Primary cell cultures from Cod (*Gadus morhua*) and Atlantic Halibut (*Hippoglossus hippoglossus*).

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A thesis submitted in the fulfilment of the requirements of Stirling University for the degree of Master of Philosophy

Stirling University
In collaboration with
Fisheries Research Services Aberdeen

March 2007

Acknowledgements

I would like to thank both David Smail for all his help and support, and William Starkey for his supervision throughout this project. I would also like to thank all my colleagues at the FRS laboratory especially in Virology for putting up with me throughout as without their help none of this would have been possible.

Abstract

This project investigated the development of primary cell cultures from cod and Atlantic halibut tissues (spleen, heart, optic nerve, brain, liver and tail). By using a range of culture techniques it was concluded that enzymatic disaggregation was the most efficient technique to isolate cells and develop confluent cultures. Almost seventy attempts were made in total to establish a culture. Primary cultures were produced for all organs except liver.

Eagle's Minimal Essential Medium (E-MEM) supplemented with 10% foetal bovine serum was the best all-purpose media of use. Collagen coated tissue culture flasks greatly enhanced attachment of heart cells and poly-D-lysine was beneficial to brain cell attachment. Use of the growth factors epidermal growth factor and endothelial cell growth supplement did not increase the growth of primary cultures but did increase growth in the established cell line CHSE-214 (Chinook salmon embryo). The affect of the chemical mutagen 4-nitroquinoline-N-oxide (4NQO) and two mitogens (calcium ionophore and concanavalin A) on primary cultures was inconclusive. Unusually a few of the heart cultures exhibited spontaneous contractile ability.

Unfortunately although the primary cultures of tail, brain and heart were sub-cultured up to four times, they lost their proliferative potential and eventually died.

1. Introduction

1.1 Primary Culture

A primary culture is that stage of the culture following isolation of the cells but before the first subculture (Freshney, 2000). The first fish cell line was described in 1962, and there is much information available regarding primary culture of fish cell lines as well as those derived from other animals. Fish tissue culture is very similar to the techniques used in mammalian and avian tissue culture and there is much overlap (Wolf and Ahne, 1982). Both Wolf and Quimby (1969) and Wolf and Ahne (1982) provide a comprehensive guide to primary culture specifically relating to fish.

Fish cells seem to be cultured somewhat easily (Wolf and Quimby, 1969). There are nevertheless specific tissues which are cultured more readily than others. Embryonic and larval cells are the most easy to cultivate being in an active a state of mitosis. In the past it was difficult to obtain eggs or fry of some fish species because they are pelagic spawners (Wolf and Quimby, 1969). However due to recent advances, many species which were previously unavailable in embryonic form are now routinely cultivated within the aquaculture industry.

The second most common tissue used for cultivation is fin, due to its high regenerative ability (Fryer and Lannan, 1994). Surprisingly there are not a high number of cell lines originating from gonadal or ovarian tissues since these tissues would also exhibit high levels of mitosis. Almost every other organ is represented in the current list of fish cell lines (Fryer and Lannan, 1994). There are also macrophage cell lines available. Wang *et al.* (1995) have maintained macrophages in culture for more than two years and channel catfish leukocytes have also been maintained as long term suspension cultures (Vallejo *et al.*, 1991). Ultimately the choice of tissue and species will be dependent on what the cell line is required for.

Organ culture could be also used to isolate viruses but is not ideal. Organ cultures are more difficult to prepare, cannot be propagated and therefore are not reproducible (Freshney, 2000). Organ culture is more suited for studies of cell biology than virus propagation.

1.2 Tissue Isolation

The initial stage to consider in fish primary culture is the isolation of the appropriate tissue. One of the main risks faced when isolating tissue is contamination from microbes and/or fungi. Internal organs are considered sterile with the exception of the digestive tract therefore as long as organs are removed aseptically the contamination risk is low. It is prudent however; to disinfect the area of incision or even the entire fish before dissection (Wolf and Quimby, 1969). There are numerous examples of disinfection procedures. The most common reagents used for decontamination include strong disinfectants (chlorine based), 70% alcohol solution and balanced salt solutions (BSS) containing high levels of antibiotics (Wolf and Quimby, 1969). The fish is either immersed or bathed in such solutions for several minutes. To further reduce the risk of contamination from fecal matter, food can be withheld for several days prior to dissection.

External organs and larvae or whole fry pose a greater risk of contamination. Gill tissue can also be especially difficult to decontaminate. Butler *et al.* (2004) detail a method that prevented contamination of their primary gill cultures. Treating the fish in a similar manner as for internal organs appears to be the standard method for decontamination. Methods vary from complete immersion for a few minutes to immersion for hours. Meguro *et al.* (1991) used a series of disinfection steps to ensure no contamination in their fin cultures. Generally there is a greater use of antibiotic solutions to decontaminate tissue opposed to strong disinfectants as these can damage cells (Wolf and Ahne, 1982; Chang *et al.,* 2001; Lai *et al.,* 2003; Chi *et al.,* 1999; Ristow and Avila, 1994).

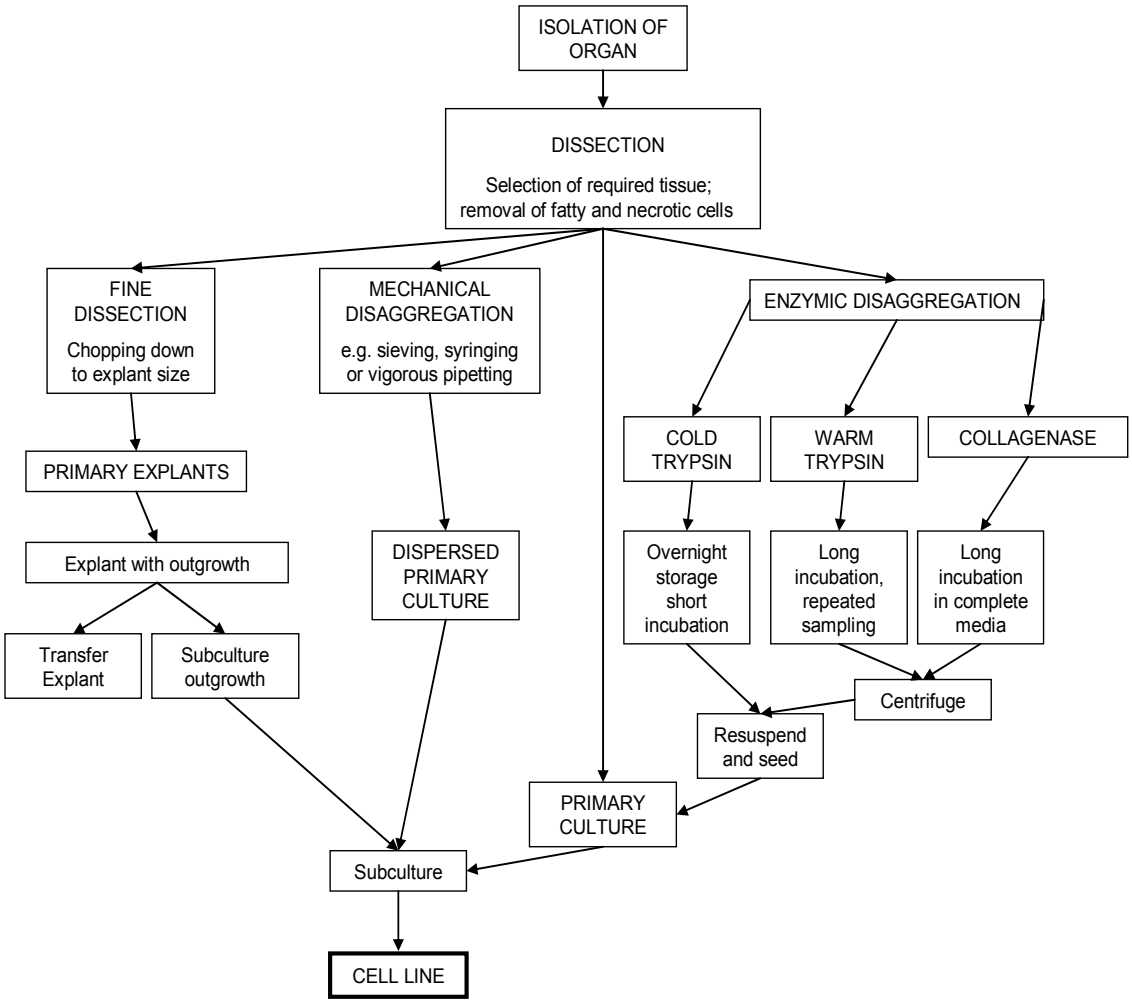

Figure 1.1 Options for Primary culture. (Reproduced from Freshney, 2000)

1.3 Explant

The next stage to consider is the disaggregation of the isolated tissue into individual or small clumps of cells. Figure 1.1 (Freshney, 2000) is a summary of the main techniques of establishing primary cultures. The simplest procedure is the explant method which involves finely chopping the tissue into fragments no larger than 1-2mm^3. These fragments are left to adhere to a tissue culture vessel with the culture vessel positioned so that excess fluid drains away. After the fragments have adhered, further media can be gently added to cover them completely (Wolf and Ahne, 1982). Cells outgrow from the explant eventually forming a culture. Freshney

(2000) suggests enhancing adherence of the explants by using serum or other attachment factors. Several cell lines have been established using this method (Tong *et al.*, 1997; Fernandez-Puentes *et al.,* 1993; Bejar *et al.,* 1997; Tong *et al.,* 1998).

1.4. Mechanical Disaggregation

Another common method of cell isolation is mechanical dispersion. By forcing the tissue through a sieve or syringe individual or small clumps of cells are released from the bulk of the tissue. However this procedure causes a great deal of mechanical damage and produces lower yields than any other method (Freshney, 2000). Furthermore it is only recommended for softer tissues *e.g.* brain, spleen. The only cell lines to be established this way are lines which originate from leukocytes isolated from kidney using a sieve (Wang *et al.*, 1995)

1.5 Enzymatic Disaggregation

The most common method of isolation is through enzymatic disaggregation. The basic method is detailed as follows: tissues are minced and suspended in a digestion mixture, usually trypsin, but other proteolytic enzymes may be used (*e.g.* collagenase or pronase) (Wolf and Ahne, 1982). Cells are harvested from the trypsin by neutralizing the enzyme with media containing serum, then pelleting the cells by centrifugation and re-suspending in fresh media.

There is a great deal of variation to this basic method. Wolf and Quimby (1969) detail numerous variations of temperature, duration of digestion and digestive mixtures. Digestions can be short, extended (*i.e.* overnight) and range from temperatures of 4˚C to 20˚C. A higher temperature results in a faster digestion of the tissue and vice versa a colder temperature will take longer to disassociate cells from the tissue. Lai *et al.* (2001, 2003) used a trypsin solution for digestion at 4°C for one hour to establish cultures. Faisal *et al.* (1995) used a similar trypsin solution for thirty minutes at room temperature. 0.25% trypsin with EDTA is a

common choice as an enzymatic dissociation solution (Lai *et al.,* 2001; 2003; Faisal *et al.,* 1995; Chi *et al.,* 1999; Ristow and Avila*,* 1994; Chang *et al.,* 2001). There is also a great deal of variation in the harvesting method. There may be one harvest or numerous harvests throughout the digestive period resulting in several pellets of cells which are then pooled. Chi *et al.* (1999) repeatedly harvested the cells at thirty minute intervals and pooled all harvests together at the end. Chang *et al.* (2001) also had more than one harvest with trypsinization continuing for any remaining undigested tissue. Butler *et al.* (2004) established a method to isolate gill tissue in which two different enzyme solutions are used alternatively at thirty minute intervals. Cells are harvested from the supernatant after centrifuging rather than the pellet being re-suspended.

It appears that an optimized method must be developed for each fish species as well as specifically for each tissue type. Temperature may well be dependent on the species' natural physiological range. Assessing the viability of yield as well as the resulting cell recovery and attachment will indicate the best isolation method. Viability assessment is fairly standard and Freshney (2000) details a basic procedure using trypan blue and a haemocytometer.

