translation Morpholino prevents mRNA interaction with ribosome and this way inhibits protein synthesis. In splicing blocking Morpholino inhibits normal mRNA splicing when formed mRNA is not anymore specific to target protein. In protein synthesis this abnormal mRNA produces a protein which does not have same function in the body as normal protein. (Draper et al. 2001; Gene Tools LLC. USA.)

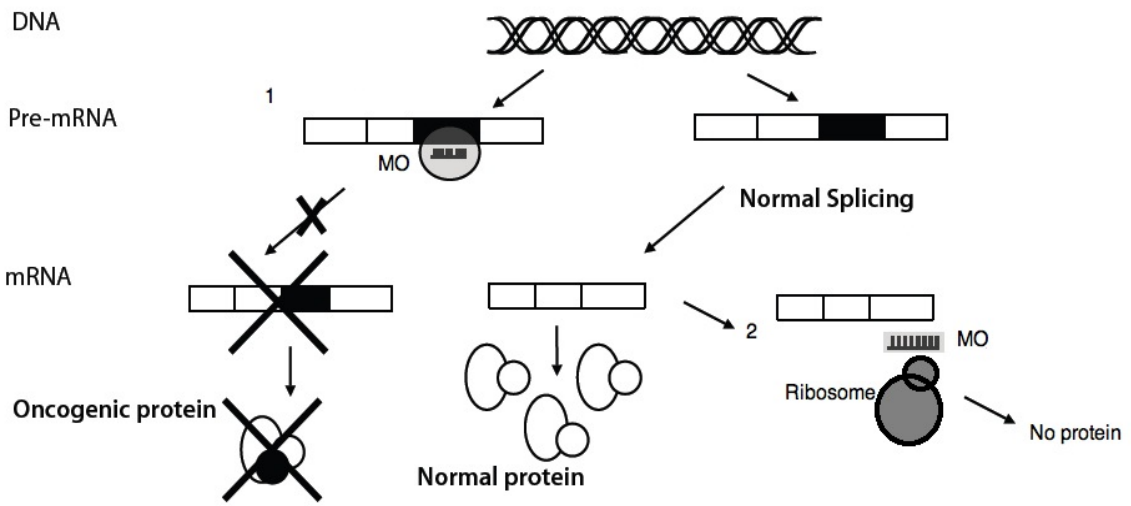

FIGURE 6. Gene knockdown mechanisms with Morpholinos. 1) Morpholino (MO) sterically blocks Pre-mRNA splicing and prevents normal mRNA formation and protein synthesis products oncogenic protein. 2) Morpholino inhibits mRNA translation in ribosome. Ribosome can not interact with mRNA and protein synthesis is prevented. (Modified from Carling et al. 2009)

Morpholino oligonucleotides are available only from Gene-Tools LLC. They also do the design work for the customer and for that, the target sequence is needed. (Gene-Tools LLC, USA.) Sequences are available in online genome browsers such as ensebl! (www.ensembl.org) but they can also be determined from polymerase chain reaction (PCR) products (Bevan et al. 1992). PCR is a common laboratory method to amplify spesific DNA sequences. Method is based on the ability of DNA polymerase entzyme to produce new DNA. Process needs primer for wanted DNA sequence, because DNA polymerase can add nuckleotides only if there is 3'-OH group. Primers are short single stranded DNA pieces. Reaction also needs sample DNA template which contains the target sequence and nucleotides to bild new DNA strands. (NCBI, http://www.ncbi.n-lm.nih.gov/projects/genome/probe/doc/TechPCR.shtml.) Figure 7 explains the steps of PCR reaction. First sample DNA is denaturated and single stranded DNA is formed. Primers help enzyme (TAQ polymerase) to start the synthesis of new double stranded DNA. (Bio-Rad, http://www3.bio-rad.com/.)

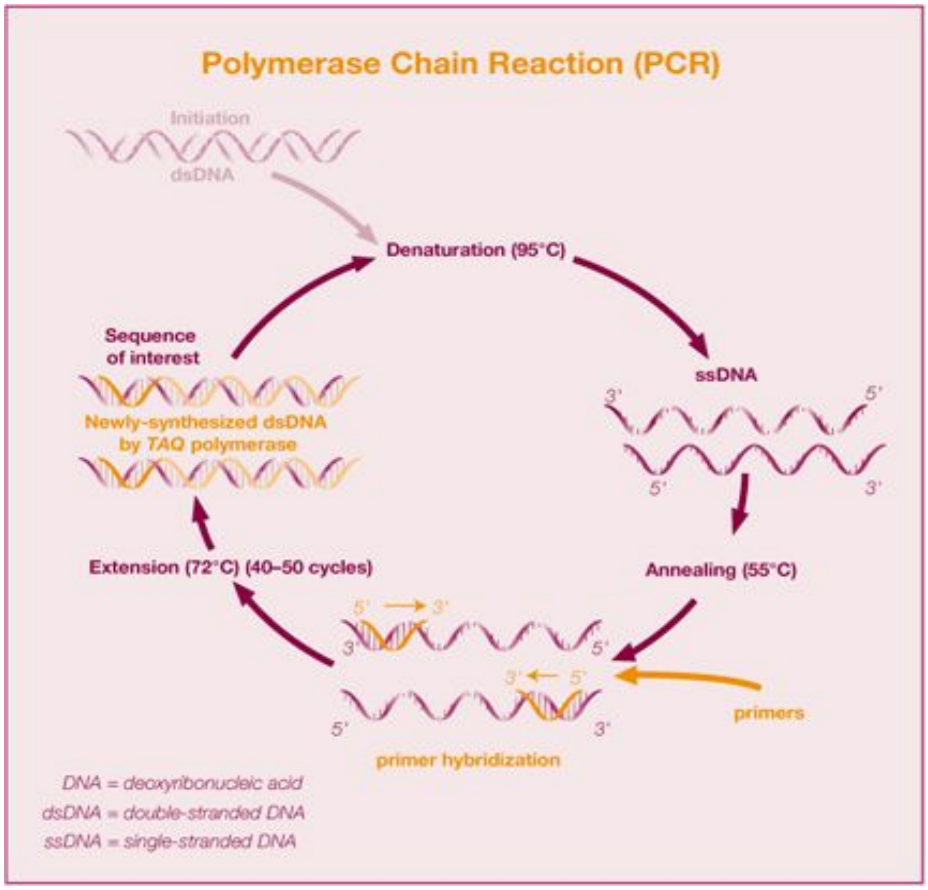

Figure 7. Polymerase chain reaction. TAQ polymerase synthesize new double-stranded DNA from denaturated sample DNA (picture from Bio-Rad, http://www3.bio-rad.com/).

4.2 Lipid droplet staining

Greenspan et al. (1985) have studied many different staining agents for lipid droplet staining and they found out that Nile Red, 9-diethylamino-5H-benzo[α]phenoxazine-5-one, has excellent properties for LD staining and microscopy. Nile Red, chemical structure shown in figure 8, is very hydrophobic, uncharged heterocyclic molecule and for this reason it is poorly soluble in water, but it dissolves in many organic solvents and lipids. (Fowler and Greenspan 1985; Greenspan et al. 1985.)

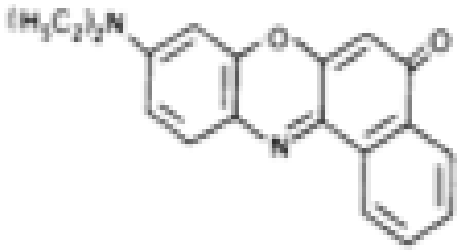

FIGURE 8. The chemical structure of Nile Red (Greenspan et al. 1985).

Nile Red is highly fluorescent in all organic solvents and it fluorescence various colors from golden yellow to deep red. Fluorescence color depends on relative hydrophobicity of the environment. Nile Red interacts with very hydrophobic lipid droplets and when viewed at wavelengths ≤570 nm the fluorescence of LDs is preferentially detected. This selectively will be lost if wavelength of fluorescent emission is over 590 nm. (Fowler and Greenspan 1985; Greenspan et al. 1985.)

When dissolved in lipids like triacylglyserol, Nile Red fluorescence yellow-gold and in more polar environment such as phospholipids or ethanol the color of fluorescence is red. In aqueous media fluorescence is prevented. To use Nile Red does not require any solvents because it dissolves easily in lipids in tissue or cell culture and could be applied in aqueous medium. The stain free from organic solvents is better for the sample, because organic solvents can change the lipid distribution of the tissue sample. Many other lipid stains must be dissolved in organic solvents. These properties make possible to view stained parts without removing staining solution and the color of fluorescence could be used to divide stained sections to neutral lipids and phospholipids. (Fowler and Greenspan 1985; Greenspan et al. 1985.)

Fukumoto and Fujimoto (2002) have compared Nile Red to two other widely used and well known lipid stains, Sudan III and oil red O. These two stains need organic solvents when applied and researchers found out that the solvents cause lipid droplet fusion and deformation. Based on the results of this study, Nile Red seems to be better stain for LDs than oil red O or Sudan III. (Fukumoto and Fujimoto 2002.) One major advantage of Nile Red is that it can be used with living zebrafish by adding the dye directly in zebrafish breeding media. In figure 9. there is a example of lipid staining in living

zebrafish. Lipid increasing and decreasing drugs have been used and amount of lipids could be seen with fluorescence microscope. (Jones et al. 2008.)

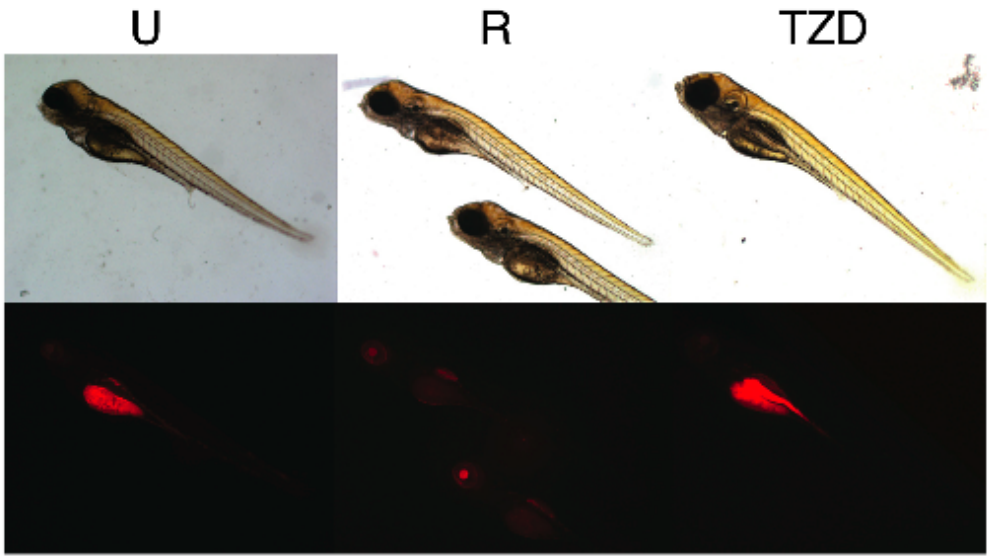

FIGURE 9. Example of Nile Red staining when studying living zebrafish. U means untreated, normal fish, R is fish treated with lipid decreasing drug and TZD is fish treated with lipid increasing drug. Lipid content in each case can been seen as red fluorescence. (Jones et al. 2008.)

Spandl et al. (2009) have used another stain, LD540 succesfully for living cells. LD540 is a lipophilic dye and it has been developed for lipid droplet imaging. LD540 also fluorescents red but it produces brighter fluorescence than Nile Red. The chemical formula of LD540 is 4,4-difluoro-2.3,5.6-bis-tetramethylene-4-bora-3a,4a-diaza-s-indacene and the structure of LD540 is shown in figure 10. (Spandl et al. 2009.)

FIGURE 10. Chemical structure of LD540 (Spandl et al. 2009).

LD540 is effective with living cells (Spandl et al. 2009) but any evidence of using it with living zebrafish could not be found.

5 ZEBRA FISH AS AN ANIMAL MODEL

Animal models are important in biomedical research to understand the pathogenesis of human diseases in cellular and molecular level. Animal models are also needed when testing new drugs and therapies. Mouse models have been commonly used, but they have limitations when large-scale genetic or therapeutic screening is needed. (Lieschke and Currie 2007.)

Zebra fish *(Danio rerio)* (figure 11.) has numerous advantages to be used as an animal model. Zebra fish embryos and larvae are optically clear so visualization of developmental processes is easy. Fertilization is carried out ex vivo, fertility is high and development is rapid. Maintenance costs per animal are relatively low compared to bigger mammalians. Large-scale studies are possible and high-density genomic maps and other genomic resources are available for zebra fish. (Lieschke and Currie 2007.)

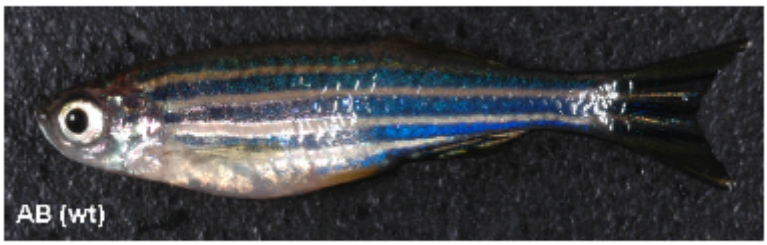

FIGURE 11. Adult zebra fish (White et al. 2008).

There are several mutant lines available which help in therapy and drug development (Lieschke and Currie 2007). One interesting example of mutant zebra fish is transparent *casper* mutant (shown in figure 12) by White et al. (2008). This mutant is transparent not only as embryo and larvae state but also as an adult. Transparency gives new opportunities for visualization and monitoring biological processes. (White et al. 2008.) Liu et al. (2010) used fat-free mutant in their study about lipid and glucose metabolism.

FIGURE 12. Transparent *casper* mutant zebra fish helps in monitoring biological processes (White et al. 2008).

Jones et al. (2008) have studied the effect of common mammalian fat metabolism regulating molecules on zebra fish. They found out that zebra fish Nile Red fluorescence fat metabolism assay could be useful in future studies to develop new therapies for clinical obesity.

For zebra fish husbandry, there is comprehensive book available. The Zebra fish book (http://zfin.org/zf_info/zfbook/zfbk.html) gives all directions for basic caring, breeding and various different research methods.

6 PURPOSE OF THE STUDY

Aim of this study was to find out the effects of perilipins on the phenotype of zebra fish (*Danio rerio*) and compare them with already published results. Genes for PLIN1, PLIN2 and PLIN3 had been previously located in zebra fish genome (Ensembl genome browser, release 61) and in this study we aimed to knock down these genes one by one using anti-sense morpholino oligonucleotides. Amount of lipids and phenotype changes of the whole zebra fish was detected by fluorescence staining and microscopy. Well known fluorescent lipid stain Nile Red (Jones et al. 2008) was used and compared to more recent LD540 stain (Spandl et al. 2009).

Laboratory experiments with zebra fish will be done in the laboratory of University of Tampere which is under permission of State Provincial Office of Western Finland. Finnish Laboratory Animal Welfare Act 62/2006 and Laboratory Animal Welfare Ordinance 36/2006 are followed in the facility.