1.6 Media and Additives

Further consideration must also be given to media and possible additives. Factors such as pH, media, sodium chloride concentration, temperature, serum, etc. should also be considered as part of the primary culture procedure. Physiologic salines are used in most tissue culture procedures. Within fish culture the available salt solutions Earle's balanced salt solution (BSS), Hanks BSS and phosphate buffered saline (PBS) have been widely used (Wolf and Quimby, 1969). BSS forms the basis of many complete media and provides the necessary inorganic salts (Freshney, 2000). PBS is a common choice in primary isolations (Meguro *et al.,* 1991; Lai *et al.,* 2001; Chang *et al.,* 2001). PBS is also used

regularly in the subculture of established fish cell lines such as CHSE-214 to rinse cultures before passage.

Eagle's Minimal Essential Medium (E-MEM) supplemented with foetal bovine serum (FBS) comes close to being an all purpose culture medium for the cells of mammals, birds, reptiles, amphibians and of course fish (Wolf and Quimby, 1969). Fernandez *et al.* (1993a) found that although initial culture was in L-15, optimal growth was achieved in E-MEM. Other media routinely used in fish culture are Glasgow MEM, Hank's MEM (H-MEM) and Leibovitz L-15 medium (L-15). Complete media contains essential amino acids, vitamins and salts in varying amounts and combinations (Freshney, 2000). In general most fish cultures use media developed for mammalian cell culture. However some primary cell lines have had specific culture medium designed to optimize growth during development of the primary culture (Wang *et al.,* 1995; Kumar *et al.,* 2001).

The most common addition to basic media is serum. Serum contains growth factors, adhesion factors and is also a source of minerals, lipids and hormones. Serum also acts as a pH buffer (Cheng *et al.*, 1993). Recent work seems to rely on FBS as opposed to fish, horse, sheep, or human sera. Wolf and Quimby (1969) quote instances in which other types of serum have been used but the results appear to be of mixed benefit. FBS seems to be the most popular choice being inexpensive and easy to obtain in large volumes. Chen *et al.* (2004) and Diago *et al.* (1995) both used fish serum (<1%) in combination with FBS. Serum concentration can also have an affect on primary cultures. Throughout the literature concentration varies from 5% to as high as 20%. Serum concentrations are not usually much higher than this, as there is evidence that high serum concentrations may inhibit cell growth. (Freshney, 2000; Cheng *et al.*, 1993; Loo *et al.*, 1987). After the addition of FBS the main additive to media appears to be L-glutamine. L-glutamine is the main source of carbon for established cells (Freshney, 2000). As

serum provides much of the necessary growth factors, attachment factors, hormones and lipids there is often no requirement for further additives apart from L-Glutamine. Very few established fish lines have any other additions to the media. Most examples of additives to media are in serum-free or reduced media replacing substances that serum would normally provide (Miller *et al.,* 1994; Wang *et al.,* 1995; Cheng *et al.,* 1993). Both of these cell lines are also leukocyte based. However that does not entirely discount the possibility of using further additives to media already supplemented with serum. Kumar *et al.* (2001) detail a large list of additives for example fish muscle extract, sucrose, prawn shell extract which were explored when developing a primary culture.

This leads us to consider the use of attachment factors. Tissue culture vessels are hydrophilic so as to attract cells but have no other coating. Cells attach to a flask through an extra-cellular matrix (ECM) of which collagen is a major component. Cells secrete this matrix of proteins and proteoglycans themselves. The matrix binds to the substrate and the cells bind to the matrix via adhesion molecules such as fibronectin and laminin (Freshney, 2000). This is why used glassware supports growth better than un-used glassware. Presently there are numerous attachment factors and ready coated flasks available to purchase. Many of these are commonly used in mammalian primary culture to enhance attachment. Butler and Nowak (2004) and Bols et al. (1994) investigated the use of several different attachment factors in an attempt to improve the substrate. There is definite scope for further research into the use of attachment factors in fish primary culture.

In early fish cell culture there is little mention of the addition of growth factors to media already containing serum. Again this is because growth factors are more often added to serum-free media. Miller *et al.* (1994) detailed the use of chemical mitogens used to establish suspension cultures from leukocytes. Cell cultures exhibited a strong proliferative response after exposure to the mitogens. Faisal *et*

al. (1995) exposed cultured liver cells to plant derived mitogens stimulating DNA synthesis (indicative of cell proliferation). One of the earlier uses of growth factors was by Watanabe *et al.* (1987) who used a mammalian epidermal growth factor. Chen *et al.* (2004) used a basic fibroblast growth factor to stimulate growth. Although both established a cell line, it was inconclusive as to whether this was a result of the added growth factors.

Wolf and Quimby (1969) quoted pH as being uncritical in establishment of a culture as most cells fare well in the range of 7.2-7.8. Review of literature reveals that there is little concern for the affect of pH on cell growth. Basic media provides the correct pH for cell proliferation and establishment. The use of buffering solutions or CO_2 incubators to control pH is common in routine tissue culture and should not be considered unusual.

Sodium Chloride (NaCl) concentration may be important for cell survival particularly in marine fishes. Fernendez *et al.* (1993b) presented information on NaCl concentrations for freshwater fish showing that although high NaCl concentrations were used for establishment these levels were not required in successive passages. Fernendez *et al.* (1993a) also demonstrated optimum growth at lower NaCl concentrations for marine fish. This is further supported by Chang *et al.* (2001). Most cell lines do not rely on NaCl to be added to the media.

1.7 Cell Line Establishment

All these factors and more play an important role in the initial stages of primary culture and in the attachment, survival, and finally growth of the primary culture. Regardless of the method employed to isolate cells, primary culture is the first in the series of selective processes that may or may not give rise to an established cell line (Table 1.1 Freshney, 2000).

Table 1.1 Selection in Cell Line Development
(reproduced from Freshney, 2000)

	Factors influencing selection	
Stage	Primary Explant	Enzymatic disaggregation
Isolation	Mechanical Damage	Enzymatic damage
Primary Culture	Adhesion of explant and outgrowth	Cell adhesion and spreading
First Subculture	Trypsin sensitivity; nutrient, hormone, and substrate limitations	
Propagation as a cell line	Relative growth rates of different cells; selective overgrowth of one lineage Nutrient, hormone and substrate limitations Effect of cell density on predominance of normal or transformed phenotype	

Only those cells which survive isolation and adhere will form the basis of a culture. Cells which then proliferate are selected over those cells which survive but do not grow. Once confluence is reached and the culture is passaged the same selection process is repeated.

Unfortunately this will not change the fact that most cell lines are still finite. Normal cells can only divide a limited number of times (Freshney, 2000). This is known as the Hayflick limit. Finite cell lines only have 20-80 population doubles before cell death occurs. The protective ends of the chromosomes, the telomeres, gradually shorten with each cell cycle and when a critical telomere length is reached, the cell is unable to divide. The cell enters an irreversible state of quiescence (Barker *et al.*, 2000). This is thought to involve the tumor suppressor gene p53 which arrests cell cycle progression. The gene p53 arrests the cell cycle in order to allow the cell to survey its DNA for damage; if DNA is undamaged the normal cycle resumes (Luft *et al.*, 1998). This progression of events whereby cells move from actively dividing to a non-dividing state is known as senescence.

Figure 1.2 details the evolution of a cell line (Freshney, 2000). Note that for a cell line to become established or continuous it must undergo transformation or immortalization. Transformation usually refers to the genetic alteration of a cell resulting from the introduction, uptake and expression of foreign DNA. The use of the term transformation within cell culture has slightly different meaning to this. Generally it refers to an alteration in growth characteristics (*e.g.* loss of contact inhibition, low serum requirement, continuous growth) which may or may not correlate with immortalization (Freshney, 2000). Normal cells are converted into cells that will divide without limit hence bypassing senescence and cell death.

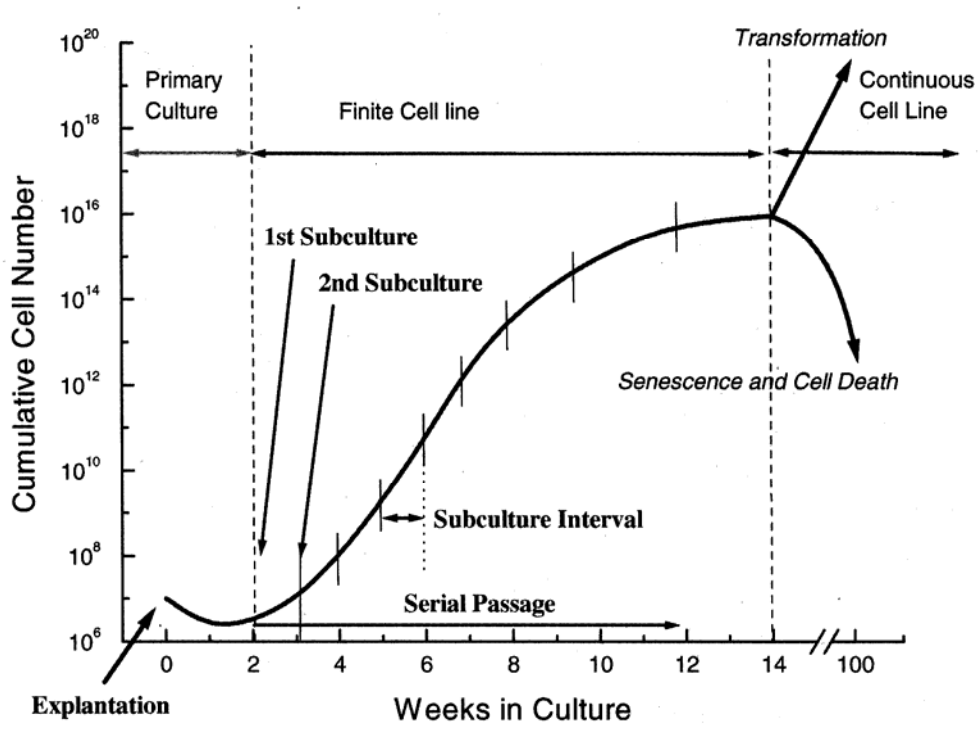

Figure 1.2 Evolution of a Cell Line (reproduced from Freshney, 2000)

The majority of established cell lines have developed from normal tissue which has spontaneously transformed (Fryer and Lannan, 1994; Butler and Nowak, 2004). However transformation can also be induced through chemical mutagens, infection with a transforming virus or through transfection (Freshney, 2000; Butler and Nowak, 2004; Guo *et al.,* 2003; Takarada *et al.,* 1989). Media, growth factors

and other supplements will simply optimize the conditions for growth and provide more opportunities to develop a continuous cell line, but do not ensure development of an immortalized cell line.

1.8 Current Fish Cell Line Listing

The first established fish cell line reported was the Rainbow Trout Gonad (RTG-2) line established by Wolf and Quimby (1962). Since then many more cell lines have been established. The most current listing of fish cell lines is authored by Fryer and Lannan (1994) listing one hundred and twenty-five cell lines from fifty-two different species. Further investigation of the literature reveals another thirty-six new established cell lines since Fryer and Lannan (1994) (Table 1.2).

By far the most common use of fish cell lines is for the diagnosis of viral fish diseases by culture in a monolayer (Fryer and Lannan, 1994). There are numerous other uses for fish cell lines, for instance biochemical, physiological and immunological studies (Hightower and Renfro, 1988). There is also a growing interest in the use of fish cell lines for cytotoxicity tests. The most common fish species used for establishing primary cultures tend to be from commercially important species in aquaculture (Hightower and Renfro, 1988). Hence there are forty-six fish cell lines from the family Salmonidae, whilst marine species are less well represented. Forty-eight of the one hundred-eighty plus cells are from marine species the rest originate from freshwater species.

Table 1.2 Fish Cell Lines Developed since 1994

Species	Designation	Common Name	Tissue of Origin	Reference
Anguilla japonica	EP-1	Japanese Eel	Elver	Kou *et al.,* 1995.
Caranx melampygus	BTMS	Bluefin Trevally	Muscle	Zhao and Lu, 2006.
Caranx melampygus	BTF	Bluefin Trevally	Fins	Zhao and Lu, 2006.
Carassius sp.		Goldfish	Macrophages	Wang *et al.,* 1995.
Clarias gariepinus		African catfish	Ovary	Kumar *et al.,* 2001.
Epinephelus awoara	GB	Yellow Grouper	Brain	Lai *et al.,* 2001.
Epinephelus awoara	GSB	Yellow grouper	Swimbladder	Lai *et al.,* 2003.
Epinephelus awoara	GH	Yellow Grouper	Heart	Lai *et al.,* 2003.
Epinephelus awoara	GE	Yellow Grouper	Eye	Lai *et al.,* 2003.
Epinephelus awoara	GF	Yellow Grouper	Fin	Lai *et al.,* 2003.
Epinephelus coioides	GF-1	Grouper	Fin	Chi *et al.,* 1999.
Epinephelus coioides	GS	Orange Spotted Grouper	Spleen	Qin *et al.,* 2006.
Lateolabrax japonicus	SPH	Sea perch	Heart	Tong *et al.,* 1998.
Lateolabrax japonicus	SPS	Sea perch	Spleen	Tong *et al.,* 1998.
Lates calcarifer	SF	Asian Sea bass	Fry	Chang *et al.,* 2001.
Lates calcarifer	SISE	Sea Bass	Blastula (embryo)	Parameswaran *et al.,* 2006.
Leiostomus xanthurus		Spot	Liver	Faisal *et al.,* 1995.
Mylopharyngodon piceus	SBC	Black Carp	Swimbladder	Chen *et al.,* 1995.
Oncorhynchus kisutch	CoE45	Coho salmon	Embryo	Ristow and Avila, 1994.
Oncorhynchus kisutch	CoE 115	Coho salmon	Embryo	Ristow and Avila, 1994.
Oncorhynchus kisutch	CoE 345	Coho salmon	Embryo	Ristow and Avila, 1994.
Oncorhynchus mykiss	RBTE 45	Rainbow trout	Embryo	Ristow and Avila, 1994.
Oncorhynchus mykiss	RT-gill W1	Rainbow trout	Gill	Bols *et al.,* 1994.
Oncorhynchus mykiss W.	TPS	Rainbow trout	Pronephus	Diago *et al.,* 1995.

Ophicepalus striatus	E-11	Striped snakehead	Fry (Clone of SSN-1)	Iwamoto *et al.,* 2000.
Pagrosomus major	RSBF	Red sea bream	Fin	Tong *et al.,* 1998.
Paralichthys olivaceus	FEC	Japanese Flounder	Embryo	Chen *et al.,* 2004.
Paralichthys olivaceus	FSP	Flounder	Spleen	Kang *et al.,* 2003.
Paralichthys olivaceus	FFN	Flounder	Fin	Kang *et al.,* 2003.
Paralichthys olivaceus	FG-9307	Flounder	Gill	Tong *et al.*, 1997.
Salmo salar	RGF	Atlantic Salmon	Gill	Bulter and Nowak, 2004.
Salmo salar	RGE-2	Atlantic Salmon	Gill	Bulter and Nowak, 2004.
Salmo salar	ASK	Atlantic Salmon	Head Kidney	Devold *et al.*, 2000.
Salmo salar L	SHK-1	Atlantic Salmon	Head Kidney	Dannevig *et al.,* 1997.
Salmo salar L	TO	Atlantic Salmon	Head Kidney	Wergeland and Jakobsen, 2001.
Sparus aurata	SAF-1	Gilt head Seabream	Fin	Bejar *et al.,* 1997.

The principle aim was to establish a continuous cell line, with the hope that it could be used to culture emerging fish viruses. Two different species were investigated: cod (*Gadus morhua*) and Atlantic halibut (*Hippoglossus hippoglossus*). Both species are increasingly important commercially. In particular cod production has increased from eighty-two tonnes in 2003 to a potential of eight hundred and fifty-one tonnes in 2006. Halibut production has almost doubled from two hundred and thirty-one tonnes in 2003 to a potential of four hundred and twenty-three tonnes in 2006. As a consequence, novel diseases *e.g.* nodavirus are now being seen in both these species (Starkey *et al*., 2000 & 2001) as well as currently established diseases *e.g.* infectious pancreatic disease in halibut (Wood *et al*., 1996) and viral haemorrhagic septicaemia in cod (Snow *et al*., 2000) . Most cell lines were developed because currently available cell lines are not suitable for isolating a new virus or disease. As less than thirty fish cell lines are available commercially, this provides further reason to develop new cell lines. There is definite scope for development of new cell lines from less well represented families and newly cultivated species. Additionally, most of the current cell lines originate from fin or embryonic tissue with relatively few being derived from specific organs. The initial purpose for establishing a new cell line was to replace the existing cell lines susceptible to nodavirus. The current cell lines available are E-11 and SSN-1. Both these cell lines however; are persistently infected with a retrovirus. As nodavirus specifically infects the optic nerve, brain and heart these organs were targeted in preference over kidney.

Developing a new cell line for these reasons also provides an opportunity to investigate the use of attachment and growth factors when attempting to establish a primary culture. Attachment and growth factors have not been widely used in fish cell culture. Furthermore, the potential for artificial means of transforming primary cultures is open to investigation.

2. Materials and Methods

2.1 Experimental Animals

Atlantic halibut (*Hippoglossus hippoglossus*) and cod (*Gadus morhua*) were obtained from commercial hatcheries. Halibut weight ranged from 100 to 500g (approximately two years of age), cod weight ranged from 200-500g (approximately two years of age). The larvae fish were from freshly hatched eggs between one and two days old. All fish were euthanized by an overdose of the anaesthetic MS-222 (tricaine methane sulphonate) (Sigma).

2.2 Reagents

Table 2.1 Reagents

Reagents	Supplier
Dulbecco's phosphate buffered saline (PBS)	Invitrogen
Gentamicin	Invitrogen
Neomycin	Sigma
Foetal Bovine Serum (FBS) European Origin	PAA
L-Glutamine	Invitrogen
Trypsin EDTA	Invitrogen
Earle's Minimum Essential Media (E-MEM)	Invitrogen
Hank's Minimum Essential Media (H-MEM)	Perbio
Leibovitz Media (L-15)	Perbio

2.3 Isolation of tissue

Fish were sprayed and wiped using 70% ethanol. The heart, liver, spleen, brain, and a section of tail were removed aseptically within a class II biological safety cabinet. Additionally from Atlantic halibut, the optic nerve was removed. The tissues were immersed in Dulbecco's phosphate buffered saline (Invitrogen) supplemented with 40mg of gentamicin (Invitrogen) and 200mg of neomycin (Sigma) per litre (Table 2.2). This will be referred to as PBS mixture. All tissue was then removed from the PBS mixture to a clean Petri dish and minced using a scalpel into smaller sections of 2-5mm.

Table 2.2 Components of Mixtures and Media

Mixture	Reagents
PBS Mixture	Dulbecco's phosphate buffered saline Neomycin 40mg/L Gentamicin 200mg/L
Trypsin Mixture	Trypsin EDTA
Basic Culture Media	Media (E-MEM, H-MEM, or L-15) 10% FBS 1% L-Glutamine (200mM) Neomycin 10µg/L Gentamicin 50µg/L

The primary culture of the isolated tissue was undertaken using three main techniques: explant, mechanical and enzymatic disaggregation. The first attempts at isolation were done using all three methods on both species to determine which was the most successful for each organ and species.

2.4 Explant method

After mincing, tissue sections were re-suspended in less than 1ml of basic culture media. The basic culture media consisted of E-MEM supplemented with 10% FBS (PAA), 1% L-glutamine (Invitrogen), gentamicin and neomycin (Table 2.2). The re-suspended tissue was transferred to a tissue culture flask (a 25cm^2 Primaria flask- BD Biosciences). The tissue sections were then allowed to settle for a minimum period of 24 hours. Once the sections had adhered further culture media was added to take the complete amount up to 5ml in a 25cm^2 flask or 2.5 ml in a 12.5cm^2 flask.

The cod larvae were treated in a similar way. The larvae were immersed in 70% ethanol and then PBS mixture for a minimum of five minutes. They were minced into smaller sections within the PBS mixture in a Petri dish and left to adhere in a flask. As before media was added to the flask to bring the total volume up to the correct amount.

2.5 Mechanical Disaggregation

After mincing tissue sections were forced through a fine sieve of 100µm (BD Biosciences) with a small amount of basic culture media to disassociate the cells. The resulting solution was then transferred to a 25cm^2 tissue culture (Primaria flask- BD Biosciences) and allowed to attach to the substrate for 24-72 hours. After this adhering period, the media was removed and the flasks washed with PBS and the flasks refilled with fresh culture media.

2.6 Enzymatic Disaggregation (Enzymatic method)

After mincing, tissue sections were re-suspended in approximately 5ml of enzyme (trypsin-EDTA (1X) or collagenase both Invitrogen). Collagenase was re-hydrated in Hank's BSS (Invitrogen) the final dilution being approximately 150 mg per ml. The same method was repeated in cod and halibut using trypsin and collagenase as the digestive enzymes. The enzyme that produced the highest harvest and attachment of cells was used for all remaining tests.

In order to determine the optimum method of harvesting cells, two different methods of mixing the enzyme and tissue were investigated, using either a plate shaker or magnetic stirrer. The total time period of digestion and number of harvests during this digestion period were also investigated. The influence of temperature on harvest was examined at ambient (20-22ºC) and at 4ºC. For each tissue the resulting procedure that delivered the highest yield and resulted in the highest attachment of cells was used hence forth. The final procedure is detailed below.

For the softer tissues (*i.e.* spleen, heart, liver and brain) the suspended tissue was mixed at room temperature (20-22ºC) on a plate

shaker. Every 45 to 60 minutes the cells were harvested by removing the trypsin containing any disassociated cells. The remaining pieces of tissue were re-suspended in fresh trypsin. The activity of the removed trypsin was stopped by the addition of an equal amount of basic culture media. The harvested cells were then centrifuged at 1000rpm (200g) for 10 minutes at 4ºC with no brake. The supernatant was removed and the pellet re-suspended in a small amount of culture media. The re-suspended pellet was then stored at 4ºC until another 4/5 pellets were obtained, after which all pellets were pooled and seeded into 25cm^2 tissue culture flasks. Twenty-five cm^2 Primaria flasks were always used except were specified different.

For the tail sections and optic nerve the trypsin mixture was mixed at room temperature on a magnetic stirrer. This trypsin mixture was harvested after 4-5 hours of mixing and a pellet obtained in the same fashion as for the softer tissues. The mixture was re-suspended with culture media and seeded into 25cm^2 tissue culture flasks.

The disaggregated cells were left to adhere to the tissue culture flasks for a minimum of 24 to maximum of 72 hours before the original media was removed and replaced with fresh culture media so as to remove any debris and unattached cells.

2.7 Temperature Comparison

Cell attachment and growth were investigated at five different temperatures. Disassociated cells were seeded into four flasks (25cm^2). Each of the four flasks was incubated at a different temperature (6ºC, 10ºC, 15ºC, 20ºC, 25ºC) and the resulting cultures examined and graded for confluence. The temperature that produced the best attachment and growth was used as the optimum temperature for all remaining tests.

2.8 Media Comparison

Attachment and growth response were investigated using three kinds of culture media. Disassociated cells were seeded into three flasks. Each of the three flasks was incubated with a different culture media (E-MEM, Invitrogen; L-15, and H-MEM both Perbio) supplemented as the basic culture media (Table 2.2). The resulting cultures were examined and graded for confluency. The optimal media for each organ was used as the basic culture media for all remaining tests.

2.9 Attachment Factors

In order to investigate the possible benefits of using coated tissue culture flasks on cell attachment, three different attachment factors were investigated: collagen, laminin and poly-D-lysine. Disassociated cells from spleen, heart and tail were seeded on to pre-coated collagen and laminin 25cm^2 flasks (Greiner Bio-one). Disassociated brain cells were seeded on to pre-coated poly-D-lysine 25cm^2 flasks (Greiner Bio-one). Each pool of cells was divided equally between the coated flask and a standard 25cm^2 tissue culture flask (Greiner Bio-one) which was used as a control. Cultures were examined and graded for confluency after the 24-72 hour adherence period.