Hypothesis for knocking down PLIN1 is, that it should increase lipolysis and produce fish with lower lipid content, because PLIN1 inhibits lipolysis and increases fat storage. (Brasaemle et al. 2000; Tansey et al. 2001; Wolins et al. 2005, 2006a; Xu et al. 2006.) Knocking down PLIN2 probably produces leaner phenotype as well, because it also acts as a stabilizer of stored fat (Bonggi et al. 2009; Dalen et al. 2004; Imai et al. 2007; Masuda et al. 2006). Knocking down PLIN3 may not have any effect on lipid content (Ohsaki et al. 2006).

7 MATERIALS AND METHODS

The basic protocol of the study is shown in the table 2. In preliminary studies Morpholinos were designed and the staining protocol tested and optimized. Morpholino injections take place in few hours after fertilization when embryos are under 8 cell stage. In the first day post fertilization (dpf) embryos will be checked with fluorescence microscope to if injections were successful. At 3 dpf embryos will be stained with Nile Red and LD540.

TABLE 2. The schedule of the study.

Preliminary studies	-Determining the concentration of staining solution and staining protocol (Jones et al. 2008) -PCR studies and gene sequencing for morpholino design
Fertilization	-Morpholino injections in the first few hours, with fluorescence marker
1 dpf	-Checking if injections were successful (fluorescence), incubation in E3 water, removing dead embryos
2 dpf	-Refreshing the E3 water, removing dead embryos
3 dpf	-Staining begins (Jones et al. 2008), removing dead embryos
4 dpf	-Visualization with fluorescence microscope, removing dead embryos
5 dpf	-Visualization with fluorescence microscope -Effect of morpholino starts weakening -End of experiment

7.1 Morpholino design

Primers for PLIN1-3 genes were purchased from Oligomer Oy (Helsinki, Finland) and are shown in table 3. Solid primers were dissolved in sterile H_2O to form stock solutions, which have concentrations of 100 µM. Stock solutions were diluted with sterile H_2O to form 10 µM working solutions.

TABLE 3. Sequencing primers for PLIN1-3 genes. F 1-3 are forward primers and R1-3 are reverse ones.

F1, PLIN3	5'-TAC ATC ATC GGG GTG ACC TT-3'
R1, PLIN3	5'-TGA TTG TCC TAT GTG TTA ACA TCT TTT-3'
F2, PLIN1	5'-GCA GCA GCA CTC ATG ATC TAA-3'
R2, PLIN1	5'-TGA CCC TCA AGA AAG AAC AGG-3'
F3, PLIN2	5'-AAG CGT TTT TGT TCG GTT TC-3'
R3, PLIN2	5'-CCT TGC TTT GTC AAC CTA CCA-3'

Eight different wild type AB zebra fish DNA templates were used as samples in PCR study. Master Mix for PCR was made for each forward-reverse primer couple as follows: 10x PCR Buffer 25 µl, dNTP's (2 µM)30,2 µl, MgCl2 30 µl, Primer (forw.) (10 µM) 8,0 µl, Primer (rev.) (10 µM) 8,0 µl, Taq. Enzyme (5 U/ml) 1,4 µl, H_2O (sterile) 130 µl.

23 µl Master Mix and 2 µl DNA template was added in each reaction. Samples were mixed and centrifuged and PCR was carried out with PTC-0200 DNA Engine (MJ Research Inc. USA). Products were tested with gel electrophoresis to ensure the quality. With primers 1 and 2 quality of PCR products was good, but with primer couple 3 results were not successful.

PCR products were cleaned by mixing 55 µl PCR product with 5,5 µl exonuclease I, E.-coli and 11 µl FastAP™ thermo sensitive alkaline phosphatase. Mixture were centrifuged and incubated in 37 °C for 15 min. Reaction was stopped by heating in 85 °C for 15 min. After cleaning the sequencing PCR was carried out. Samples were made by mixing 1 µl cleaned PCR product and 9 µl master mix (Big Dye 10 µl, Dilution buffer

(x5) 20 µl, primer forw. or rev. 10 µl and H_2O 50 µl). Master mix were made for both forward and reverse primers. Sequencing PCR program was carried out. After PCR the samples were precipitated.

Precipitation was carried out by incubating samples at room temperature for 10 minutes. After the incubation 90 µl 70% ethylene alcohol was added in every sample. After 15 minutes incubation at room temperature, samples were centrifuged for 45 min in 2000 g and alcohol was removed. 150 µl ethylene alcohol was added and samples were centrifuged again in 2000 g for 10 minutes. After removing alcohol, sample strips were centrifuged upside down without caps in 700 g for one minute to remove all possibly remaining alcohol.

15 µl Hi-Di™ formamide was added in each sample and samples were mixed well. Samples were incubated for 2 minutes at 95 °C and cooled down in ice bath. Cooled samples were delivered to the sequencing service (core facility of IMT).

Primer for PLIN2 got positive reaction on agarose gel only with one DNA sample out of eight and whole process was repeated. Results on gel were still negative and temperature gradient PCR was carried out with Bio-Rad CFX96 Real-Time system C1000 Thermal Cycler device. The sample giving the positive reaction was used as a control and two different amounts of MgCl2 (30 µl and 45 µl) were used in four different annealing temperatures (64°C, 60,4°C, 56°C and 54°C). On agarose gel, reactions were still negative. Reason for that could be that there are differences between individuals and thus the primer could not bind to all of them.

Gene sequences were compared to the sequences published in Ensembl genome browser and samples were compared to each other to see if there are some differences between individuals. Sequence scanner v1.0 software (Applied Biosystems) was used to analyze sequences. An example of sequence analysis is shown in appendix 1. The browser was updated since the last search and in new release 62 there were some changes. Sequence for PLIN2 was updated and that's why the primer designed with old knowledge did not work. PLIN3 was corrected to be PLIN5 so according to release 62 (April 2011) PLIN1, PLIN2 and PLIN5 could be found in the genome of zebrafish. The new primers for the PLIN2 were designed with Primer3 software and purchased from Oligomer Oy (Helsinki, Finland). The whole PCR study was repeated. This time the results were positive with all samples and sequencing was performed.

When the sequences for all three genes were successful and there were no differences between individuals the sequences were sent to Gene-Tools LLC (USA) for morpholino design. Morpholinos were available only for PLIN1 and PLIN5 and they were ordered. For PLIN2 the company could not produce suitable morpholino.

Therefore the study was done by silencing PLIN1 and PLIN5 genes. PLIN5 regulates fatty acid oxidation and knocking it down may produce phenotype which has increased lipid level. (Bosma et al. 2011; Wolins et al. 2006b)

7.2 Optimizing the staining protocol

Staining protocol was optimized and tested before actual experiments. 3 dpf wild type zebra fish were incubated at 28 °C for 2 hours and 24 hours with different concentrations of Nile Red stain and with LD540 stain. Nile red was saturated solution in ethanol. Three different concentrations of nile red were tested: 1, 3 and 5 µl per 30 ml E3 water which is standard embryo medium containing NaCl, KCL, $CuCl_2$, $MgSO_4$ and methylene blue. 3 µl LD540 was used per 30 ml E3 water. LD540 was used in concentration of 3 µl per 30 ml E3 water.

Fresh E3 water was added on petri dishes with about 25 zebra fish larvae. Nile red was pipeted directly on the dish and stirred carefully. After 2 h and 24 h incubation, pictures (figures 13 and 14) were taken with fluorescence microscope using rhodamine lamp and exposure time of 36,6 ms. Unstained fish were used as a control.

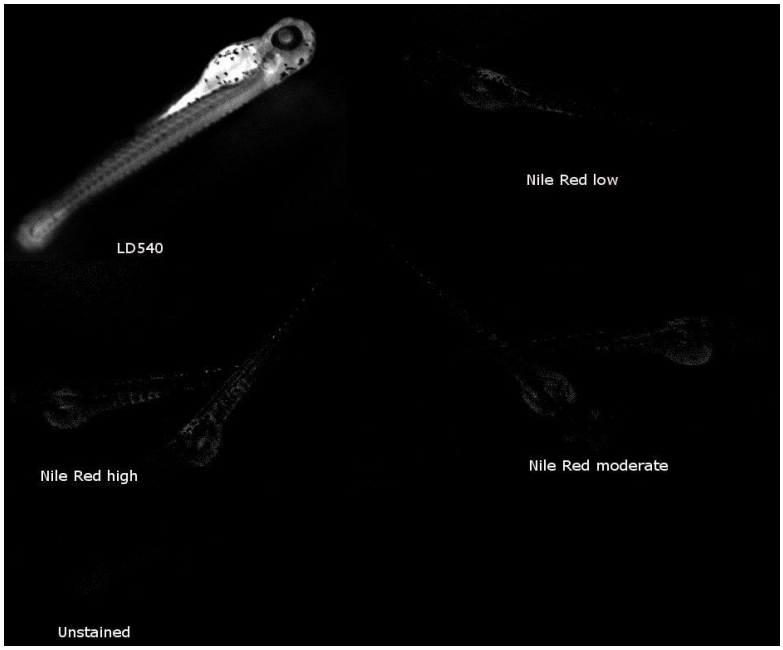

FIGURE 13. 3 dpf wild type zebrafish after 2 h incubation in nile red or LD540 stain. Exposure time of the camera was 36,6 ms.

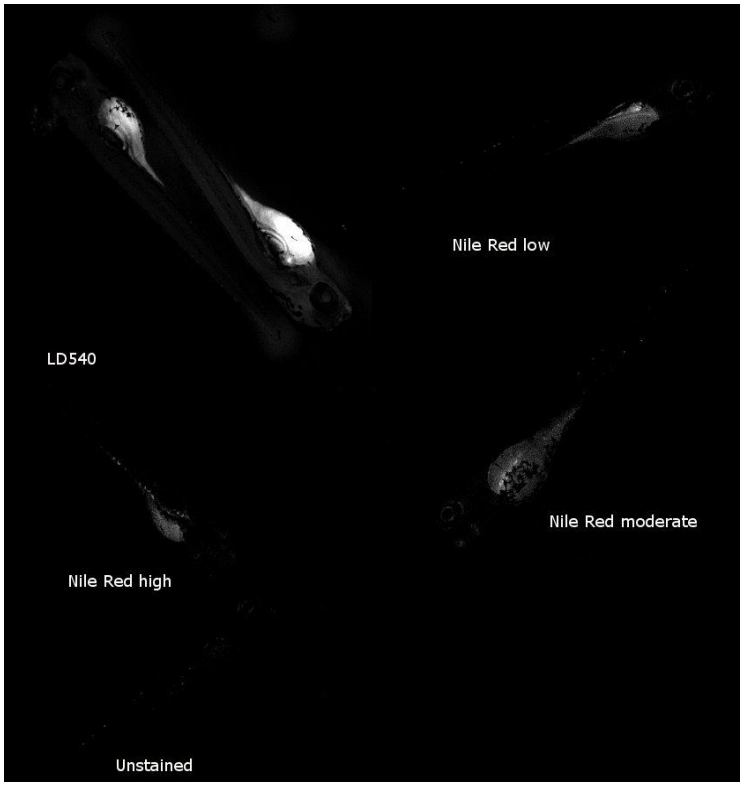

FIGURE 14. 4 dpf wild type zebrafish incubated 24 h in nile red or LD540 stain. Exposure time of the camera was 36,6 ms.

The figure 13 shows that LD540 gave very strong fluorescence after 2 h incubation. After 24 h (figure 14) fluorescence was still strong, but more centered in the yolk sac.

Based on these studies and experience from tests with cells, the first incubation time was shortened to 30 min. LD540 has been used in cultured cells and then methods were different (Spandl et al. 2009). Spandl et al. (2009) noted that LD540 produces brighter fluorescence than Nile Red. LD540 seems to be suitable dye for living zebra fish as well and gives strong fluorescence which is easy to detect.

7.3 Morpholino injection

Morpholinos were dissolved in sterile water according to manufacturer's instructions to get 1 mM stock solution. Stock solutions were stored in freezer. Before use the stock was heated 5 min in 65°C to make sure that all Morpholino was dissolved. For injection, 2,5 µl 1 mM stock solution was mixed with 1 µl 2% rhodamine dextran and 6,5 µl 0,2 M KCl to get 10 µl injection solution with Morpholino concentration of 250 ng/nl. Rhodamine dextran is red, fluorescent agent which helps to see if the injection is successful.

Borosilicate needles were cut and filled with injection solution (shown in figure 15). Microinjector (WPI Pneumatic Picopump microinjector and Narishige micromanipulator) was calibrated to give 1 nl of solution in one egg. Wild type AB zebra fish eggs were collected from fertilization tanks and put on petri dish as shown in the figure 16. Morpholino was injected in the nucleus of embryos and they were put in the 28°C incubator in E3 water. Uninjected controls were put in incubator as well.

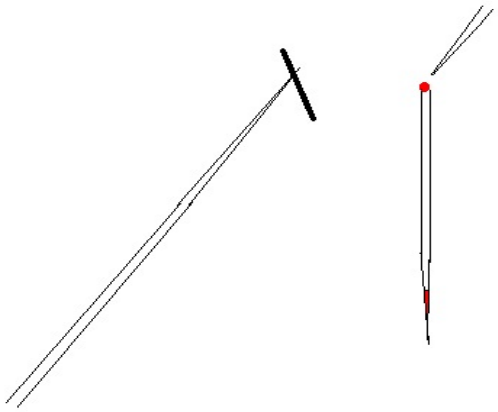

FIGURE 15. Borosilicate glass needles were sharpened by cutting a small piece off with scalpel. Needles were filled by pipeting a little drop of Morpholino solution on top of it and let it settle down.

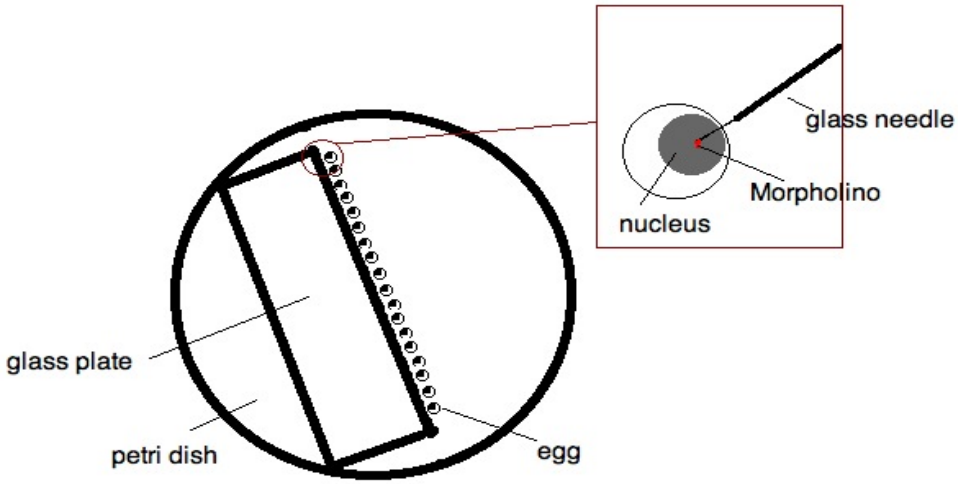

FIGURE 16. Schematic picture of microinjection. Zebra fish eggs are in row on a petri dish and Morpholino solution is injected in the nucleus.

In 1st dpf injected embryos were checked with fluorescence microscope to see if the injection has been successful. In successful injection the whole embryo shows the red fluorescence. In the figure 17 there is a successfully injected embryo. Dead embryos were removed and counted. Embryos were monitored every day and dead ones were removed and E3 water refreshed.