2.10 Growth Factors

Attempts to improve cell growth with the use of bovine derived growth factors were undertaken using both an established cell line and primary cultures.

2.10.1 CHSE

The established cell line CHSE-214 (Chinook Salmon Embryo available from ECACC) was used to establish growth response after the addition of bovine growth factors to basic culture media. CHSE are routinely cultured at 20°C using E-MEM supplemented with 5% foetal bovine serum

and 0.01% L-glutamine (Invitrogen). For the experiment 25cm^2 flasks were seeded with 1.7 x 10^5 cells/ml using media supplemented with one of two growth factors either 50ng/ml of epidermal growth factor (EGF) (BD Biosciences) or 100µg/ml endothelial cell growth supplement (ECGS) (BD Bioscience). The same number of flasks were seeded using un-supplemented media to provide a typical growth curve as a control.

In order to plot a basic growth curve for each set of flasks, cells were counted using a haemocytometer as detailed in Freshney (2000). One to two microlitres of cell suspension was inserted into a haemocytometer chamber, the cells counted using a microscope and the cell concentration calculated from this. Hence the number of cells per millilitre was calculated for each flask. Five replicate counts for two flasks were taken for each time point over one week. The two flasks and replicate counts from each were then averaged. The information was used to plot a basic growth curve for the growth supplemented cultures and the control cultures.

2.10.2 Primary culture

Disassociated cells obtained from each organ were seeded into two standard 25cm^2 tissue culture flasks. After the adherence period the flasks were washed and the media was replaced. One 25cm^2 flask used basic culture media supplemented with either EGF or ECGS the other flask used just basic culture media as a control. Both flasks were observed after a week and graded for confluency. This was only investigated for cod cultures.

Fish serum was also investigated as a potential growth factor/supplement. This was only attempted on cod. The fish was bled using a vacuette tube (Greiner Bio-one) and needle before dissection. The blood was then centrifuged at 1000rpm (200g) for 10 min. to remove the red blood

cells and the resulting supernatant removed and frozen at -20°C for future use. Disassociated cells were seeded into two 25cm^2 flask, one to be used as a control and the other which was supplemented with 2% fish serum. Both flasks were examined after 7 days and graded for confluency.

2.11 Mitogens

Further attempts to improve cell growth were made with using two types of mitogens: concanavalin A (Sigma-C5275) and calcium ionophore (Sigma-A23187). Each mitogen was prepared based on Miller and Chem's (1988) method. One milligram of concanavalin A (con A) was dissolved in 1ml of sterile PBS to produce a stock solution which was stored at -20°C. One milligram of calcium ionophore (A23187) was dissolved in 400µl of Dimethyl Sulfoxide (DMSO) to create a stock solution which was also stored at -20°C.

To determine the optimum concentrations for use, a cytotoxicity test was run using six serial dilutions. CHSE were sub-cultured as detailed before in 24-well plate (Greiner Bio-one) with 1ml of media in each well. Fifty microlitres of con A was added to the first column of wells rows A-C to give a final dilution of 50µg/ml in each well. One hundred microlitres was then transferred from each well to the well in the next column and repeated across all columns to give 6 serial dilutions with 3 replicates each. Row D was kept as a cell control (Figure 2.1). The same procedure was followed for calcium ionophore; 4µl of stock solution was added to the first column to give a final concentration of 10µg/ml.

The plates were read after 7 days and the first dilution that did not cause complete cytopathic effect (CPE) was used as the optimum working dilution.

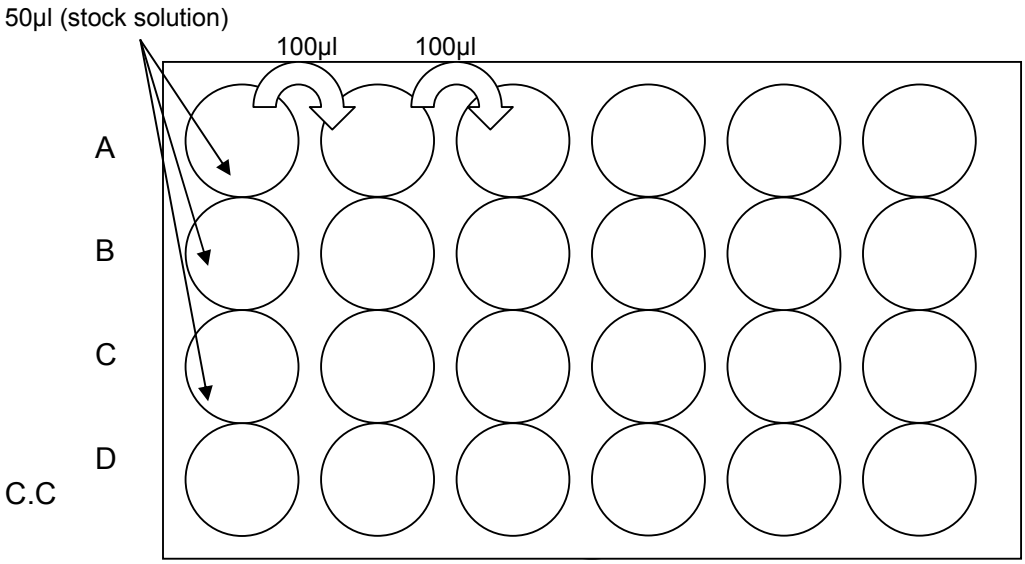

Figure 2.1. Set-up of plate for cytotoxicity test

Two flasks of disassociated cells were seeded and after initial attachment the media was replaced with basic culture media supplemented with the optimum working concentration of either con A or calcium ionophore. The second flask was kept as a control. The cells were observed and graded for confluency after 7 days. This was only investigated for cod cultures.

2.12 Chemical Mutagen

The optimum working concentration for the chemical mutagen 4-nitroquinoline-N-oxide (4NQO) was likewise investigated. Before using 4NQO a risk assessment was carried out as it is a toxic chemical. CHSE were sub-cultured as detailed before in a 24-well plate with 1ml of media in each well. Two hundred-fifty milligrams of 4NQO was dissolved in 3ml of DMSO to give an approximate 80mg/ml working concentration. Ten microlitres of stock solution was added to the first column and transferred across the plate. The cells were exposed to the chemical for 3 hours after

which the media was then removed and replaced with fresh media. The first dilution not to cause CPE was used as the optimum working concentration.

Two flasks of disassociated cells were seeded. After initial attachment the cells were exposed to media supplemented with 4NQO for 3 hours. This media was then removed and replaced with fresh basic cell culture media. The second flask was kept as a control. The cells were observed and graded for confluency after 7 days. This was only investigated for cod cultures.

2.13 Senscence Test

Cultures which did not appear to grow or reach confluency were tested to determine if they were senescent using a senescent cells staining kit from Sigma (CS0030) as per the manufacturers instructions. Briefly cells were fixed, stained and examined under a microscope. Those cells which stain blue are deemed to be senescent. The kit is based on a histochemical stain for β-galactosidase activity, at pH 6, which is unique to senescent cells.

2.14 Microscopic Examinations and Scoring

Flasks were observed under an inverted light microscope for attachment after fresh media was added. If no cell attachment was observed the flask was discarded. Any contaminated cultures were also discarded. Any flasks which did contain attached cells were examined weekly for growth and the culture media refreshed on a weekly basis. Morphology was observed and recorded using a Nikon digital camera. Fibroblastic-like cells were defined as cells which were bipolar, the length of which is usually twice its width. Generally epithelial-like cells are defined as a cell which is polygonal with regular dimensions when cells are confluent (Freshney,

2000). However epithelial cells may appear to be more fibroblastic in shape when present in low numbers.

It was presumed that a high harvest of viable cells gave rise to greater attachment. The harvest and subsequent attachment of cells were graded using a scale (Table 2.3). All cultures which were used for comparison tests were examined and graded with this scale after the initial 24-72 hour attachment period.

Table 2.3. Scoring system to record confluency

Score	Percentage of substrate coverage (confluency)
-	0%
+/-	<1% (individual cells)
+	5-30%
++	30%-50%
+++	>50%

2.15 Passage

Confluent cultures were sub-cultured. A confluent culture was considered to be that in which all cells are in contact with other cells around their periphery and no available substrate is left uncovered (Freshney, 2000). The media was removed and the flask washed with PBS. The cells were exposed to trypsin-EDTA (0.5ml per 25cm^2 culture flask) until the cells began to round up and lifting of the cell monolayer occurred. An equal amount of basic culture media was added to the suspended culture to neutralize the trypsin. Cells were dispersed by gently pipetting over the surface of the flask. The resulting suspension of cells was seeded into a tissue culture flask of the same size or of no more than twice the surface area. These cultures were examined weekly as before and sub-cultured again when confluent.

3. Results

3.1 Explant Method

Attempts to produce cultures using the explant method had limited success. There was no attachment or outgrowth from either cod or halibut brain and optic nerve tissues. There was greater success for spleen, heart and tail sections from halibut; however none of these outgrowths ever produced a confluent culture and eventually cell death occurred.

Figure 3.1 depicts explant growths of spleen and heart. These cultures failed to grow to confluency although the cells appeared to remain viable for 4-6 weeks. Figure 3.2 shows a tail explant with the resulting outgrowth at various time points. The explanted tissue can clearly be seen as well as subsequent outgrowth of cells. Figure 3.2e shows the cells once the explant has been removed or detached during washing. Several of these growths were eventually lost to contamination whilst others failed to reach confluency and lifted after the initial burst of growth. Figure 3.2e also shows the beginning of typical cell necrosis observed in the cultures.

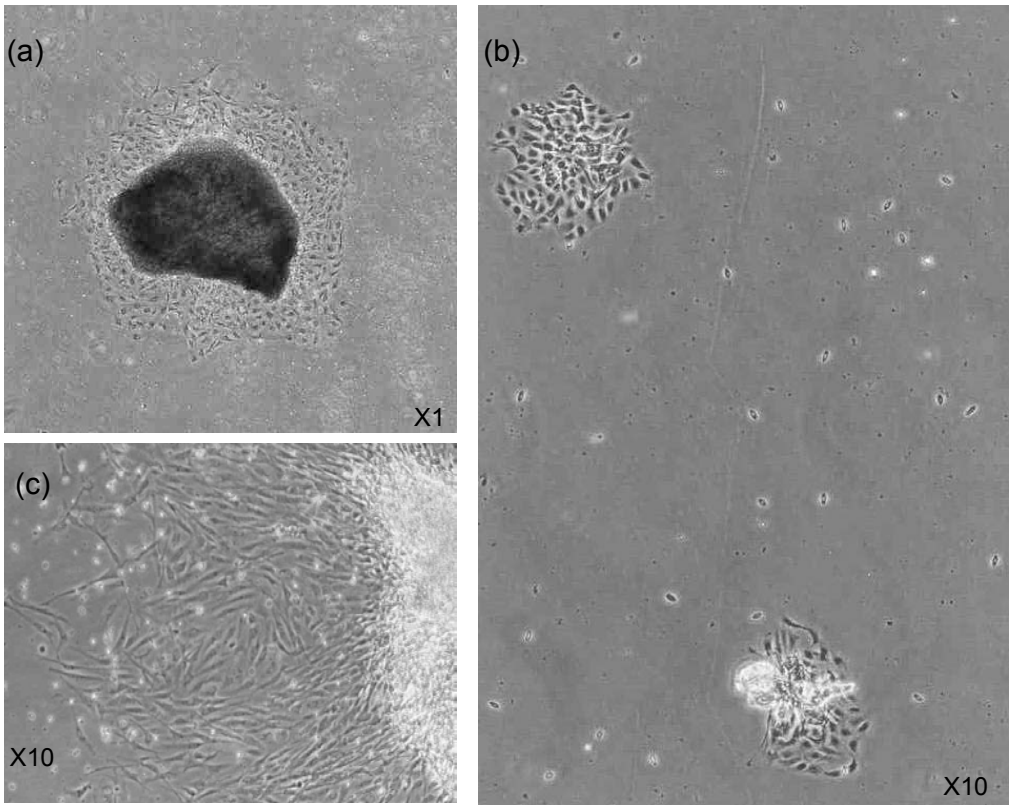

Figure 3.1. (a)-(b) Atlantic halibut heart explants and growth (day three). (c) Atlantic halibut spleen outgrowth from explant (day three).

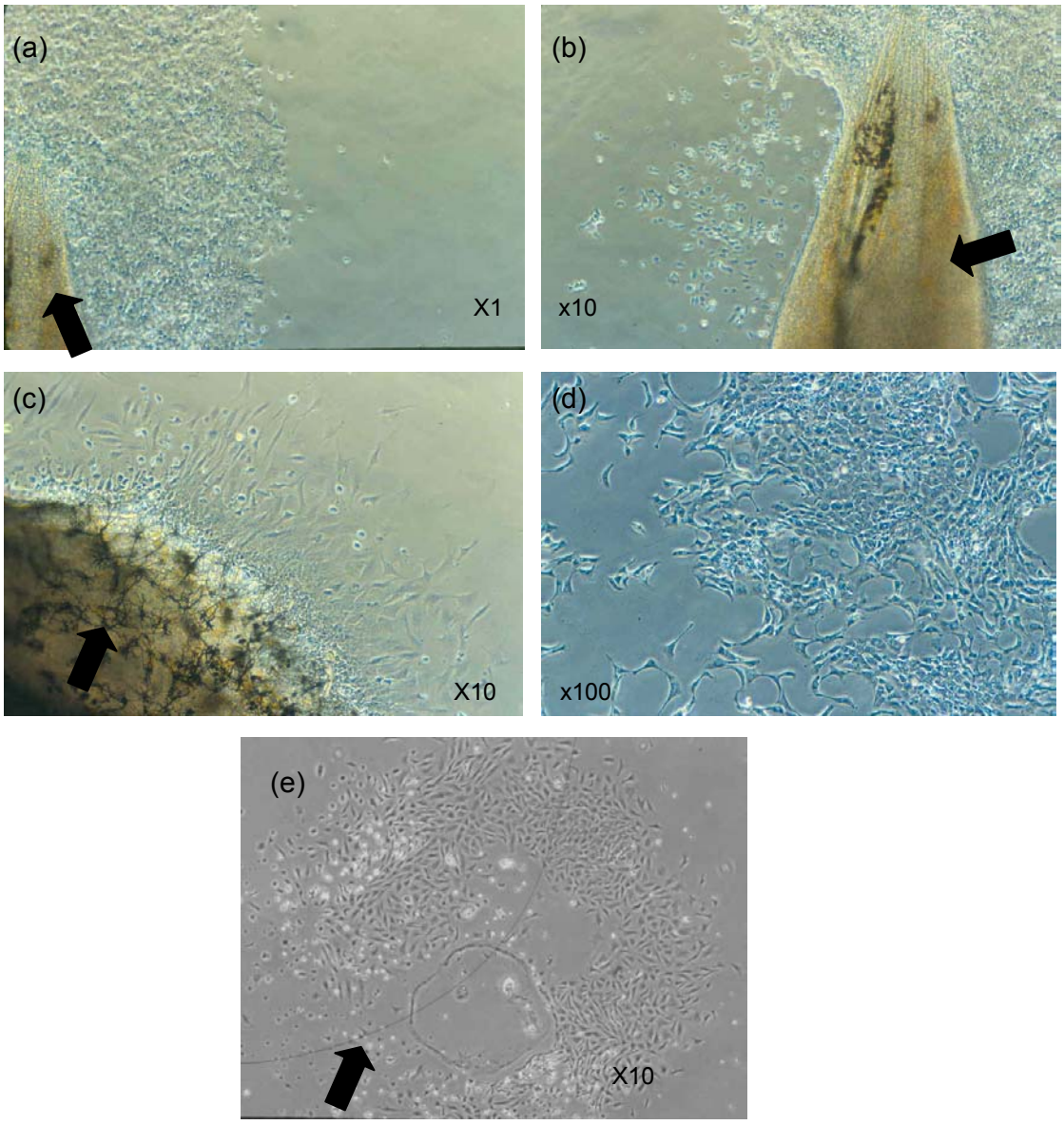

Figure 3.2 (a)-(c) One day old tail explant with outgrowing cells clearly visible around the explanted tissue. Arrow shows original tissue fragment. (d) The tail cells after the explant has been removed (day ten). (e) Cell growth (day seven) after explant removal, cell necrosis is already visible in left of culture (arrow).

3.2 Cod Larvae

There was success with the larvae explants. The larvae attached and quick outgrowth of cells was observed. However contamination was so severe, even after treatment with antibiotics, that the cells were overwhelmed. As a result the cultures had to be discarded. Figure 3.3 displays an explant of cod larvae in which bacterial contamination is already visible.

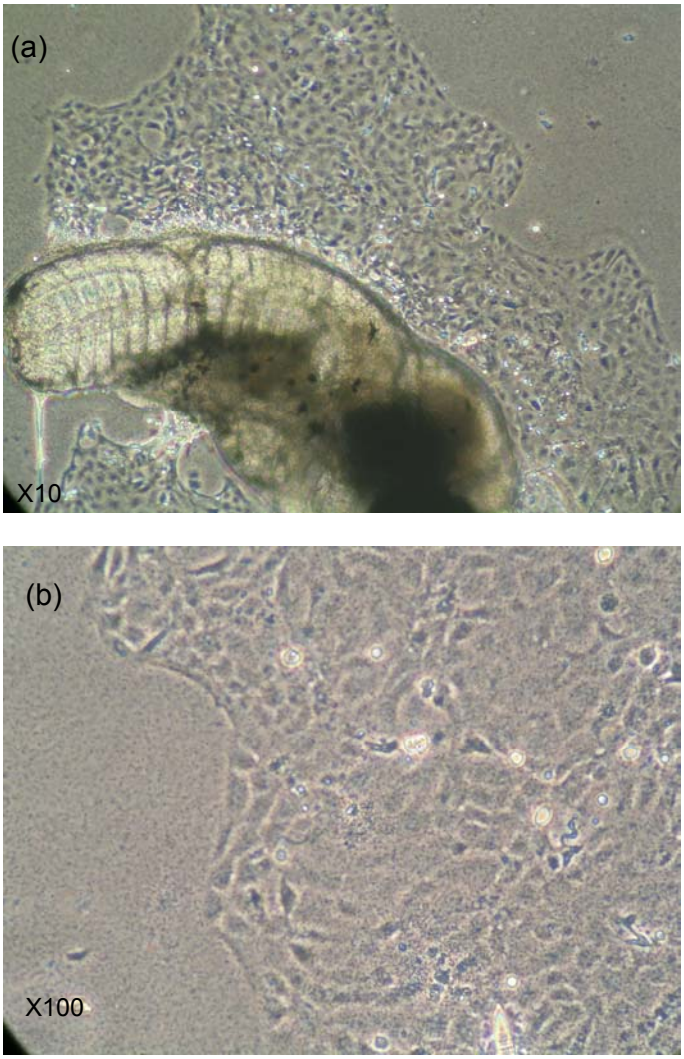

Figure 3.3 (a) Cod larvae explant and outgrowth of cells (day one). (b) Same explant and growth (day three) with contamination already becoming visible.

3.3 Mechanical Disaggregation

This technique was unsuccessful. Individual cells did not adhere after disaggregation.

3.4 Enzymatic Disaggregation

Initial investigations into the best method to disaggregate tissue demonstrated that a higher harvest of cells was obtained from the soft tissues (*i.e.* brain, spleen, and heart, liver) by using a plate mixer to agitate the enzyme mixture. The optimum temperature to maximize yield was ambient (20°-22°C). Similar isolation attempts using a magnetic stirrer either at ambient or 4°C did not produce as high a harvest of cells. Furthermore multiple harvests of cells over a 4-5 hour long digestion period resulted in a higher yield of cells than continuous digestion for 4-5 hours with one harvest. Yield from tissue digested overnight at either temperature was also less. Although the tissue was fully digested, no or very low levels of cellular attachment occurred.

Conversely for the tail and optic nerve tissue use of a magnetic stirrer to mix the tissue continuously for 4-5 hours at ambient with one harvest produced larger yields than through use of a plate shaker. Digestion overnight at 4°C did produced cultures from tail but yield was lower than that of 4-5 hours digestion. Yield from tail tissue digestion was lower on average than yield from the soft tissues.

The enzymatic method using trypsin produced the best results of the three isolation methods (Table 3.1). The use of collagenase as an enzyme did produce cultures but survival after a week was less than that of cultures produced using trypsin.

Table 3.1 Effect of collagenase and Trypsin EDTA on yield.

Organ	Collagenase		Trypsin EDTA	
	Cod	Halibut	Cod	Halibut
Spleen	++	+/-	++	+
Heart	+/-	+/-	+	+/-
Liver	-	-	+/-	-
Brain	+/-	+/-	+/-	+/-
Tail	+/-	-	+/-	-

From halibut, cultures were produced for each organ except liver. From cod, cultures were produced for each organ except optic nerve. When dissecting cod the optic nerve was difficult to extract and remove from the surrounding connective tissue and did not digest well. On the whole greater success was achieved in isolating cultures from cod than halibut.

3.5 Halibut Primary Cultures (Enzymatic Method)

Spleen cultures were obtained from halibut. Most cultures although showing initial attachment did not show high rates of growth and many did not reach confluency. For the few cultures that did reach confluency, passage was attempted but was unsuccessful. Figure 3.4a shows a culture that grew quickly and was passed when confluent. Spleen cultures remained viable for up to ten weeks before cell death occurred.

The optic nerve cells attached in high numbers and grew quite quickly reaching confluency within a week. However all passage attempts were unsuccessfully with no re-attachment seen after sub-culture.

Brain cells only attached in very small numbers and although initially successful showed no substantial outgrowth (Figure 3.4b). The clusters of cells often remained viable for 4-6 weeks after which cell death occurred. As the cells appeared to grow in groups this suggests they were of epithelial origin.

Tail cultures showed poor attachment. Cells were sparse and failed to show any substantial growth.

There was good success with heart cell attachment and initial growth. Although cells did re-attach after passage they failed to grow to confluency a second time and detached after 2-3 days. Figure 3.4c shows an early culture of cells at three weeks and 3.4d a different culture at one week. The morphology of the cells is quite different in each culture. The first culture appears to have a mix of fibroblastic and epithelial cells. The second culture has mainly fibroblastic cells. These cells grew more quickly and seemed to be more viable than the first culture of heart cells which grew more slowly and took longer to reach confluency.