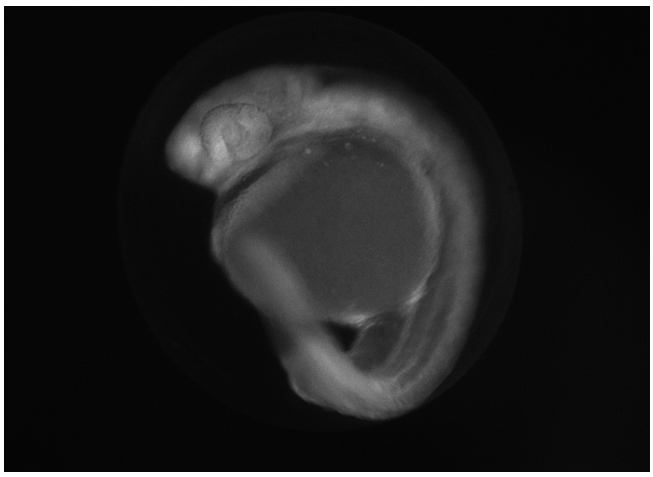

FIGURE 17. Successful injection. The whole embryo is representing fluorescence.

Injection solutions were made with different concentrations of Morpholino to see the effect of amount of Morpholino. The Morpholino for PLIN1 was injected with concentrations of 100, 175 and 250 ng/nl and the Morpholino for PLIN5 with concentrations of 250, 350 and 500 ng/nl.

7.4 Lipid staining

In 3rd dpf the embryos were stained with Nile Red or LD540. 1 µl of stain was used for 10 ml of E3 water so the relation is 1:10000. First images were taken after 30 min incubation and others after 24, 48 and 72h. Images were taken always with exposure time of 33 ms and magnification was 14x or 46x.

8 RESULTS

8.1 Perilipin 1 injected wild type AB zebrafishes

Three different concentrations of Morpholino were tested: 100, 175 and 250 ng/nl. Un-injected zebrafish were used as controls. Concentration of 250 ng/nl was tested first based on experience of other researchers at the laboratory. Other concentrations were used to see if they have any effect on results.

8.1.1 Quality of embryos

By comparing the amount of dead embryos after 24h the effect of injection on eggs could be seen and also the quality of eggs. Sometimes the eggs are not good enough to enable them to develop and for that reason there is always more or less dead embryos. In this case the quality was poor in all three experiments but injection seems not have any effect on mortality in first 24h. Results are in table 4.

TABLE 4. The quality of eggs. Mortality % after 24h incubation.

Experiment ID	Morpholino	Number of eggs	Dead after 24h	Comments
230412	PLIN1 250 ng/nl	163	40,5%	Injection does not have effect
230412control	uninjected	45	64%	Very poor quality
080512	PLIN1 100 ng/nl	130	61,5%	Injection does not have effect
080512control	uninjected	10	60%	Very poor quality
210512	PLIN1 175 ng/nl	191	29%	Injection does not have effect
210512control	uninjected	50	42%	Poor quality

Mortality of PLIN1 morpholino injected embryos during 2-5 dpf is shown in figure 18. Higher concentration seems to have significant effect on mortality and survival of embryos is very poor. Almost 90% of embryos were dead in the 4th dpf. With smaller concentrations survival rate is much better and almost all embryos were alive on the 5th dpf.

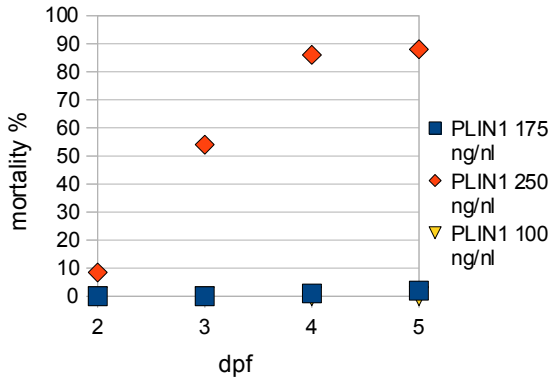

FIGURE 18. Mortality % during 2-5 dpf. 250 ng/nl concentration had significant effect on mortality.

8.1.2 Visualization

Embryos were photographed with microscope (Zeiss Lumar V1.1 fluorescence stereomicroscope) using normal light and rhodamine lamp for fluorescence pictures. Magnification was 46x or 14x and exposure time in fluorescence pictures 33 ms. PLIN1 injected embryos in 1 dpf are shown in figure 19. 100 ng/nl injected embryos look normal, but with higher concentrations some malformation could be seen. With 250 ng/nl 100% embryos were smaller and malformed and for example their eyes were much smaller than normal.

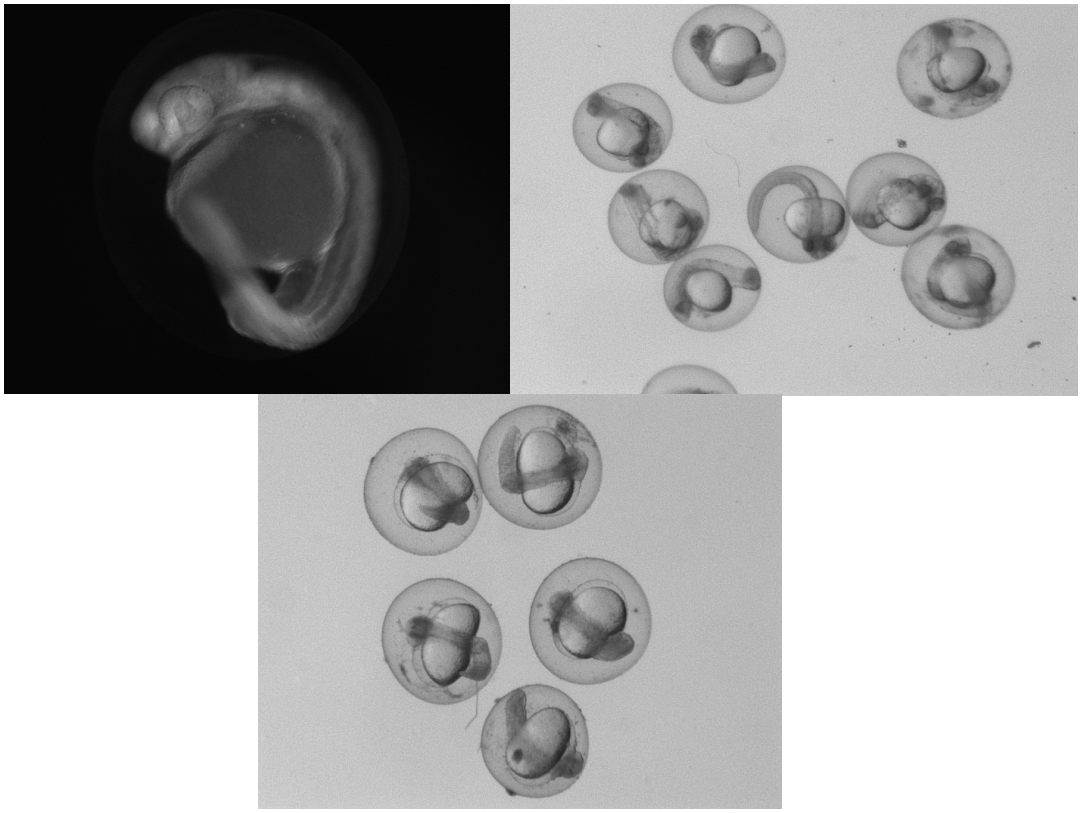

FIGURE 19. PLIN1 morpholino injected embryos in the 1st dpf. Embryos with morpholino concentration of 100 ng/nl (top left) were normal, but with concentrations of 175 ng/nl (top right) and 250 ng/nl (bottom) some malformations could be seen.

In the figure 20 2 dpf embryos have already some differences between individuals. Some of them are poorly developed and for example have smaller eyes than controls. With concentration of 250 ng/nl all embryos are underdeveloped and smaller than controls. With 175 ng/nl there is variation between individuals and several different phenotypes could bee seen. About 6% of them are extremely malformed and similar with 250 ng/nl injected embryos. With the smallest concentration 100 ng/nl embryos look like the uninjected controls.

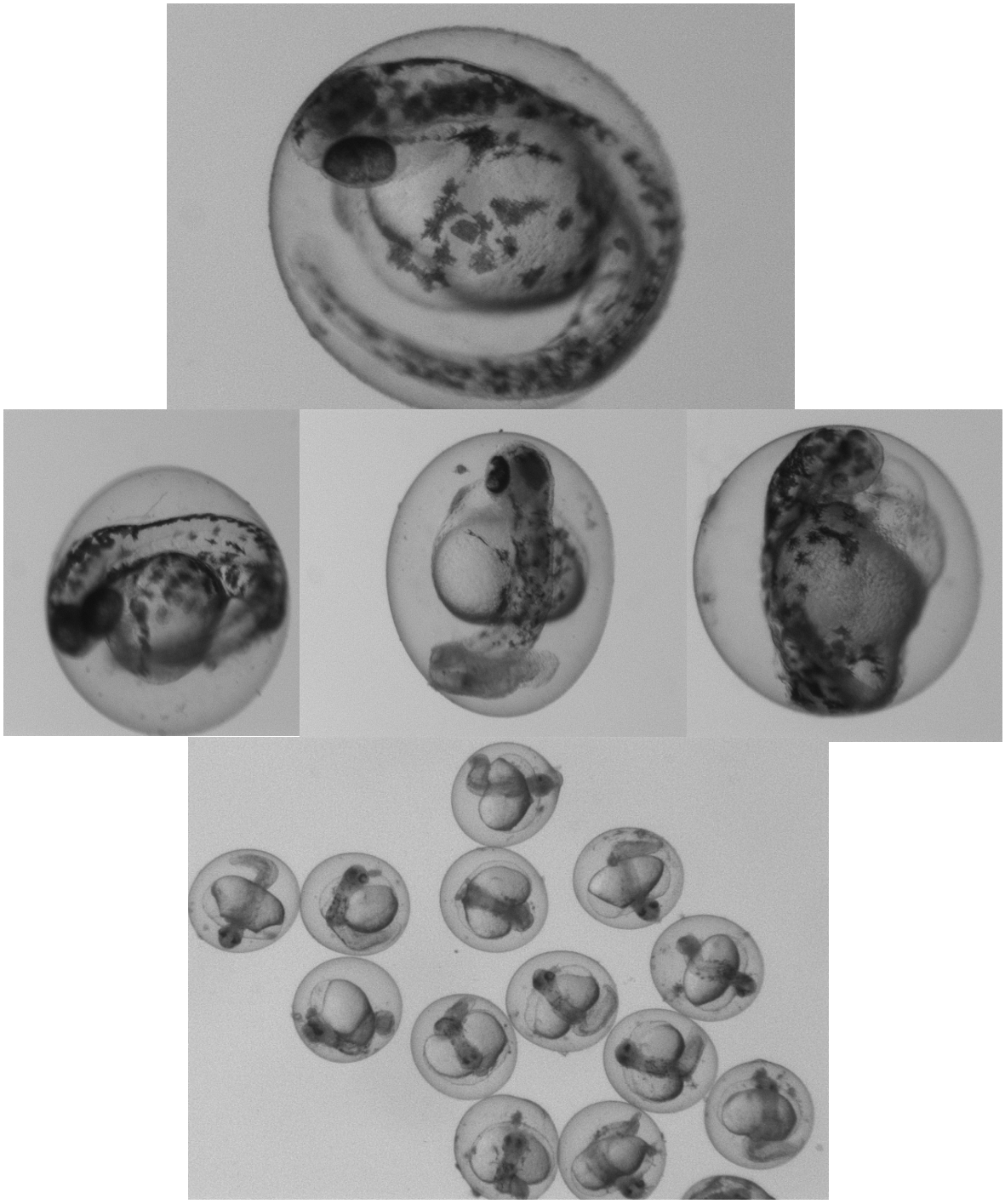

FIGURE 20. PLIN1 morpholino injected 2 dpf embryos. With the concentration of 100 ng/nl (top) embryos are normal, but with 175 ng/nl (middle) there is variation between individuals and several different phenotypes occur. With the concentration of 250 ng/nl (bottom) all embryos are poorly developed and smaller than controls.

In the 3rd dpf (figure 21) few, about 5%, underdeveloped embryos could be seen also in the group with concentration of 100 ng/nl. Most of them are still similar than controls.

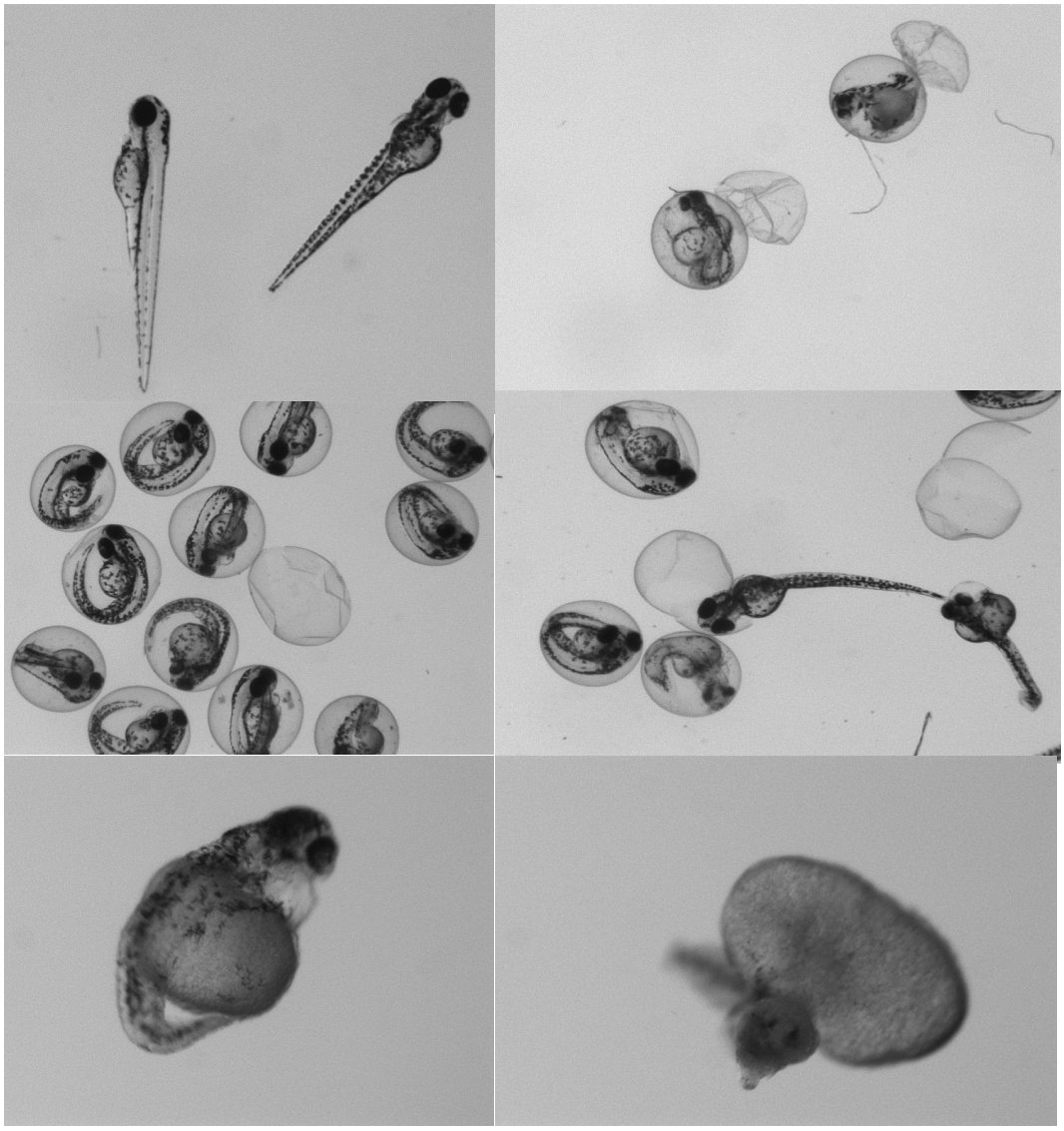

FIGURE 21. PLIN1 morpholino injected 3 dpf embryos. With the concentration of 100 ng/nl (top) few, about 5%, poorly developed embryos could be seen, but others were normal. With 175 ng/nl (middle) and 250 ng/nl (bottom) different phenotypes occur and embryos were poorly developed or malformed.