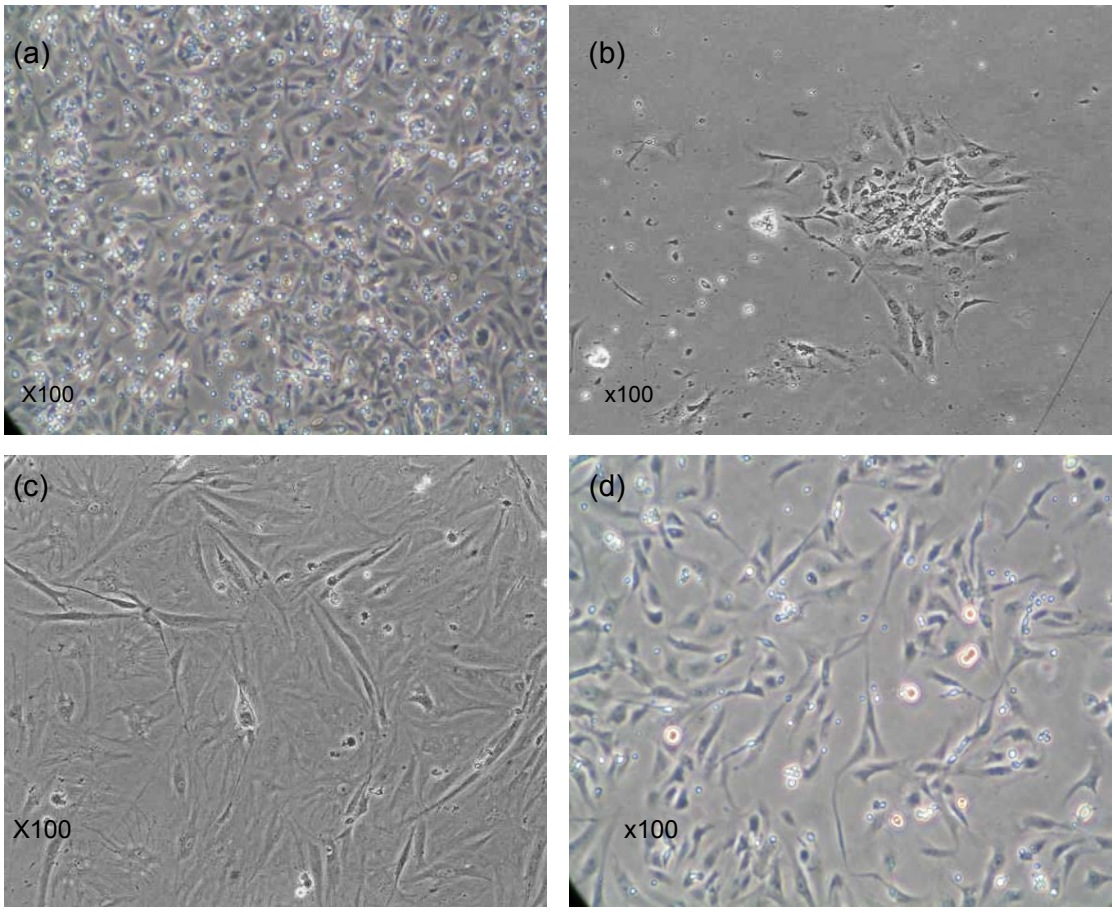

Figure 3.4 Cultures from Atlantic halibut using enzymatic method to isolate cells. (a) Spleen culture (day seven). (b) Brain culture (day seven), very little growth has occurred from original cells. (c) Heart culture (twenty-one days). (d) Different heart culture (day seven). There is a distinct difference in cell morphology between the two cultures.

3.6 Cod Primary Cultures (enzymatic method)

Cod cultures for spleen were not as successful as those produced from halibut. Although there was initial attachment (Figure 3.5) which was often quite high the cells detached quickly. The longest surviving culture lasted only 2-3 days. Many of the cultures produced appeared as in Figure 3.5a. It can be seen that there are small round cells attached to the flask, the other cells visible (small white cells) are lifting or unattached cells. It appears that they may be leukocytes. Unattached red blood cells were also prominent before and after media changes. Other spleen cultures were quite different. Cells appeared fibroblastic in shape and grew to reach confluency (Figure 3.5b). However any passage attempt on the fibroblastic cultures was unsuccessful.

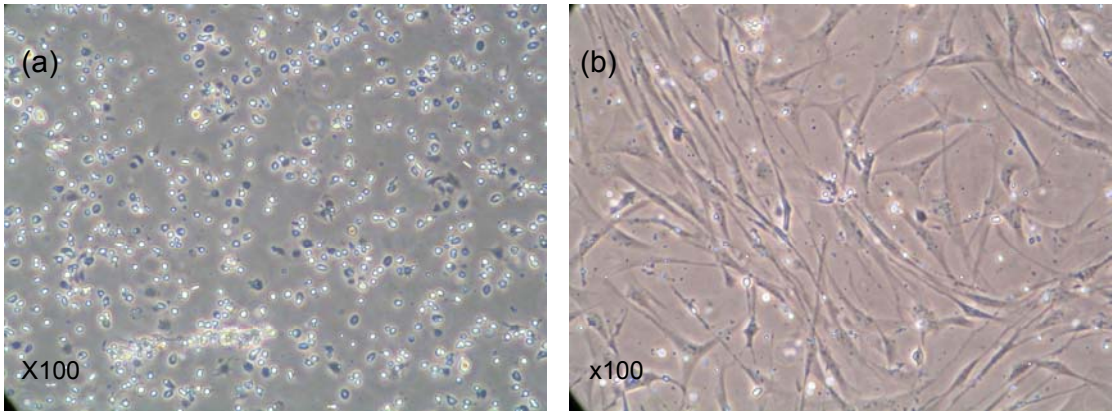

Figure 3.5 Spleen cultures from cod isolated using enzymatic disaggregation. (a) Day one. (b) Fibroblastic cod spleen cells (one week).

Cultures from brain tissue in cod were quite successful (Figure 3.6). There was varied morphology of cells between different primary cultures. Most cultures were flat monolayers of epithelial-like cells. However the cells in some cultures grew in clumps instead of a flat monolayer. These cells appeared to be fibroblastic (Figure 3.6d). The cells were particularly long and extended and grew much slower than the epithelial-like cultures. Epithelial cultures reached confluency within 2-3 weeks and were passaged up to three times before failing to re-attach and grow. Figure 3.6f shows a culture one day after passage one. Figure3.6c depicts a single cell from a brain culture after passage three. It can be seen that although the culture was confluent before passage that a low percentage of cells re-attach after passage with each sub-culture the number of cells re-attaching and growing decreased. The fibroblastic cultures never reached full confluency remaining in large clumps and sub-culturing of these was also unsuccessful.

Tail cultures were developed and reached confluency on several occasions (Figure 3.7a). Cells appeared quite uniform in morphology and were epithelial in shape. Confluency was reached anywhere between 1-3 weeks. Passage was attempted and cells passed up to three times before the cells failed to re-attach.

A few cultures were isolated from liver tissue (Figure 3.7b). It can be seen that yield was very low and no cultures reached confluency. Of all primary culture attempts using liver only one or two cultures were ever produced.

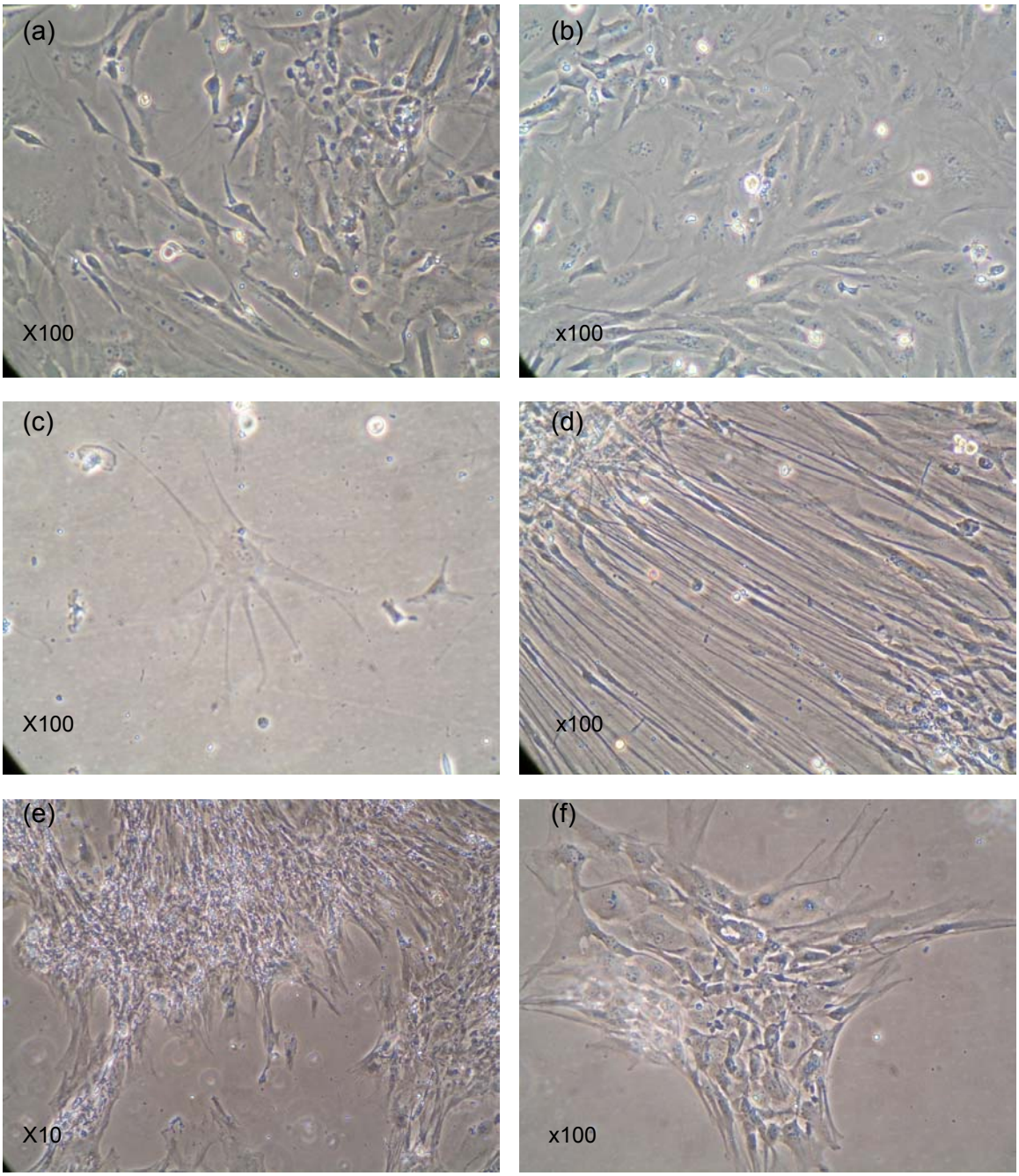

Figure 3.6 Brain cultures isolated from cod using enzymatic disaggregation. (a) Cod brain culture (one month). (b) Culture (twenty-one days). (c) Individual brain cell. (d) Brain cell (day seven). (e) Cultures (one month). (f) Brain culture after passage.

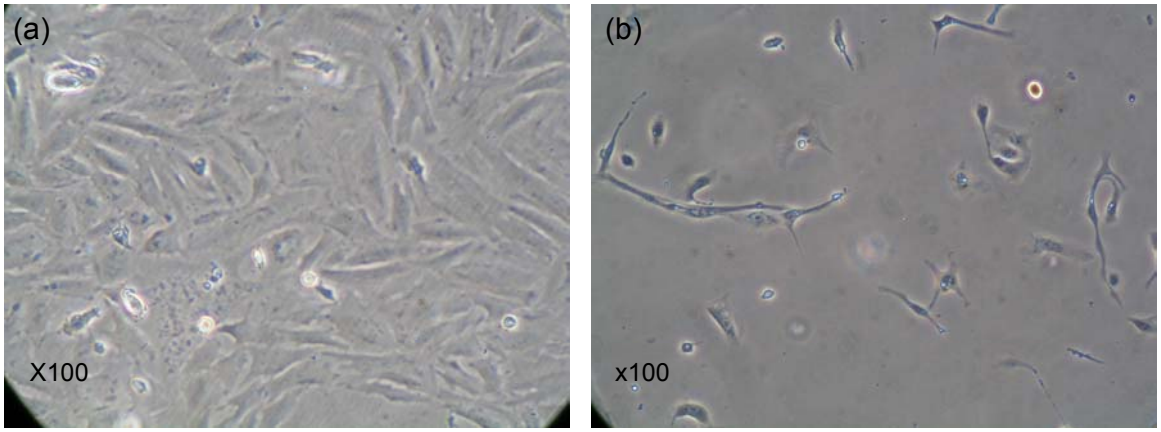

Figure 3.7 (a) Confluent culture of tail cells from cod before sub-culture. (b) Liver cells isolated from cod.

Heart cultures were not very successful on standard tissue culture flasks but greater success was obtained with the collagen coated flasks (see section 3.8). High levels of cell attachment were achieved (Figure 3.8) and cultures reached confluency (Fig 3.8e). Cells were generally epithelial in shape. Some of the heart cultures exhibited contractile ability. An underlying layer of the epithelial shaped cells was covered by a network of contracting fibroblastic cells. Passage was successful up to three passages before any loss in attachment and subsequent cell death was observed. After passage (Figure 3.8) it was easier to see the morphology of the cells. The cells appear more fibroblastic and large cells with prominent nuclei can be seen amongst the surrounding fibroblastic cells.

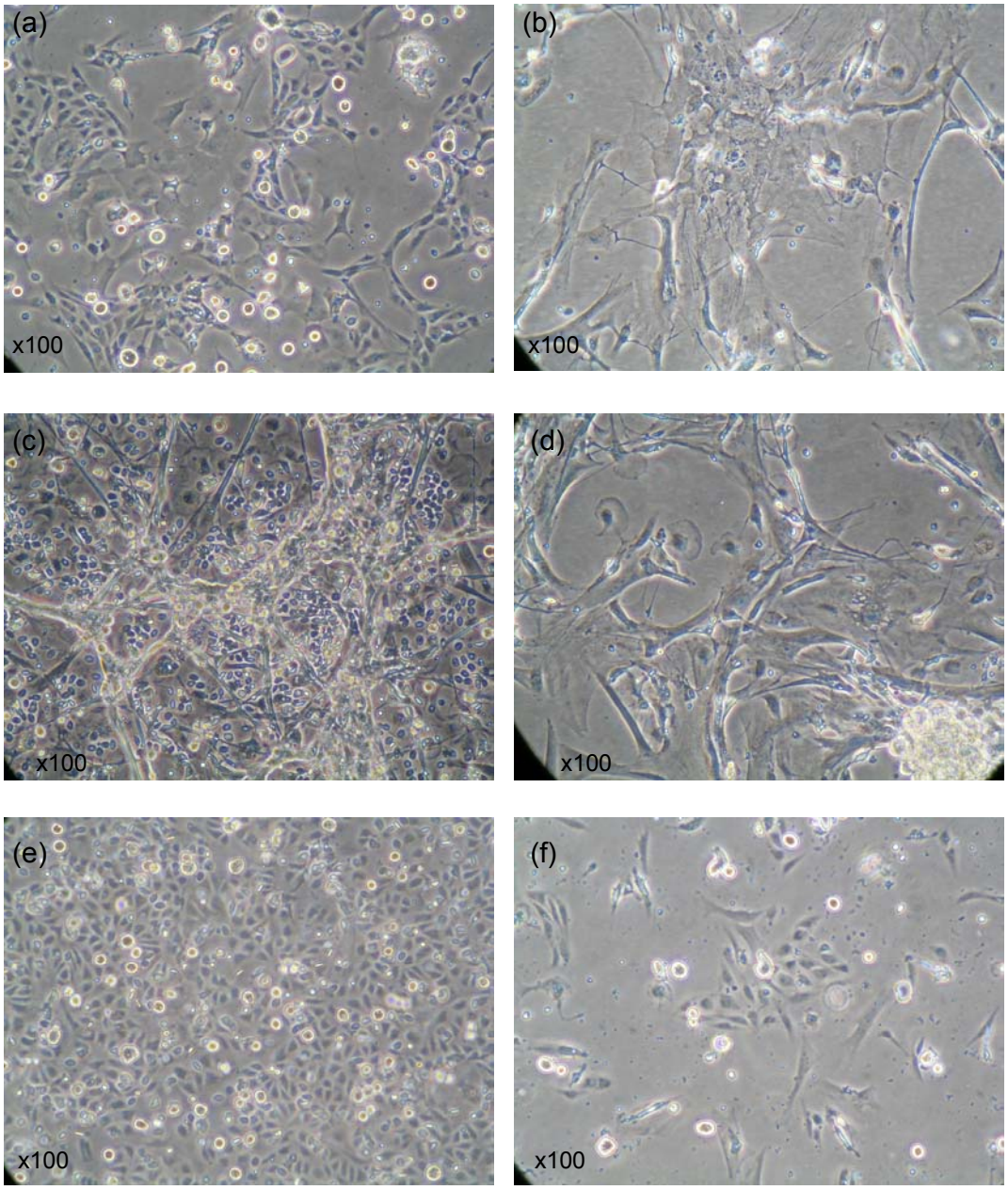

Figure 3.8 Cod heart cultures: (a)-(b) Cod heart cells. (c) Heart cells displaying contractile ability within culture. (d) Heart cells after passage. (e) Heart cell on a collagen coated flask (one day). (f) Heart cells on a normal flask (one day).

3.7 Temperature Comparison

The four different incubation temperatures resulted in attachment for all temperatures except 25°C. Survival and confluency varied depending on temperature (Table 3.2). Attachment did not vary greatly among the temperatures at and below 20°C. At 25°C attachment was poor or non-existent. The best temperature for growth was determined to be 15°C. At lower temperatures (*i.e.* 6°C and 10°C) growth was slower and at temperatures above 15°C cell longevity was less. Hence 15°C was used as the optimum temperature for attachment and growth.

Table 3.2 Effect of temperature on attachment.

Halibut	6°C		10°C		15°C		20°C		25°C	
	Trial 1	Trial 2	Trial 1	Trial 2	Trial 1	Trial 2	Trial 1	Trial 2	Trial 1	Trial 2
Spleen	+/-	+	+++	++	+++	++	++	++	+/-	-
Heart	+	+	+	++	+	+	+	+	+/-	-
Liver	-	-	-	-	-	-	-	-	-	-
Brain	+	-	+/-	+/-	+/-	+/-	+/-	+/-	-	-
Tail	-	-	+/-	+/-	+	-	+/-	+	-	-
Optic Nerve	-	-	-	-	+	-	+	+++	-	-
Cod										
Spleen	+++		+++		+++		+++		-	
Heart	+/-		+/-		+/-		+/-		-	
Liver	-		-		-		-		-	
Brain	-		+/-		+/-		+/-		-	
Tail	+/-		+/-		+/-		+/-		-	
Optic Nerve	-		-		-		-		-	

3.8 Media Comparison

There was no substantial difference in attachment between the three types of media investigated (Table 3.3). However the best growth and survival was seen with E-MEM for all tissues except tail cells which grew better in L-15.

Table 3.3 Comparison of attachment and growth using three different types of media

Halibut	L-15			H-MEM			E-MEM		
	Trial 1	Trial 2	Trial 3	Trial 1	Trial 2	Trial 3	Trial 1	Trial 2	Trial 3
Spleen	+	+		+	+		+	+	
Heart	+/-	+/-		+/-	+		+/-	+	
Liver	-	-		-	-		-	-	
Brain	+/-	+/-		+/-	+/-		+/-	+/-	
Tail	+/-	+/-		+/-	+/-		+/-	+/-	
Optic Nerve	-/+	-/+		-/+	-/+		+	++	
Cod									
Spleen	++	+/-	++	++	+/-	++	++	+/-	+/-
Heart	+	+/-	+/-	+	+/-	+	+	+/-	+
Liver	+	+/-	+/-	+	+/-	+/-	+	+/-	+/-
Brain	+/-	+/-	+	+/-	+/-	+	+/-	+/-	+
Tail	+/-	+/-	+/-	+/-	-	-	+/-	-	-
Optic Nerve	-	-	-	-	-	-	-	-	-

3.9 Attachment factors

The use of pre-coated flasks produced interesting results (Table 3.4). Cod brain cultures showed an increase in attachment on poly-D-lysine. There was no increase in attachment for halibut brain cultures. Heart cells also showed a substantial increase in attachment and growth when seeded on to laminin coated flasks. However when seeded on to collagen coated flasks, confluency was almost 100% (Figure 3.8e-f). No other tissue types showed any increase in attachment between a standard culture flask and a laminin or collagen coated flask.

Table 3.4. Effect of coating factors on attachment.

Halibut	Laminin	Collagen	Control
Spleen		N/A	N/A
Heart		+/-	+/-
Liver		+	+
Tail		+/-	+/-
	Poly-D-Lysine	Control	
Brain	+/-	+/-	
Optic Nerve	-	-	
Cod	Laminin	Collagen	Control
Spleen	+	+	+
Heart	++	+++	+/-
Liver	+/-	+/-	+/-
Tail	+/-	+/-	+/-
	Poly-D-Lysine	Control	
Brain	+	+/-	
Optic Nerve	-	-	

3.10 Growth Factors

The number of CHSE cells per millilitre grown in media supplemented with ECGS was greater than that of the control (Figure 3.9). The number of cells grown in media supplemented with EGF was also higher, although the increase in growth did not overtake the CHSE control until the final reading. However in the primary cultures neither the EGF nor ECGS prompted any visual increase in cell numbers.

The addition of fish serum to the basic cell culture media did not improve growth and in some cases was actually cytotoxic to the cells.

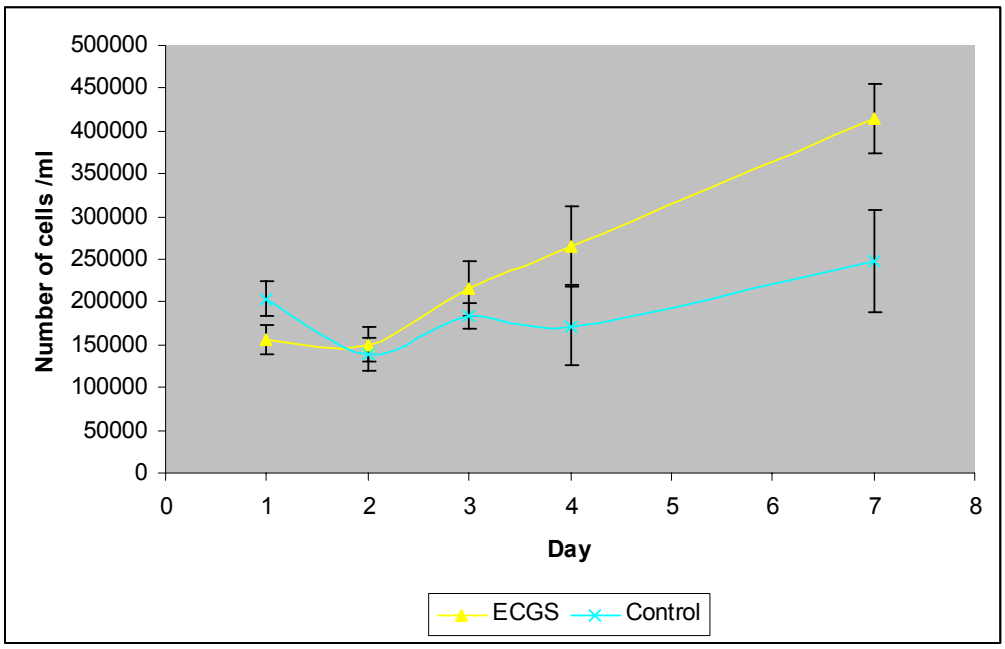

Figure 3.9. CHSE Growth over one week supplemented with ECGS. (Vertical bars show standard deviation).

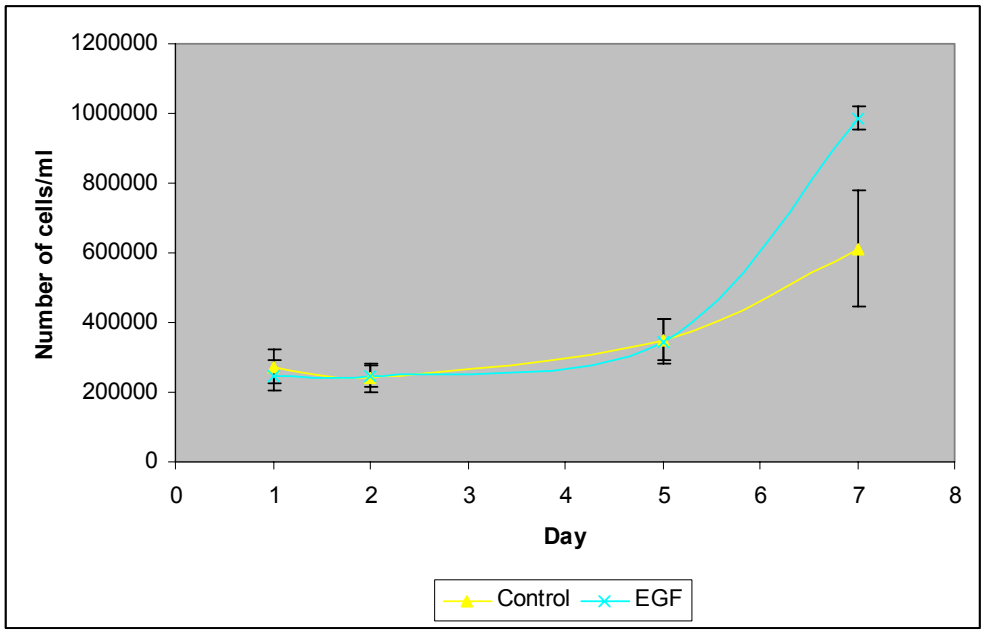

Figure 3.10. CHSE Growth over one week supplement with EGF. (Vertical bars show standard deviation).