In the 3rd dpf 250 ng/nl injected embryos were still expressing the fluorescence of the Morpholino (see fig. 22). This has to be concerned when comparing the fluorescence of stained embryos with others. With concentration of 100 ng/nl, Morpholino does not fluorescent anymore in 3rd dpf and with 175 ng/nl there is only weak fluorescence left.

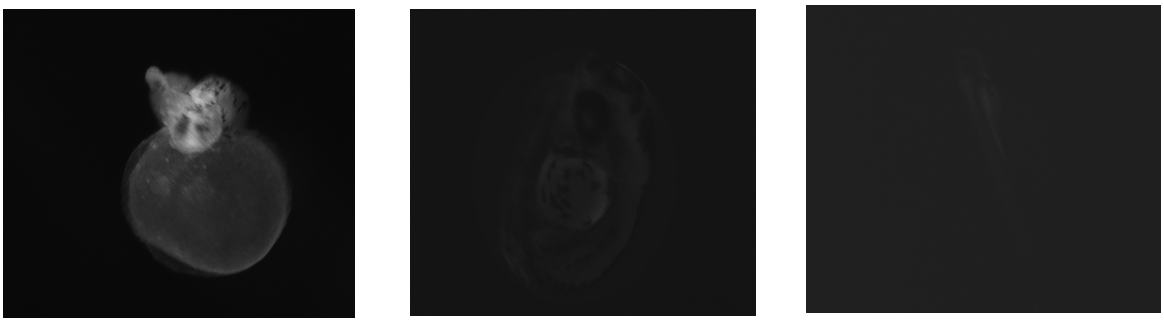

FIGURE 22. PLIN1 morpholino injected 3 dpf embryos: 250 ng/nl (left), 175 ng/nl (middle) and 100 ng/nl (right), controls without lipid staining. Fluorescence of Morpholino injection solution is still strong in the 3rd dpf with 250 ng/nl and it should be taken into consideration when comparing the fluorescence of lipid stained embryos. With the concentrations of 175 ng/nl and 100 ng/nl fluorescence of injection solution was not a problem anymore.

Different phenotypes in the 4th dpf are shown in the figure 23. 250 ng/nl has still the most dramatic effect and all embryos are hardly alive and very poorly developed. With 175 ng/nl most of embryos has some differences to controls. Some of them are significantly malformed and some have only little differences. In 4th dpf also 100 ng/nl has produced some malformed individuals but most of them are still like controls.

FIGURE 23. PLIN1 morpholino injected 4 dpf embryos. With the concentration of 100 ng/nl (top) most of the embryos were normal but few of them were malformed, with 175 ng/nl (middle) most of the embryos had some differences to controls and with 250 ng/nl (bottom) all embryos were hardly alive and very poorly developed.

In the 4th dpf embryos injected with 250 ng/nl PLIN1 are representing some fluorescence due to Morpholino. This has to be taken into consideration when comparing the staining results with other concentrations. Fluorescence is shown in figure 24.

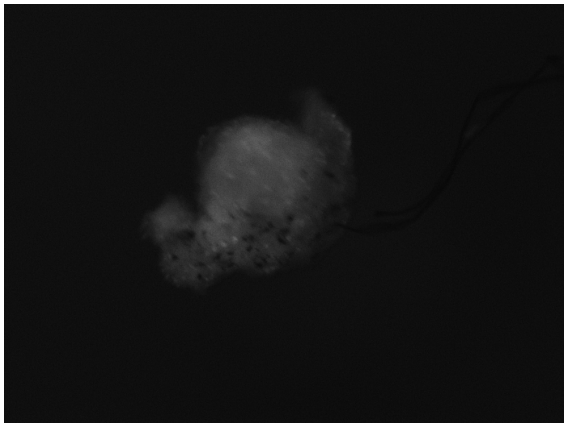

FIGURE 24. Fluorescence of PLIN1 morpholino injected embryo with the concentration of 250 ng/nl in 4th dpf without lipid staining. Embryo was still representing the fluorescence of injection solution.

In the 5th dpf experiments were ended. From 250 ng/nl injected embryos (figure 25) it was hard to say if they were alive or not, because they were so malformed. Others were alive and those looking like the controls were moving normally but some of malformed ones had difficulties in swimming and they were for example only spinning around or moving on their side. 175 ng/nl injected had about 26% very malformed individuals and 100 ng/nl injected only about 5%.

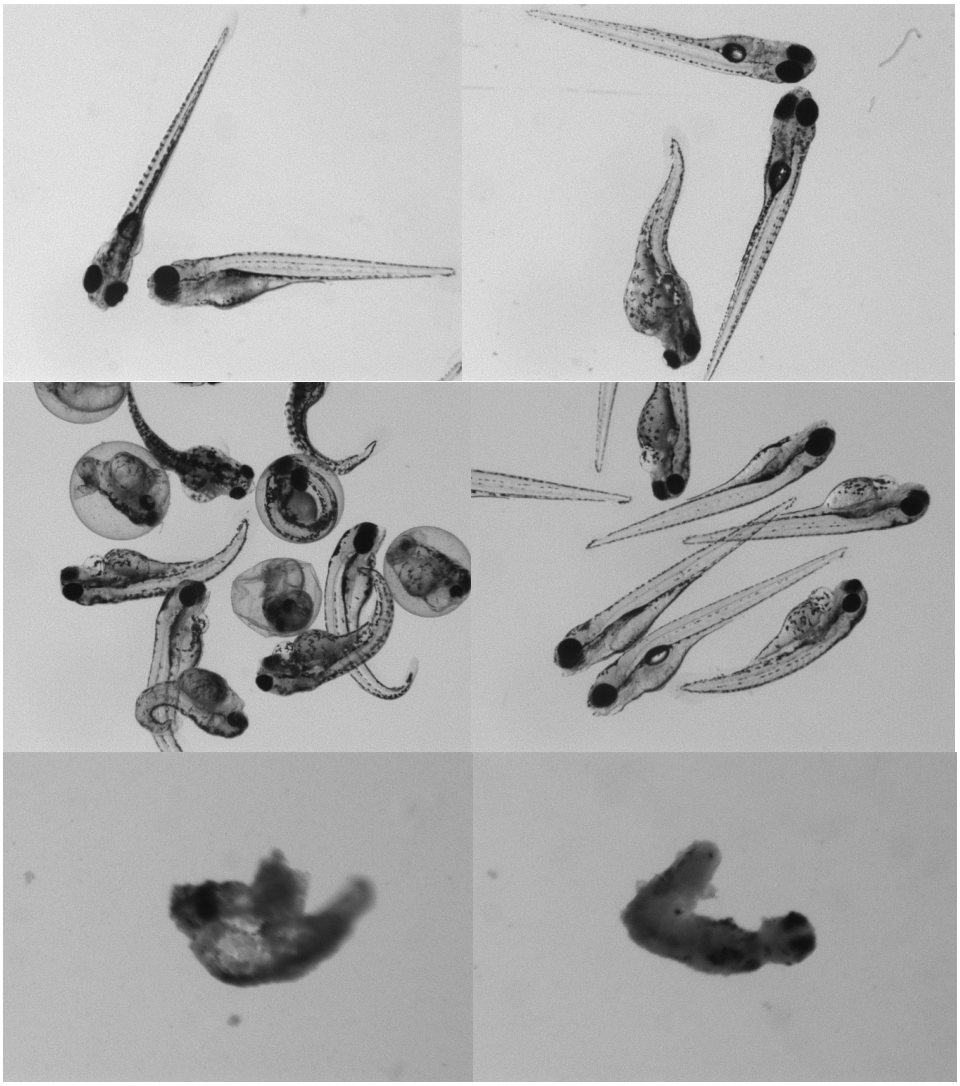

FIGURE 25. PLIN1 morpholino injected 5 dpf embryos. With the concentration of 100 ng/nl (top) only about 5% were malformed and others were normal, with 175 ng/nl (middle) about 26% were seriously malformed and they had some difficulties in swimming too. With the concentration of 250 ng/nl (bottom) it was hard to say if the embryos were even alive because they were dramatically malformed and very poorly developed.

Fluorescence staining was done using Nile Red and LD540 in 1:10 000. First images were taken after 30 min incubation. In figure 26 staining results of PLIN1 injected embryos are shown. It has to be noticed that fluorescence of 250 ng/nl injected embryos is also caused by Morpholino solution as shown in figure. Nile Red shows no fluorescence after 30 min in these embryos but LD540 has already attached on lipids.

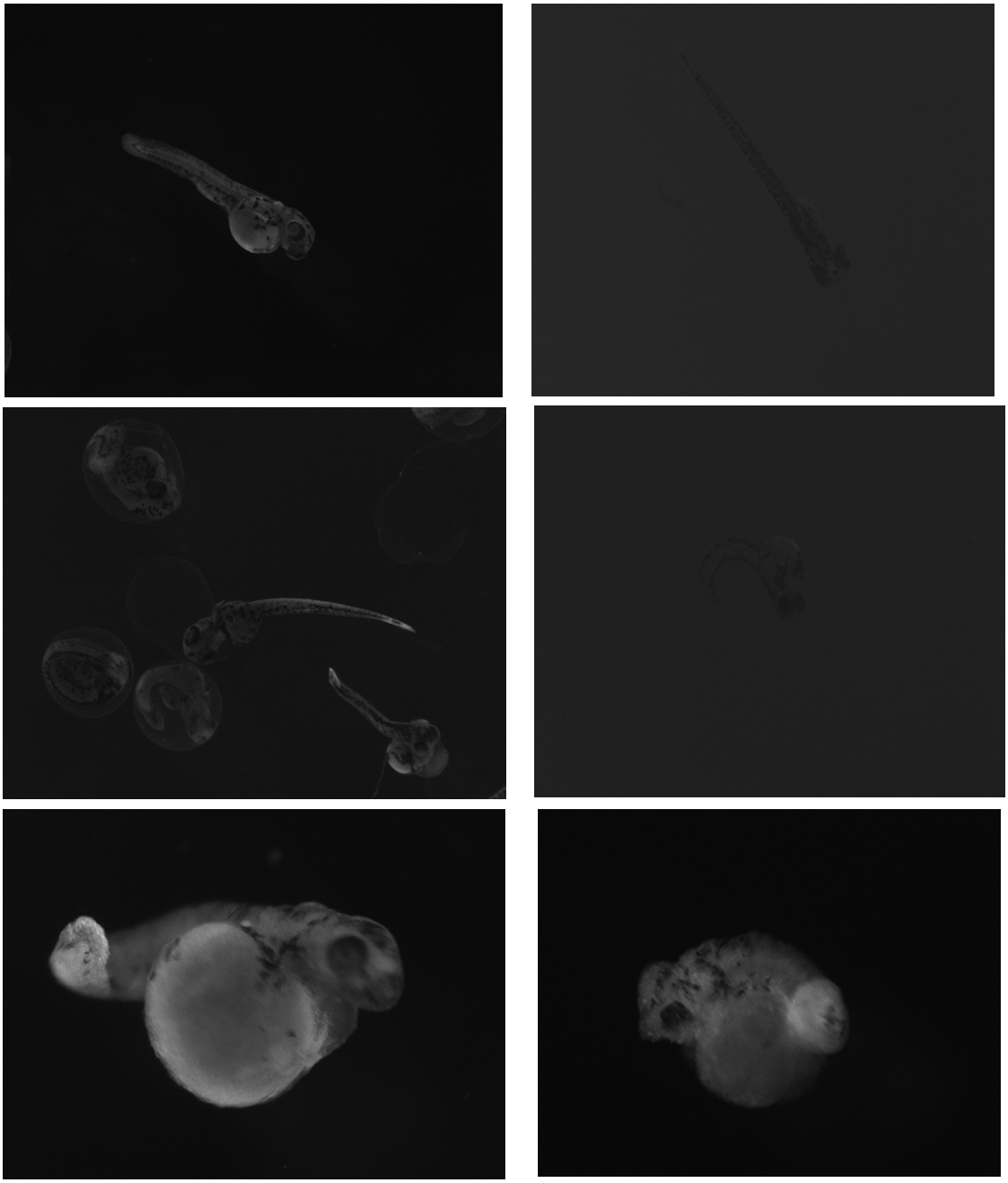

FIGURE 26. Top: LD540 (left) and Nile Red (right) lipid staining in embryos injected with 100 ng/nl PLIN1 morpholino after 30 min incubation. Middle: LD540 (left) and Nile Red (right) lipid staining in embryos injected with 175 ng/nl PLIN1 morpholino after 30 min incubation. Bottom: LD540 (left) and Nile Red (right) lipid staining in embryos injected with 250 ng/nl PLIN1 morpholino after 30 min incubation. Concentration of 250 ng/nl could not be compared to others, because the fluorescence of injection solution is still strong (see fig. 21).

After 24 h incubation (fig. 27) the lipids could be seen in the yolk sacs of embryos. LD540 stain seems to be better than Nile Red. After all, taking good fluorescence pictures with the microscope was difficult and there were problems with camera settings.

Exposure time was set on 33,0 ms every time, but background in some images are still lighter than in others.

In the 5th dpf the effect of Morpholino solution on the fluorescence of 250 ng/nl injected embryos has weakened and images in figure 28 are more comparative to others. Nile Red is also working better and intensive staining of lipids could be seen in the stomach of embryos. It is hard to say if there is any difference between concentrations. LD540 shows probably more fluorescence with 100 ng/nl that 175 ng/nl but with Nile Red the result seems opposite. Comparing 4th and 5th dpf, amount of lipids seems to decrease. In the figure 27 light, fluorescent areas are larger than in the figure 28 with LD540 stain and concentrations 100 and 175 ng/nl. And also the pictures from the 3rd dpf (fig. 26) shows more fluorescence than those from the 4th and the 5th dpf.

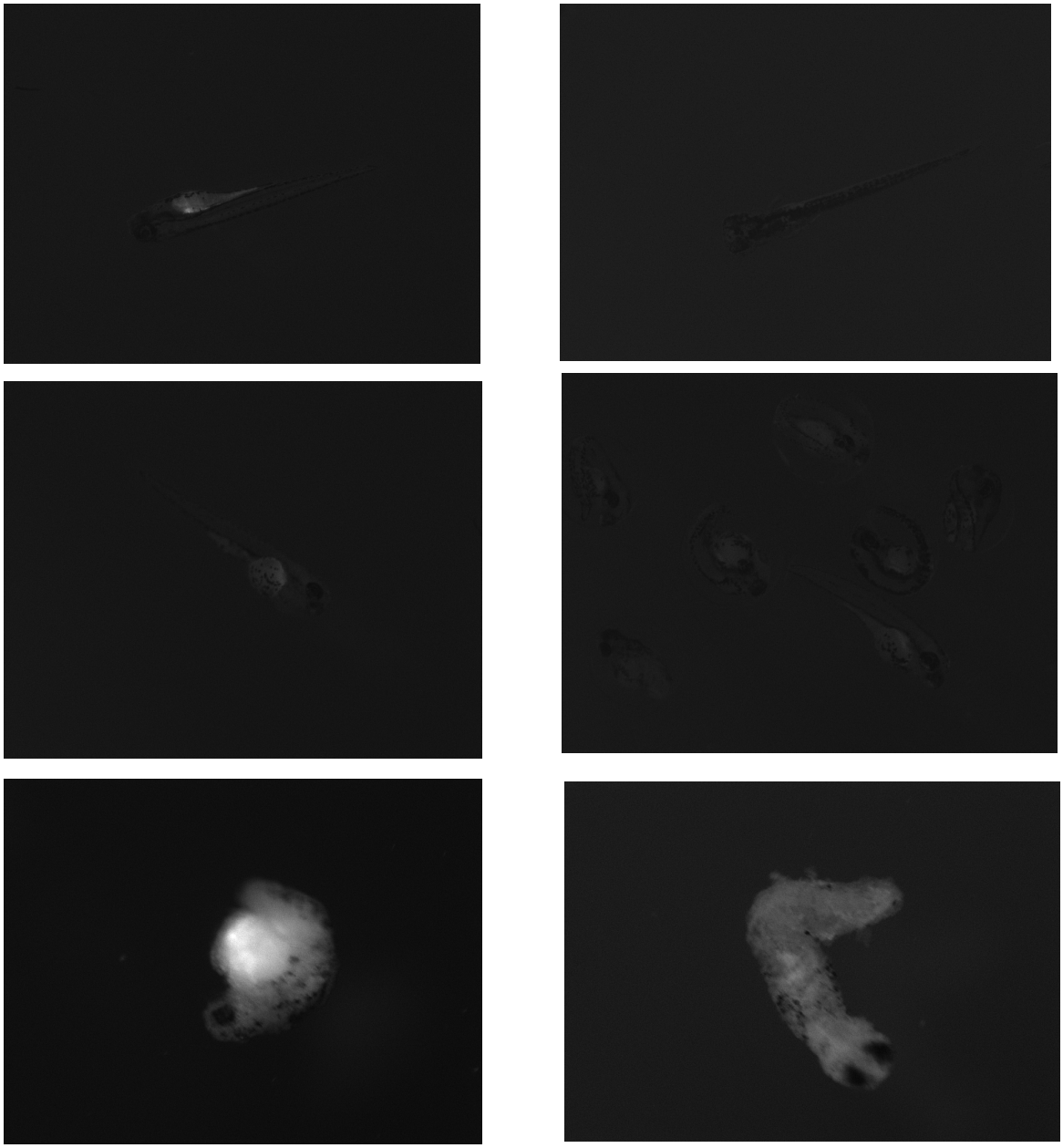

FIGURE 27. Top: LD540 (left) and Nile Red (right) lipid staining in 4 dpf embryos injected with 100 ng/nl PLIN1 morpholino after 24 h incubation. Middle: LD540 (left) and Nile Red (right) lipid staining in 4 dpf embryos injected with 175 ng/nl PLIN1 morpholino after 24 h incubation. Bottom: LD540 (left) and Nile Red (right) lipid staining in 4 dpf embryos injected with 250 ng/nl PLIN1 morpholino after 24 h incubation. Concentration of 250 ng/nl could not be compared to others, because the fluorescence of injection solution is still strong (see fig. 23)

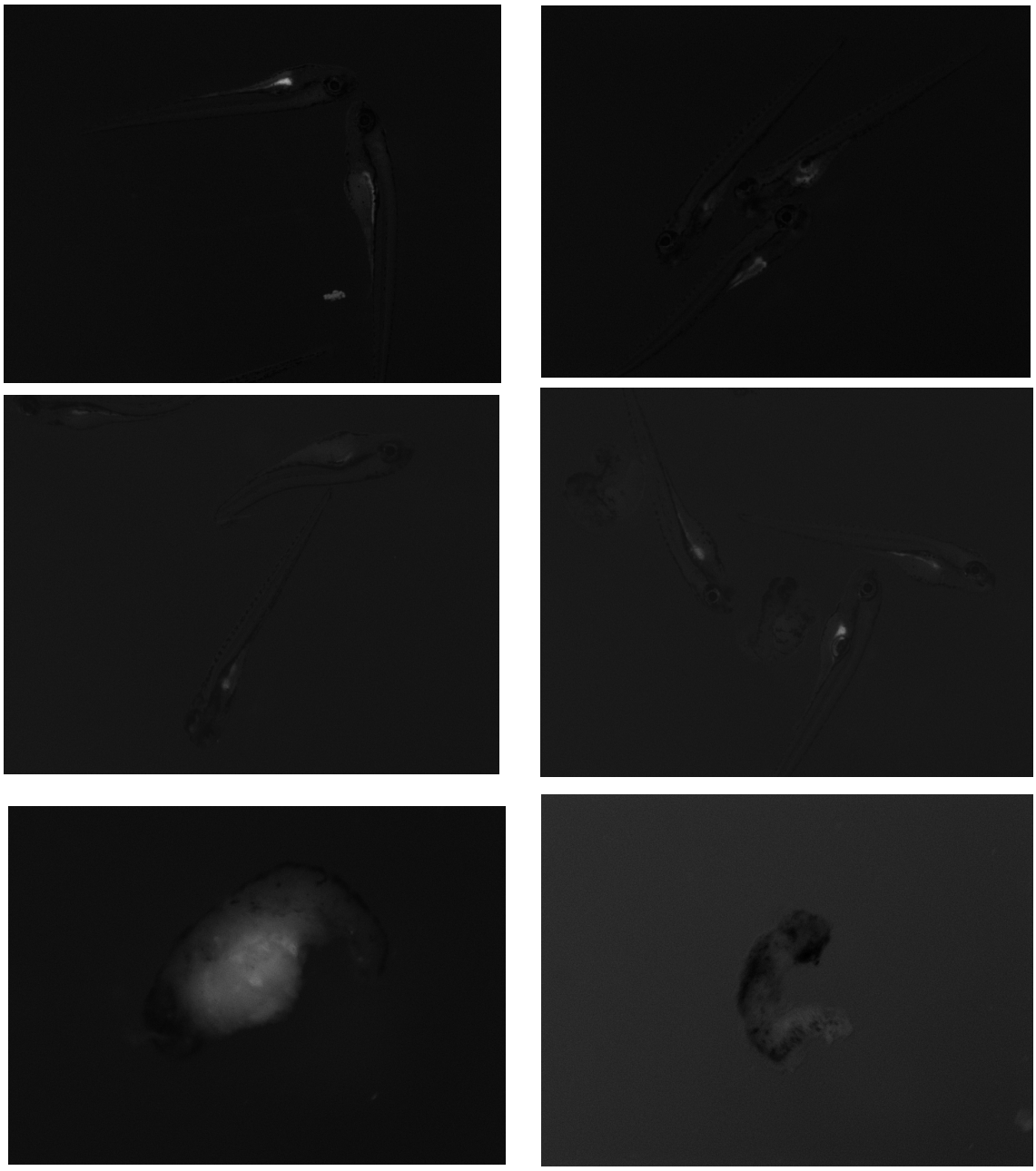

FIGURE 28. Top: LD540 (left) and Nile Red (right) lipid staining in 5 dpf embryos injected with 100 ng/nl PLIN1 morpholino after 48 h incubation. Middle: LD540 (left) and Nile Red (right) lipid staining in 5 dpf embryos injected with 175 ng/nl PLIN1 morpholino after 48 h incubation. Bottom: LD540 (left) and Nile Red (right) lipid staining in 5 dpf embryos injected with 250 ng/nl PLIN1 morpholino after 48 h incubation.

8.2 Perilipin 5 injected wild type AB zebrafishes

8.2.1 Quality of embryos

In these cases the quality of eggs was good, but mortality among injected embryos was little higher than the mortality of uninjected embryos. Table 5 shows the amount of dead embryos after 24 h.

TABLE 5. Quality of eggs was very good in these experiments.

Experiment ID	Morpholino	Number of eggs	% dead after 24h	Comments
240412	PLIN5 250 ng/nl	68	16%	
240412control	uninjected	17	0%	Very good quality
070512	PLIN5 350 ng/nl	100	26%	
070512	PLIN5 500 ng/nl	100	26%	
070512control	uninjected	50	12%	Good quality

In the figure 29 mortality % of PLIN5 injected embryos during 2-5 dpf is shown. Nothing dramatic could be seen in these percentages.

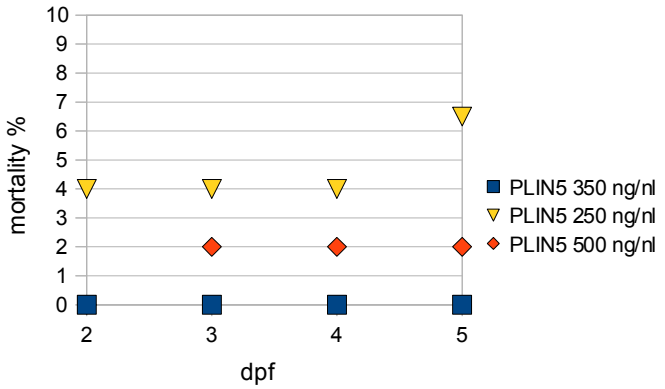

FIGURE 29. PLIN5 injected embryos, mortality % during 2-5 dpf.

8.2.2 Visualization

PLIN5 morpholino injected embryos were photographed like PLIN1 morpholino injected embryos. Figure 30 shows images from the 1st dpf of 250, 350 and 500 ng/nl injected embryos. All of them look very normal and similar to uninjected controls.

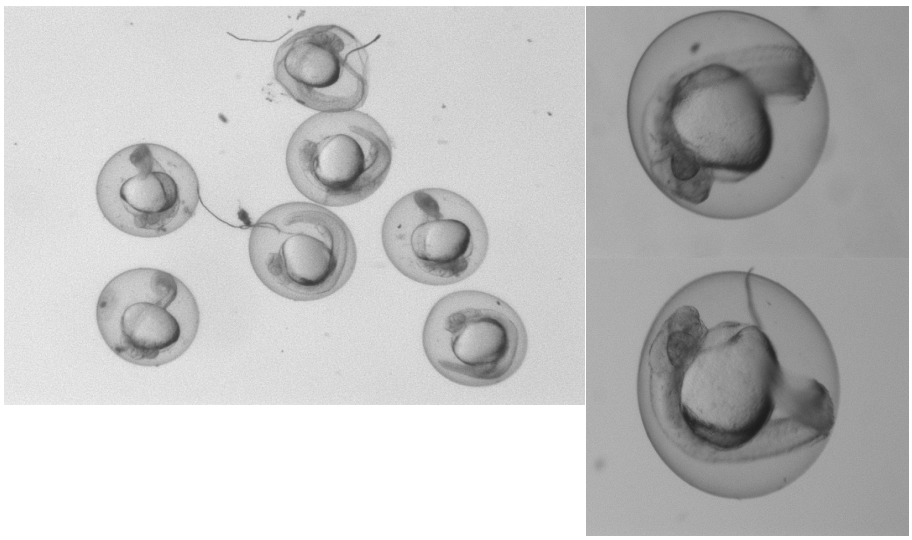

FIGURE 30. PLIN5 morpholino injected 1 dpf embryos with concentrations of 250 ng/nl (left), 350 ng/nl (right upper), 500 ng/nl (right bottom). No visible differences between concentrations.

Figure 31 shows 2 dpf PLIN5 morpholino injected embryos. They are still looking normal and moving lively. No differences between the concentrations could be seen. At 3rd dpf some differences appeared. Pictures from 3rd dpf are in the figure 32. With 500 ng/nl about 10% of embryos had visible malformations. They had some kind of bubbles beside the yolk sac.

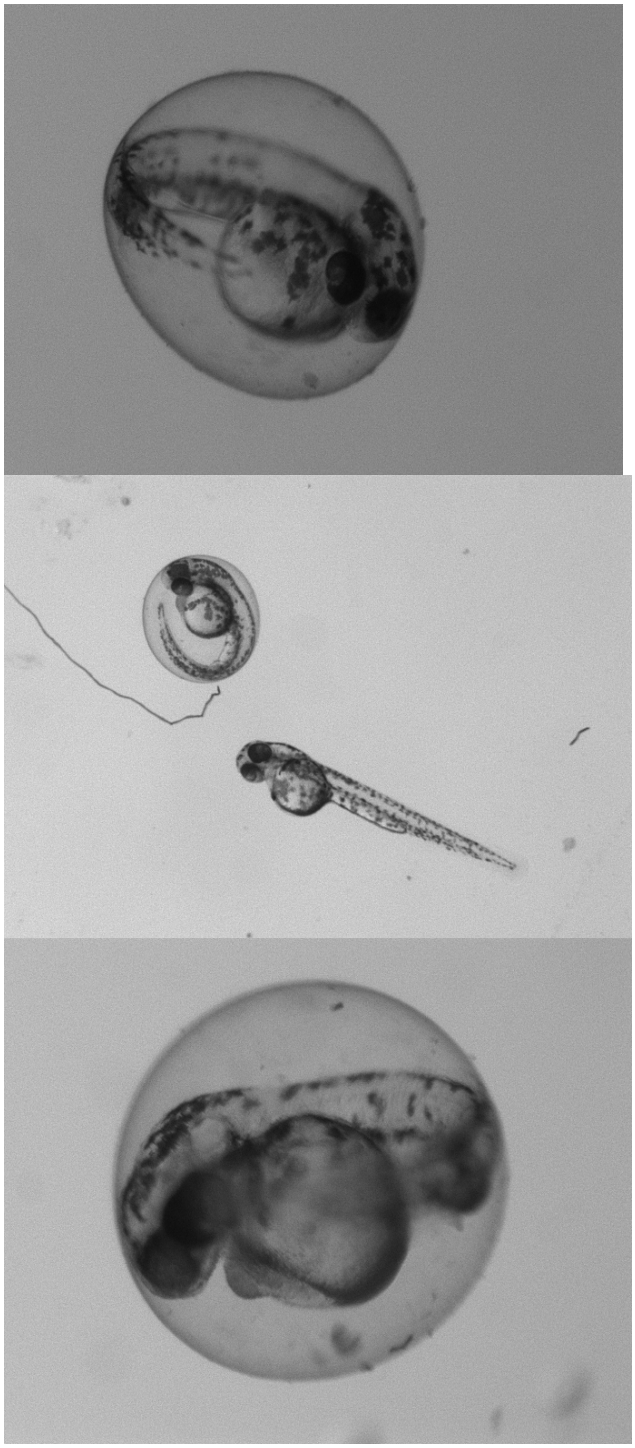

FIGURE 31. PLIN5 morpholino injected 2 dpf embryos with concentrations of 250 ng/nl (top), 350 ng/nl (middle) and 500 ng/nl (bottom). No visible differences between concentrations.

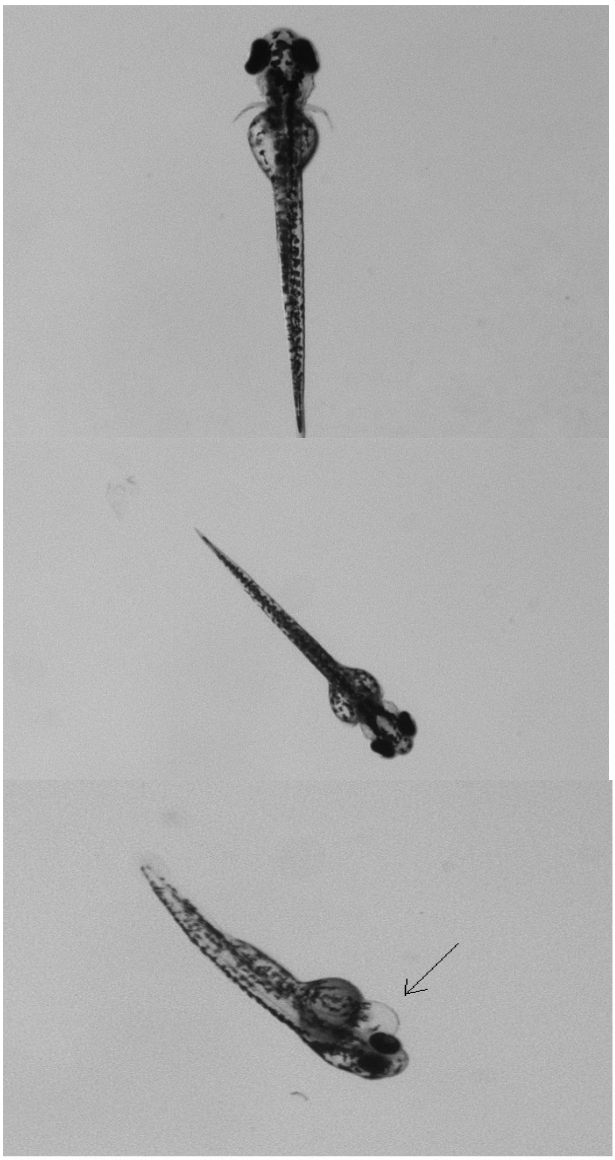

FIGURE 32. PLIN5 morpholino injected 3 dpf embryos with concentrations of 250 ng/nl (top), 350 ng/nl (middle) and 500 ng/nl (bottom). Some differences with the concentration o0f 500 ng/nl could be seen.

At 3rd dpf 500 ng/nl injected embryos were still representing the fluorescence of Morpholino injection solution. 350 ng/nl injected had only slight fluorescence left at that point. Fluorescence photographs are shown in the figure 33.

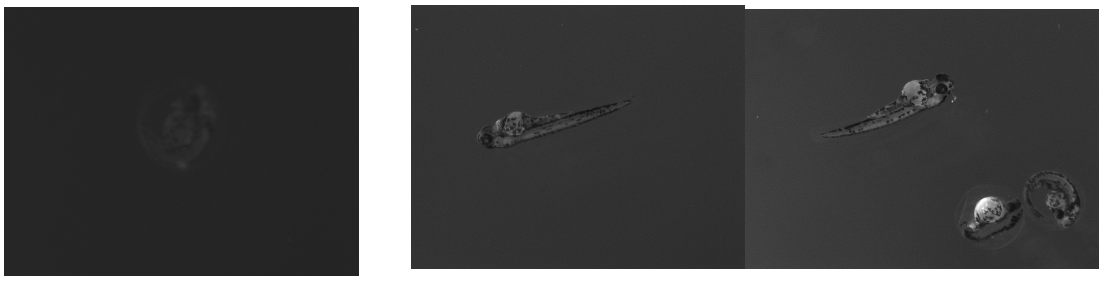

FIGURE 33. Fluorescence pictures without lipid staining in 3rd dpf. PLIN5 morpholino injected embryos with concentrations of 250 ng/nl (left), 350 ng/nl (middle) and 500 ng/nl (right). With the concentration of 500 ng/nl some fluorescence from injection solution could be seen

At 4th dpf 20% of 500 ng/nl injected embryos were visibly malformed (figure 34). Among 350 ng/nl injected fish same kind of malformation could be seen in 11% of embryos. Malformed fish had bubble like structures and their abdominal area seemed to be bigger than controls had. 250 ng/nl injected fish looked very normal, maybe slightly bigger than uninjected controls.

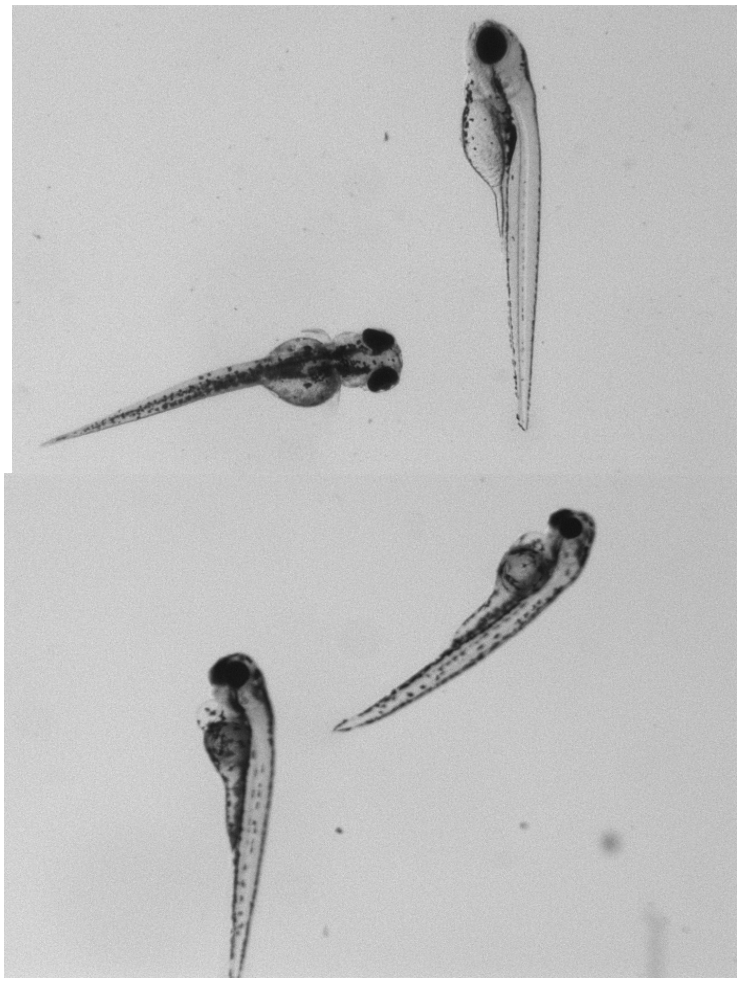

FIGURE 34. PLIN5 morpholino injected 4 dpf embryos. With the concentration of 250 ng/nl (top) embryos were normal, but with 500 ng/nl (bottom) some abnormalities could be seen.

At 5th dpf (figure 35) over 55% of 500 ng/nl injected embryos were significantly malformed and practically all of them slightly. 11% of 350 ng/nl injected had malformations and this point even 9% of 250 ng/nl injected had bubble like structures and were looking obese. Differences between individual were significant in all groups. Some embryos were like controls and did not have any bubbles.

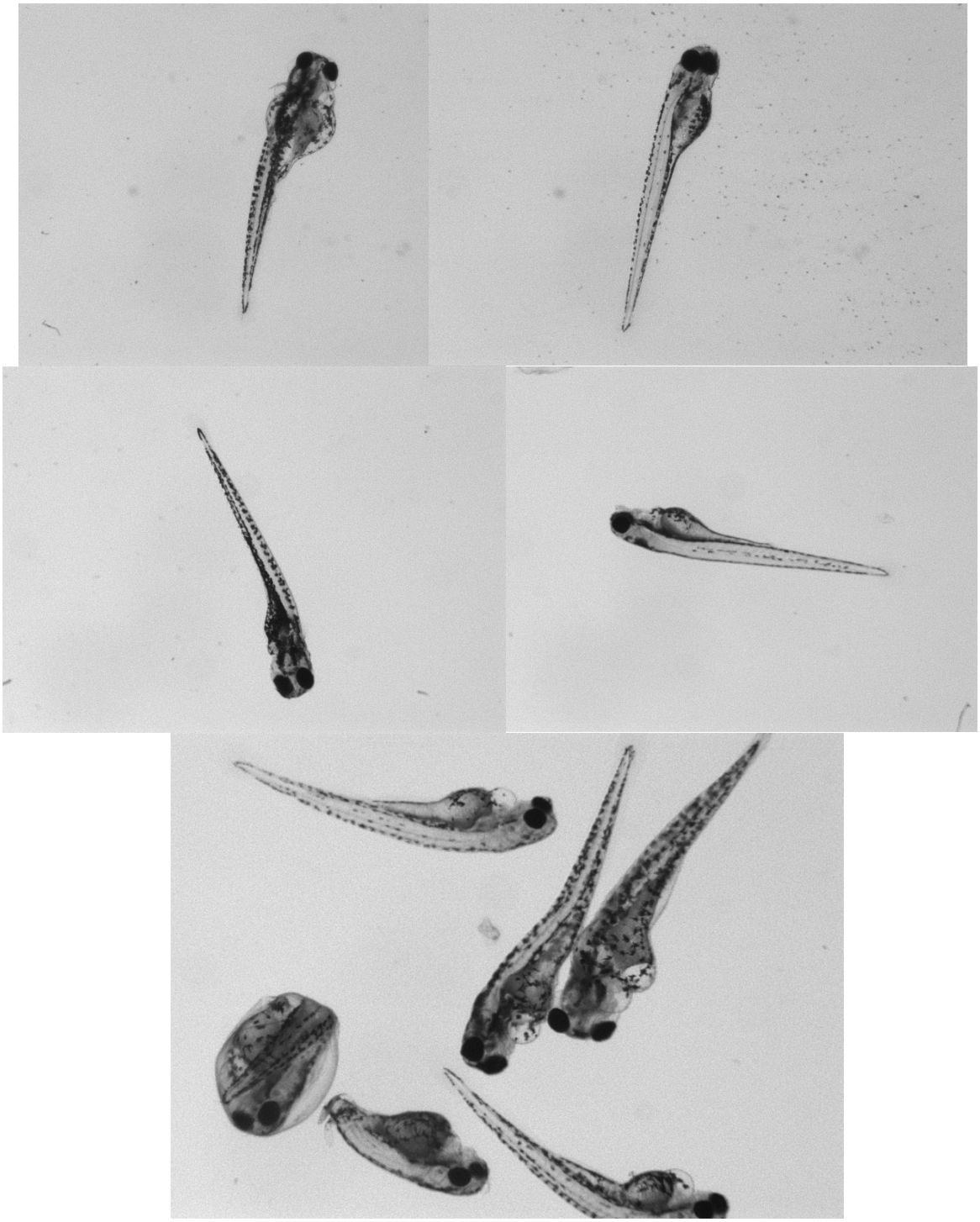

FIGURE 35. PLIN5 morpholino injected 5 dpf embryos. With the concentration of 250 ng/nl (top) 9% of embryos had abnormalities, with 350 ng/nl (middle) 11% and with 500 ng/nl (bottom) over 55% were malformed. Differences between individuals were significant in all concentration groups.

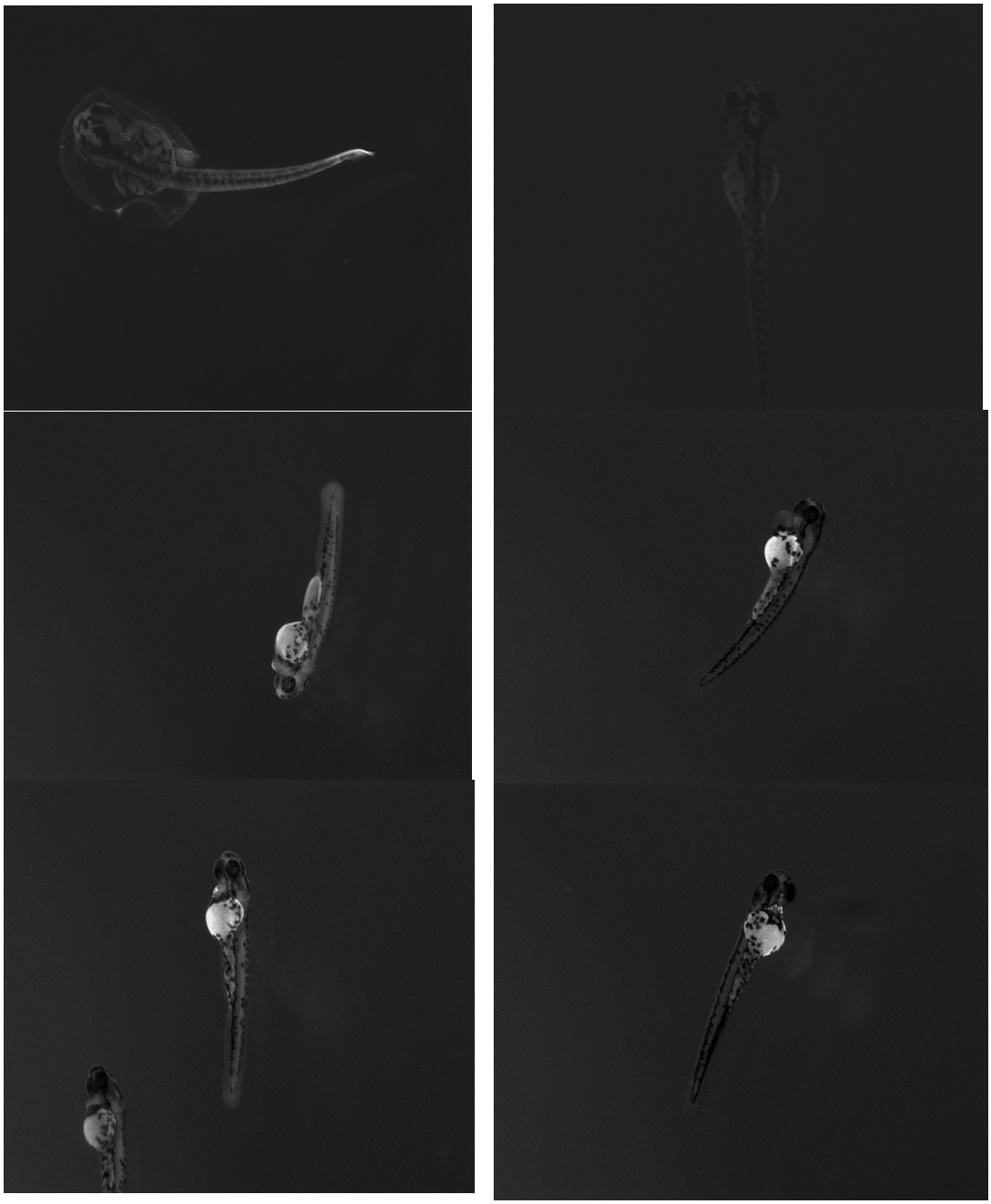

FIGURE 36. Top: LD540 (left) and Nile Red (right) lipid staining in embryos injected with 250 ng/nl PLIN5 morpholino after 30 min incubation. Middle: LD540 (left) and Nile Red (right) lipid staining in embryos injected with 350 ng/nl PLIN5 morpholino after 30 min incubation. Bottom: LD540 (left) and Nile Red (right) lipid staining in embryos injected with 500 ng/nl PLIN5 morpholino after 30 min incubation.

Embryos were stained with Nile Red and LD540 in the 3rd dpf and pictures were taken with fluorescence microscope after 30 min, 24h and 48h incubation. In the figure 36

strong fluorescence in the yolk sac of embryos injected with 350 and 500 ng/nl could be seen with both dyes. 250 ng/nl did not give significant fluorescence.

Lipid staining from the 4th dpf are shown in the figure 37. Now 250 ng/nl injected embryos show good fluorescence with LD540. Image of Nile Red stained embryo is not perfect but the same fluorescence could be seen. Fluorescences of 350 and 500 ng/nl injected embryos could be seen but were not very strong. Results with both dyes were similar.

Results from the 5th dpf are shown in the figure 38. Picture of LD540 stained 500 ng/nl injected embryo shows that there is some extra lipids on abdominal area of the fish. The bubbles seen in pictures before does not fluorescence so those are not lipid bubbles. According to figure 38, PLIN5 has had an effect on lipid content and injected fish have more lipids than uninjected controls (see fig. 43).

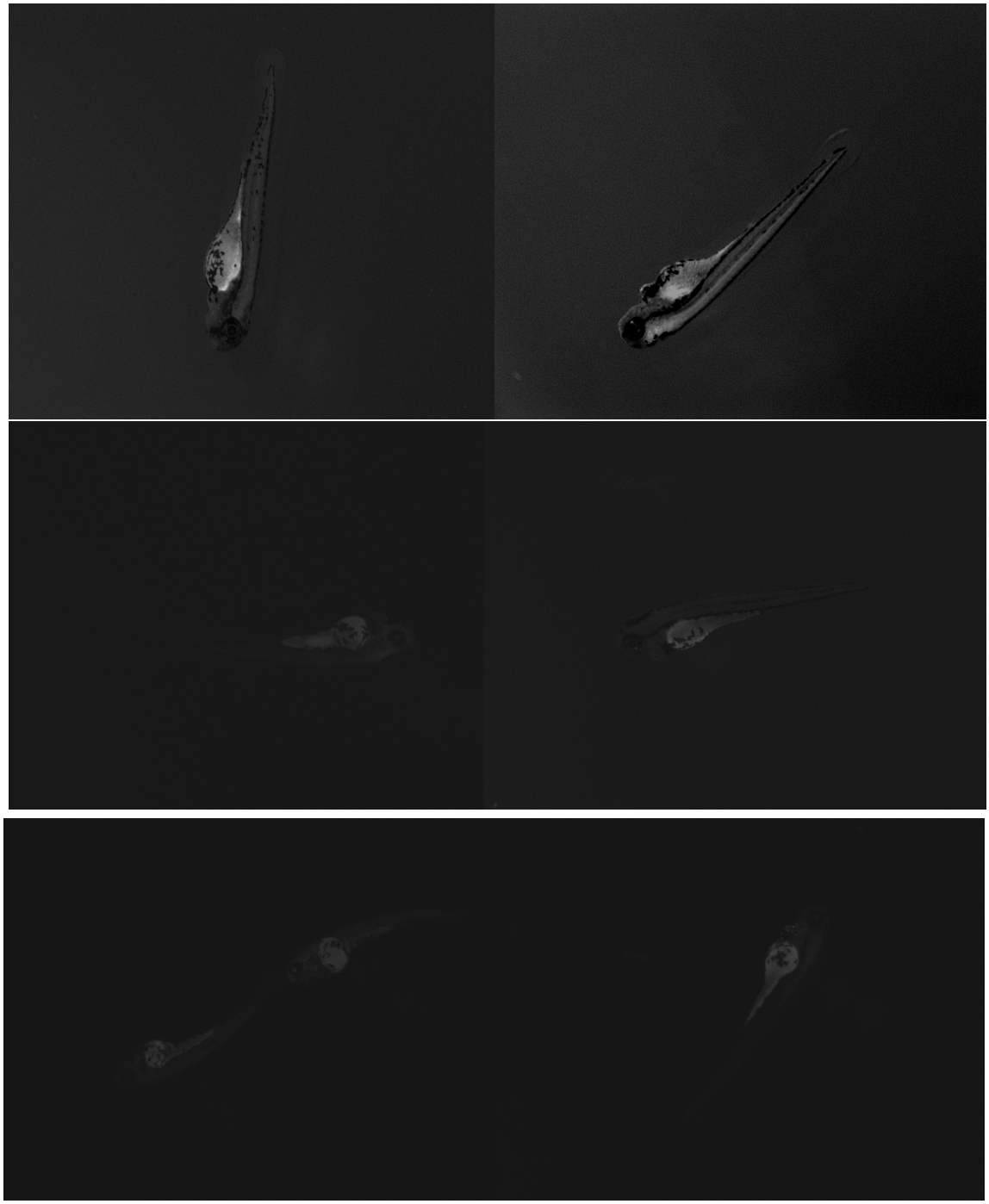

FIGURE 37. Top: LD540 (left) and Nile Red (right) lipid staining in 4 dpf embryos injected with 250 ng/nl PLIN5 morpholino after 24 h incubation. Middle: LD540 (left) and Nile Red (right) lipid staining in 4 dpf embryos injected with 350 ng/nl PLIN5 morpholino after 24 h incubation. Bottom: LD540 (left) and Nile Red (right) lipid staining in 4 dpf embryos injected with 500 ng/nl PLIN5 morpholino after 24 h incubation.

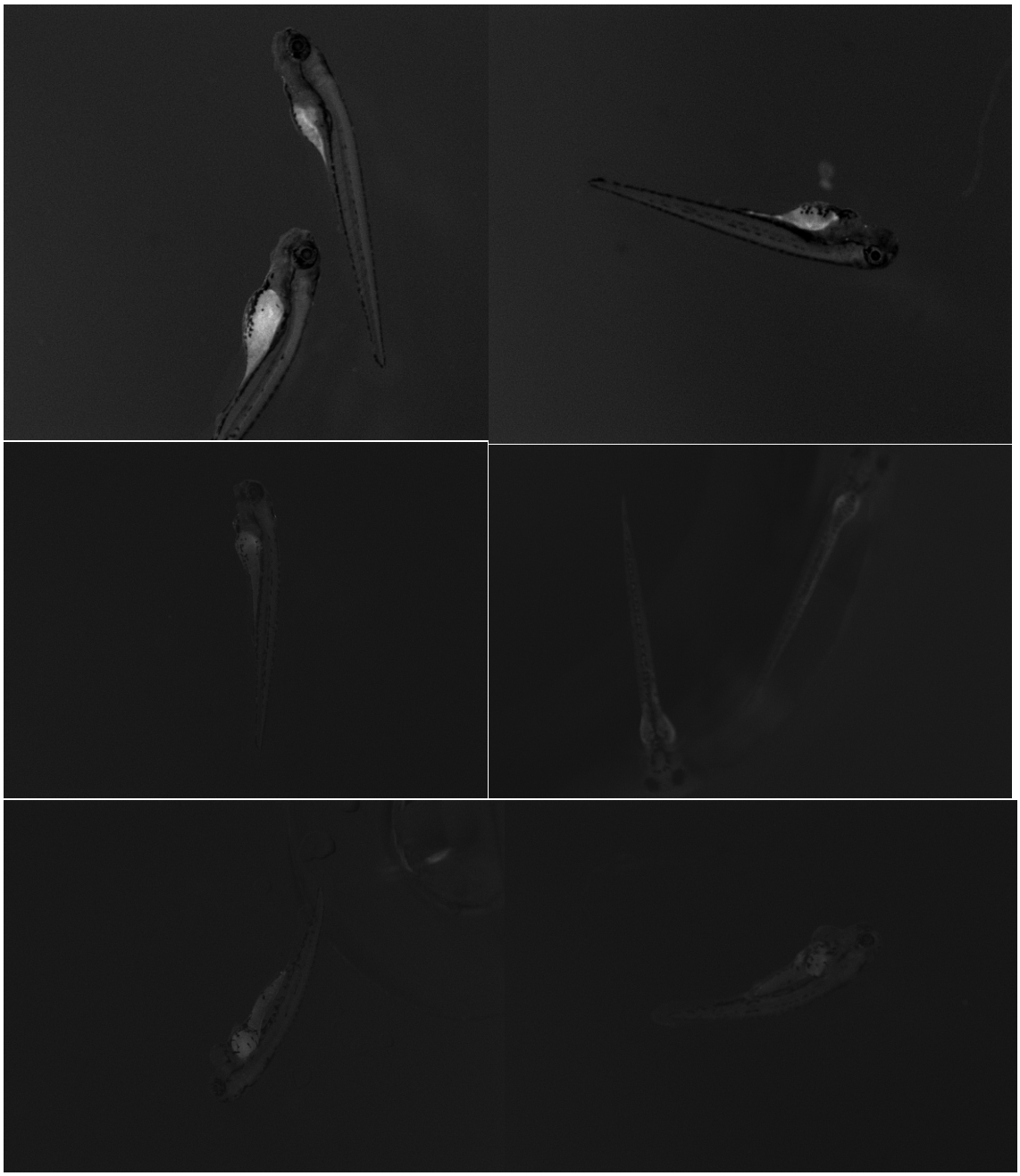

FIGURE 38. Top: LD540 (left) and Nile Red (right) lipid staining in 5 dpf embryos injected with 250 ng/nl PLIN5 morpholino after 48 h incubation. Middle: LD540 (left) and Nile Red (right) lipid staining in 5 dpf embryos injected with 350 ng/nl PLIN5 morpholino after 48 h incubation. Bottom: LD540 (left) and Nile Red (right) lipid staining in 5 dpf embryos injected with 500 ng/nl PLIN5 morpholino after 48 h incubation.

8.3 Uninjected controls

Uninjected wild type AB zebrafish were used as controls. Embryos were photographed with light, stained with Nile Red and LD540 and photographed with fluorescence as well as Morpholino injected embryos. The control phenotype of zebra fish is shown in following figures. In the figure 39 there are control embryos at 1 dpf.

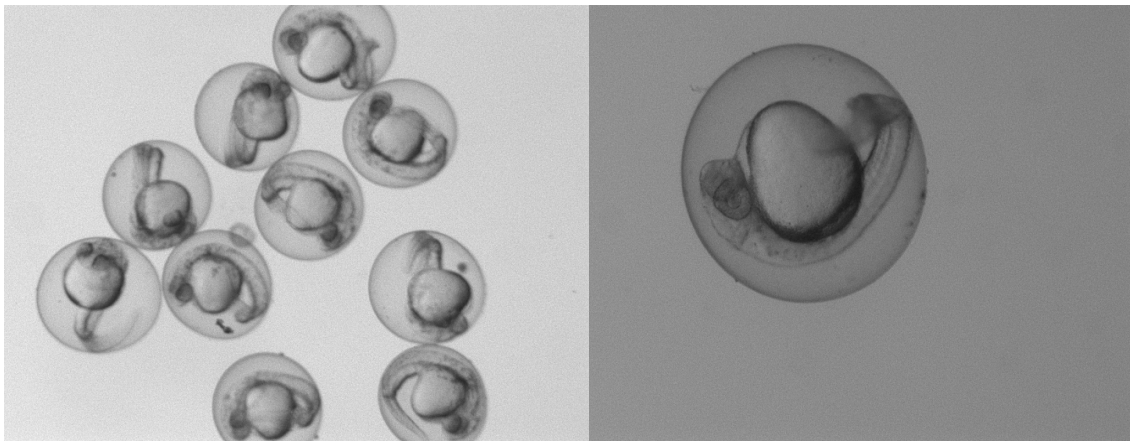

FIGURE 39. Uninjected 1 dpf control embryos. Magnifications 14x and 46x.

In the 2nd dpf some of control embryos had already hatched and they were moving lively. Figure 40 shows uninjected controls at 2 dpf.

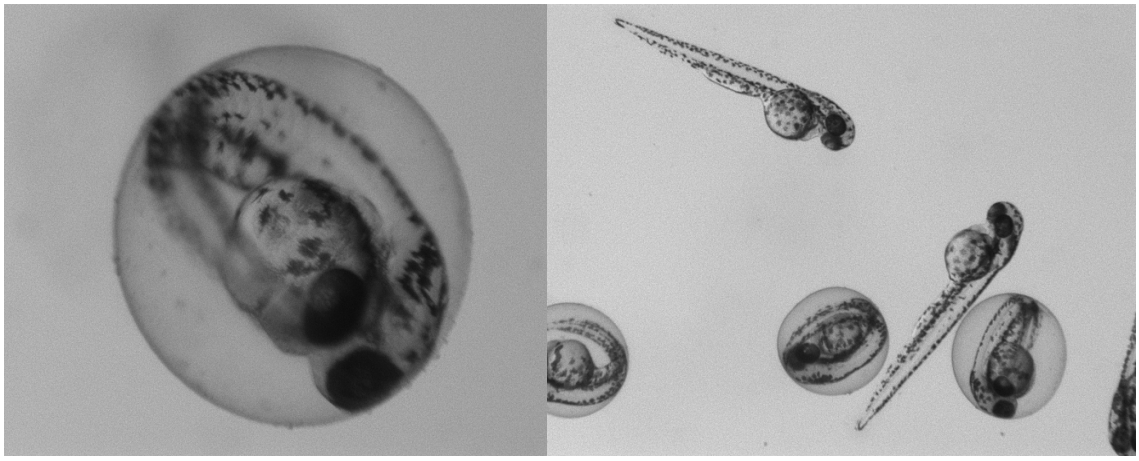

FIGURE 40. Uninjected 2 dpf control embryos. Magnifications 46x and 14x.

Control embryos were stained with Nile Red and LD540 in same way than Morpholino injected embryos. Figure 41 shows staining results of 3 dpf embryos.

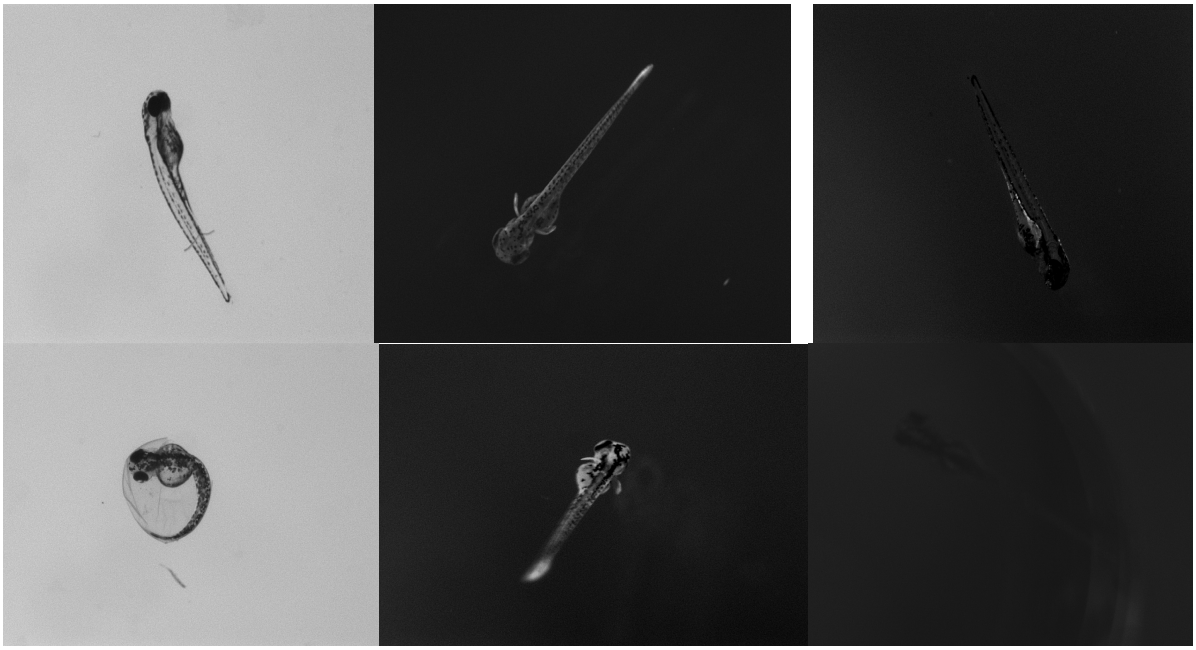

FIGURE 41. Uninjected 3 dpf control embryos. LD540 (middle) and Nile Red (right) lipid staining after 30 min incubation.

Lightmicroscope images and fluorescence staining results of 4 dpf control embryos are shown in the figure 42. Fluorescence of control embryos is weak and only little spots of lipids could be seen. Figure 43 shows some normal variation between individuals at 5th dpf. Fluorescence pictures show small lipid spots.

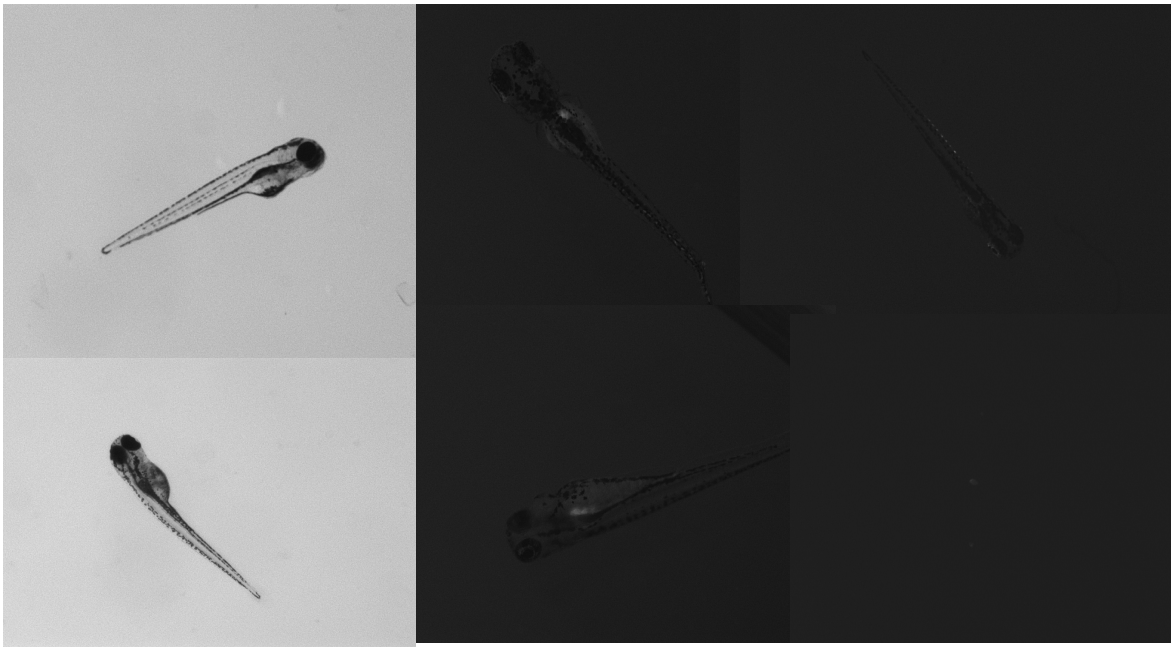

FIGURE 42. Uninjected 4 dpf control embryos. LD540 (middle) and Nile Red (right) lipid staining after 24 h incubation.

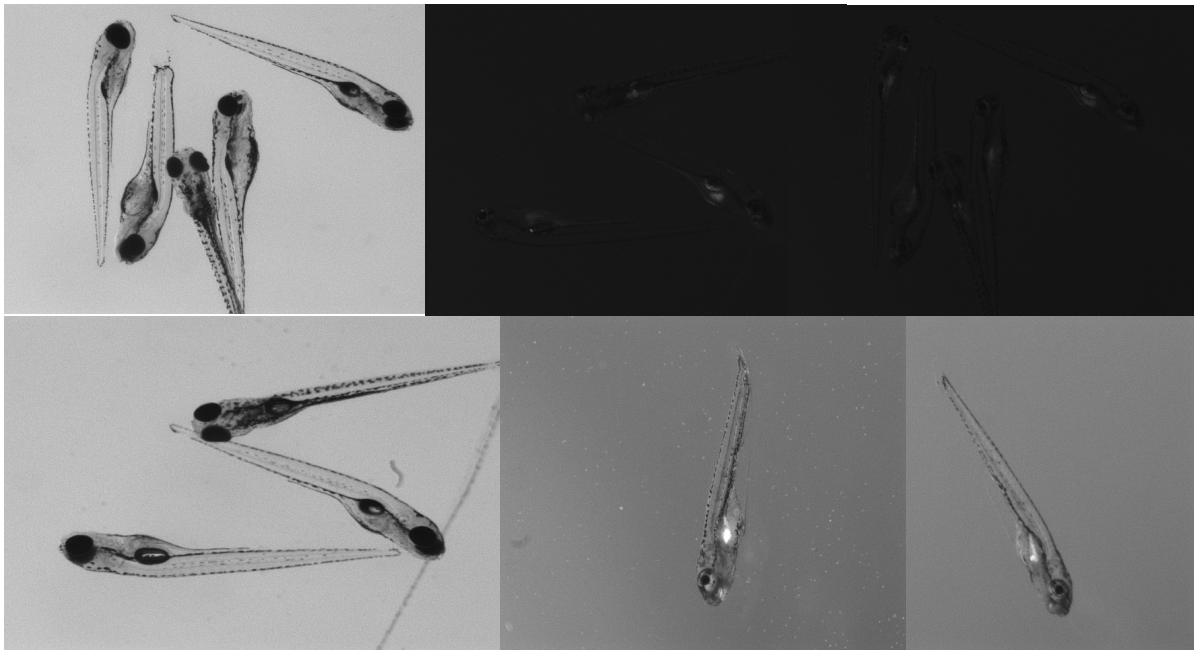

FIGURE 43. Uninjected 5 dpf control embryos. LD540 (middle) and Nile Red (right) lipid staining after 48 h incubation.

9 DISCUSSION

PLIN1 should inhibit lipolysis and increase fat storage (Brasaemle et al. 2000, Tansey et al. 2001; Wolins et al. 2005, 2006a). So knocking down PLIN1 should produce leaner phenotype as in the study of Tansey et al. (2001) in which they produced a lean phenotype in mice by eliminating PLIN1. Comparing PLIN1 pictures and controls stained with lipid dyes it's hard to say if there is some difference in fat content. In the figure 44 there are pictures of 3 dpf LD540 stained control fish and PLIN1 175 ng/nl fish. Control fish has slightly brighter fluorescence so knocking down PLIN1 probably produces leaner phenotype also in zebra fish.

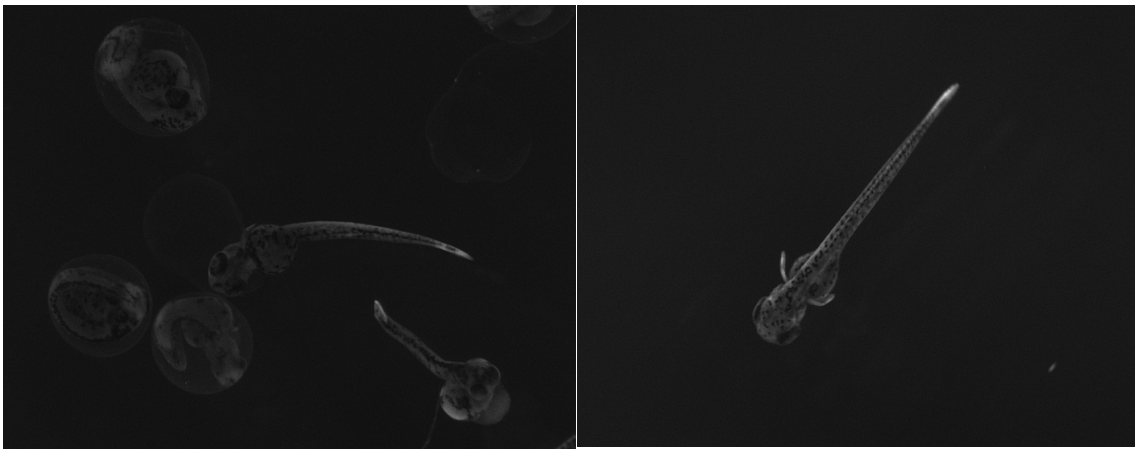

FIGURE 44. PLIN1 morpholino injected 3 dpf embryos with the concentration of 175 ng/nl and with LD540 staining on left and uninjected 3 dpf LD540 stained control on right. Control has slightly brighter fluorescence than PLIN1 morpholino injected.

In figure 26 100 ng/nl injected LD540 stained fish seems to fluorescence as much as controls so probably 100 ng/nl was not enough to reduce lipid content. Concentration of 250 ng/nl instead was too much, because survival rate with this concentration was very low. In the figure 28 it can be noticed that 250 ng/nl injected embryos do not show fluorescence with Nile Red but with LD540 there is some fluorescing areas. Therefore the poorly developed, hardly alive embryos are not totally lean. At least, the Morpholino

has obvious effect on the phenotype and it produces poorly developed fish. This poor development could be caused by the lack of essential lipids.

PLIN5 supports fatty acid oxidation and it has been noticed that in obesity PLIN5 level is decreased (Wolins et al. 2006a). So knocking down PLIN5 should increase fat content (Bosma et al. 2011; Wolins et al. 2006b). When comparing pictures of PLIN5 injected fish and controls it could be seen that injected fish have more lipids and they also look bigger. So PLIN5 acted as hypothesized and had an effect on lipid content. PLIN5 knock down did not have significant effect on survival rate and all embryos, even those with malformations were very viable.

PLIN1 and PLIN5 both produced few similar phenotypes. This phenotype with bubbles and twisted tail could be caused by Morpholino itself, not the genes knocked down. Because control Morpholino was not used in this study it could not been said which effects are caused by gene knockdown and which are side effects. In future studies control Morpholinos should be used and experiments can be done in larger scale.

This study have few problems and issues which should be considered in future studies. Quality of embryos depends on day and individuals mated. Sometimes egg production is very poor and sometimes better. It could not be known beforehand how the embryos survive during the first 24 h. For this reason the amount of successful injections can be small even if hundreds of eggs have been treated. And because injections should be done before 8-cell stage, time is one limiting factor as well. But for good results, number of injected eggs should be as high as possible so in future it might be better to do these experiments also in larger scale.

One problem is the method of determining lipid content. Visual estimation is not very accurate method and the real amount of lipids could not be measured. Quality of pictures also has an effect on results. Fluorescent pictures are all taken with 33 ms exposure time, but they are not similar. Some of them are lighter than others so there has been problem with those settings. In future studies some other methods to determine lipid content should be used to get more accurate, quantitative results for statistical analyses.

Fluorescence of Morpholino injection solution also affects the results. Injection solution has to be marked with fluorescence agent, because without that it couldn't be seen if the injections are successful or not. Problem is, that this fluorescence is mixed with fluores-

cence dye and with the method used in this study it is hard to separate these two fluorescences. But as it can be seen from results, fluorescence of Morpholino solution starts to weak from 3rd dpf and for that reason, pictures from 5th dpf are the most comparable ones especially with PLIN5 injected embryos in which 500 ng/nl produced the most significant results and also expressed the most fluorescence caused by injection solution.

Nile Red and LD540 were both suitable dyes for this study. LD540 has not reported to be used with living zebra fish before, but it worked well. Perhaps it remains in embryo longer than Nile Red and because it has brighter fluorescence (Spandl et al. 2009), it is easier to use in this kind of visual estimation.

10 CONCLUSIONS

As a conclusion perilipin 1 seems to have similar effect in zebra fish as reported in other organisms (Brasaemle et al. 2000, Tansey et al. 2001; Wolins et al. 2005, 2006a). Knock-down of PLIN1 produces leaner phenotype but also has other effects on the phenotype. It causes malformations and underdevelopment. The amount of Morpholino is crucial. Too high concentration causes extremely malformations and dramatically decreases survival of embryos and if the concentration is too low, any effects could not be seen with used method.

Knocking down perilipin 5 produce embryos with higher lipid content. This finding corresponds to previous studies with other animals (Bosma et al. 2011; Wolins et al. 2006b). Morpholino concentration of injection solution needs to be significantly higher than with PLIN1. 500 ng/nl produced visible results with this research method.

LD540 lipid stain was not used before in living zebra fish, but it seems to be suitable. And due to it is bright fluorescence and good stability in embryos it was better than widely used Nile Red in this case.

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Appendix

1. Example of sequence

PLIN1 (ENSDARG00000054048 from ensembl release 62)

Primer sequence

...CGTCTGA... gene sequence

match with sample sequence

missing from sample

Forward primer:

Sample: 2tAB1

AGCTTTATCTTGAGTATAACCTACTAAGTTTTCTGAGATTACTAGACTTAAACATGGTCT

AGTGAGCAGCAGCACTCATGATCTAATAATTCTGTCACGAGATATTTGTGTACATGTGGG

ATTAGACTAGCTGAAGTTTTTTTTTCCACGTATTTCAGCAGCTCATTGAAGACTATTGGAG

ATGGCGTCTGAGAAAAAGGACACCGGTGAAGTTTTGAAGGATCAGGTAATGTTTCATAGT

GAGACAGTTGGCGTCCTCTTGCCGACAGCTCAGTTTTTCTTTTTGAAGGTTGTTGTCTTT

CACTTTTTAATATGAATGCACCTGTTCTTTCTTGAGGGTCAGTGTTGTTTATCAGCATGT

CCAAAACTGTTCTAGTCTATTTAAAAGACAGATCACATAAGTATTACAAATTATGAAGTA

AAAACTGACTTTTTATGAACTTTTTATACATTTAACTCTTTTAATTAGCATATTTGAGCA

GTTAATTTGTATTTTATATATTAATATTCTGTTAAATTAATGGTAAAAATCATATTGTGT

Reverse primer:

sample: 2tAB2

TTCACCAATACAAACACAGATTTATGTCATTCAGAAAGAGATTTTGAAATCATGAAAGGA

TTGCCAGTGCTCATACCTGTAGGTGCAGTTGTTTCAGTCTTTCTGTGATTTGTTGATGAC

AGCTTTATCTTGAGTATAACCTACTAAGTTTTCTGAGATTACTAGACTTAAACATGGTCT

AGTGAGCAGCAGCACTCATGATCTAATAATTCTGTCACGAGATATTTGTGTACATGTGGG

ATTAGACTAGCTGAAGTTTTTTTTCCACGTATTTCAGCAGCTCATTGAAGACTATTGGAG

ATGGCGTCTGAGAAAAAGGACACCGGTGAAGTTTTGAAGGATCAGGTAATGTTTCATAGT

GAGACAGTTGGCGTCCTCTTGCCGACAGCTCAGTTTTTCTTTTTGAAGGTTGTTGTCTTT

CACTTTTTAATATGAATGCACCTGTTCTTTCTTGAGGGTCAGTGTTGTTTATCAGCATGT

CCAAAACTGTTCTAGTCTATTTAAAAGACAGATCACATAAGTATTACAAATTATGAAGTA

AAAACTGACTTTTTATGAACTTTTTATACATTTAACTCTTTTAATTAGCATATTTGAGCA