3.11 Mitogens

The cytotoxicity test determined that the optimum concentration to use to promote growth without causing cytotoxicity was 5µg/ml for concanavalin A and 0.1µg/ml for calcium ionophore. After addition to the media neither of these mitogens had any visual effect on cell growth or survival.

3.12 Chemical Mutagens

The optimum concentration for use after the cytotoxicity test was approximately 25ng/ml. However none of the cultures exposed to 4NQO showed any change in growth or other characteristics compared to the control. The time of survival of cultures exposed to 4NQO was reduced and cell death occurred earlier than the control.

3.13 Senescence Test

As the Halibut spleen and brain cultures remained viable for prolonged periods of time but showed no or little observable growth, they were tested using the senescent staining kit to determine whether the cells were viable or senescent. None of the cells in the spleen cultures stained blue. A few isolated cells of the brain cultures did exhibit a blue stain (Figure 3.11).

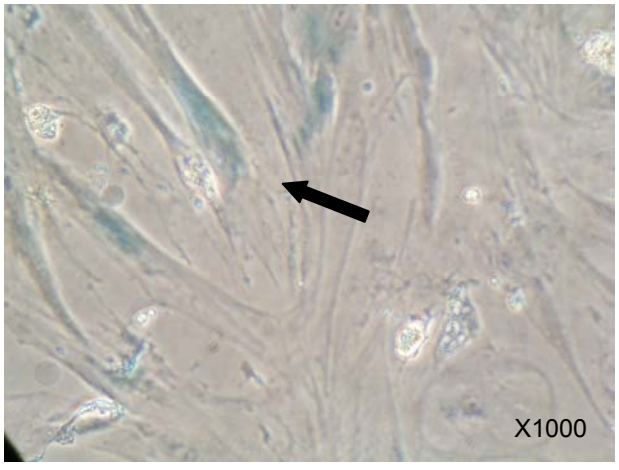

Figure 3.11 Halibut brain culture. Arrow shows stain (blue) that indicates a cell is senescent.

4. Discussion

The findings of this project provide information on several methods of producing primary cultures and other relevant factors for development. Although there have been several continuous cell lines developed using the explant procedure, this method was not particularly successful in the present study (Tong *et al.*,1997; Fernandez-Puentes *et al.,* 1993; Bejar *et al.,* 1997; Tong *et al.,* 1998; Moritomo *et al.,* 1990). It was difficult to get tissue fragments to adhere using recommended methods (Wolf and Quimby, 1969). It is likely that the flask substrate required treatment to encourage attachment of the tissue fragments, for example with serum or other attachment factors. Freshney (2000) mentions that plasma clots were used historically to increase attachment of explants. Unfortunately, those fragments that did adhere and exhibit outgrowth did not produce a culture. These cells did not survive for a substantial amount of time and hence were never sub-cultured. It may be that the conditions for growth were simply unsuitable, or that the cells were not in a favourable environment for survival, therefore cell death occurred relatively quickly.

Another possible explanation may be that there were too few cells. To an extent cell proliferation is regulated by signals from the environment. Low cell densities leave cells with free edges and so allow them to spread. High cell densities inhibit growth through cell to cell contact and the resultant change in shape of the cell (Freshney, 2000). However this does not guarantee that a low density of cells would promote growth and high density inhibit it. Low cell densities in culture can prove to be inhibiting to growth rather than stimulatory. It has been demonstrated that cell survival and growth can be improved by the presence of feeder layers or conditioned

media. Conditioned media is media which is removed from an established culture. It will contain substrate-modifying constituents, growth factors as well as other metabolites (Freshney, 2000). It provides the missing components that may encourage growth and sustain cultures. This might explain why when the explanted tissue was removed or detached after washing, cell death occurred shortly after. The use of a feeder layer of cells or conditioned media may have increased the survival time and/or encouraged the cells to grow further into a confluent culture.

The explanted cod larvae fragments were much more successful. The cells of larvae are still in an active state of mitosis and therefore, when isolated from tissue, are much more likely to proliferate. This was seen within the cultures before contamination overwhelmed the cells. Cells outgrew quickly and new growth was visible twenty-four hours after isolation. Contamination is not unusual, as it is difficult to decontaminate external tissue unless very strict procedures are followed before isolation is attempted. Although the tissue was thoroughly washed in ethanol and antibiotics before disaggregation, it was not enough to fully eliminate the bacteria which caused the contamination. Further attempts may require eggs hatched in sterile seawater to remove or reduce contamination to a manageable level.

Mechanical disaggregation did not produce any cultures. Reviewing the literature reveals that very few continuous cell lines have been established in this way. Only leukocyte-like lines derived from kidney have been developed with any success using mechanical disaggregation (Wang *et al.,* 1995; Wergeland and Jakobsen, 2001). Mechanical disaggregation causes more physical damage and produces lower yields of cells than any other method (Freshney, 2000). This is apparent in the cultures produced

using this method, which exhibited a lot of debris and no whole cells. It is likely that the sieve size was too small which as a result lysed all the cells so that none survived intact. It is unlikely that further research into the benefits of this method would be of much value.

As predicted by the literature the highest success in producing cultures was achieved using enzymatic digestion. Most continuous fish cell lines have been developed in this way. There are numerous variations to the basic method and each attempt at isolating cells from a new species and/or organ will require a certain amount of optimization. It is quite common for full experimental details to be omitted from publications as well as information on the number of attempts required to establish a cell line. This imposes a requirement for optimization at each new attempt. This project revealed that very simple differences between methods can result in improved yields. The harvest of cells produced using two different mixing techniques illustrates this point. When mixing the soft tissues with a magnetic stirrer, yield was lower, due in part perhaps to the flea causing more mechanical damage to the cells. For optic nerve and tail tissues, this extra abrasive action may have been necessary to release individual cells.

The experiments that involved using different temperatures for trypsinization are in agreement with the findings of Wolf and Quimby (1969). A higher temperature results in a faster digestion so taking less time and vice versa a colder temperature will take longer. For this project not only was it more practical to run the digestion at room temperature, it also produced slightly better yields.

The digestive enzyme trypsin-EDTA proved to work well in this case. So there was little reason to investigate further into the use of other enzymes for digestion. It is the most commonly used enzyme for digestion.

However, it is possible that by investigating other enzymes, cultures could have been produced from those organs with which there was little success *i.e.* liver. Or that by using a mixture of enzymes (Butler and Nowak, 2004) that the number of cells released from other tissues could have been increased.

After isolation cell survival and growth is influenced by environmental factors such as media, temperature, etc. The investigations within this project into temperature did demonstrate that temperatures above 20°C are not suitable for cold water fish. Most established cell lines developed from cold water species are grown at 15-20°C (Fernandez *et al*., 1993c). Wolf and Mann (1980) also report a 25°C inhibitory limit for coldwater species.

As expected E-MEM with 10% FBS added was the best media for cell survival and growth with one exception (tail cells which grew better on L-15). This is in agreement with other published studies where cells grown in E-MEM and L-15 performed the best (Fernandez *et al.*, 1993a; Tung *et al.*, 1991). Some cultures exhibited clumping of the cells in H-MEM, perhaps indicating slight toxicity (Fernandez *et al.*, 1993a). The principle differences between these three media types are based on concentration of salts and variations in the amino acids present as well as the amount. For instance E-MEM is high in bicarbonate whilst H-MEM is low. L-15 has higher levels of sodium pyruvate but lacks bicarbonate. Levels of bicarbonate can have an important influence. Divalent cations *e.g.* Ca^{2+} are required by some adhesion molecules and can also influence whether cells proliferate or differentiate (Freshney, 2000). Bicarbonate also plays a role in pH especially with the low cell numbers found in primary cultures. The culture of cells from fish has followed a similar process to that in warm-blooded animals and

hence the types of media used are similar; MEM (Earle's, Hank's, or Glasgow) being the most widely used.

The cells that attached and proliferated did not demonstrate any of the normal indicators of stress i.e. vacuoles, granulation or clumping so we can assume that the media did not create unfavourable conditions for survival. This however does not guarantee the optimum conditions for growth. Further development of specifically designed media for fish cell lines is rare as currently existing media are generally adequate. There is no need to refine media until a cell line has been established and growth needs to be optimized. There have been some cases were media has been specially developed during the primary culture stage. However the basis of the media was still E-MEM and cultures were still isolated although attachment and growth was to a lesser extent (Kumar *et al.,* 2001). In this project E-MEM supplemented with serum was sufficient for survival and growth so there was no further need to refine the media.

It is important to note that although in this case the serum used was sufficient for survival and growth, testing of different serum batches could play a role. This is observed in established fish cell lines. One cell line may grow well upon a particular serum batch whilst another cell line will show poor or little growth on the same serum batch. Investigating different serum batches as done with established cells lines may have improved growth in those cultures which exhibited lower growth rates.

The next selection process that occurs after isolation (enzymatic or explant) differentiates between cells which adhere and cells which do not (Freshney, 2000). Throughout fish primary culture attachment factors are not routinely used and most established fish cells lines were initiated without the use of any attachment factors. Cod and halibut cells adhered to normal

tissue culture flasks without the need for any special coating. However, cod heart cells did demonstrate a marked increase in attachment when seeded on to collagen coated flasks. Before using collagen yields were lower and confluent cultures less common. Butler *et al.* (2004) also found that collagen increased attachment. Collagen is a protein obtained from connective tissue (skin, tendon and bone) and it can affect adhesion, growth and morphology of a variety of cell types. Other tissue types did not show the same increase in attachment except for brain tissue on poly-D-lysine coated flasks. Poly-D-lysine is a synthetic attachment factor which improves the adhesive properties of a flask by altering the charge to positive from negative. It also enhances absorption of serum or ECM proteins. As poly-D-lysine is formulated specifically to improve neurite attachment, increased attachment of brain cells was not an unexpected result.

There is past evidence of basic improvements to the culture vessel substrate in an attempt to increase attachment of cells. Serum has been used to coat a tissue culture vessel to enhance attachment of tissue fragments (Kumar *et al.,* 2001). The same study also found that glucose and sucrose increased attachment but that other attachment factors had an inhibiting affect on attachment. Butler and Nowak (2004) and Part *et al.* (1993) also demonstrated that some attachment factors were inhibiting. Bols *et al.* (1994) investigated the use of several attachment factors finding that only collagen IV improved cell adherence. The success of attachment factors appears to vary from organ to organ and investigation may be required for every fish species studied. As the attachment of cells is one of the first obstacles to overcome when developing a culture it can prove important to primary isolation.

To achieve a confluent culture, cell growth must occur. This growth can occur naturally. As discussed earlier, cells rely on the external environment to an extent to stimulate growth. It is possible to encourage cells to proliferate by simulating these signals. However, any growth factors available commercially are primarily developed for mammalian cell culture. Although investigations into the use of bovine growth factors on fish primary cultures did not produce any significant increase in primary cell growth, there was an increase in the growth of CHSE with the addition of ECGS and EGF. This is supported by work done by Chen et al. (2004) in which basic fibroblast growth factor (bFGF) increased cell growth.

Growth factors are primarily used as a replacement for factors in serum when serum-free media is required. Under normal circumstances serum provides the necessary growth factors and so addition of further growth factors has no significant effect. Attempts to use fish serum as a growth stimulate were unsuccessful. The fish serum was toxic to the cells. Past studies also found fish serum to be inhibitory or toxic (Wolf and Quimby, 1969). While serum contains growth factors, it can also contain other substances which are cytostatic (Freshney, 2000). There has been use of specific fish-based growth factors to increase growth of isolated cells (Kumar *et al.*, 2001). There is also evidence of mitogens being used to increase cell proliferation particularly in leukocyte based cultures but also in liver cultures (Miller and Clem, 1988; Faisal *et al.,* 1995). The use of mitogens in this project had no effect on cell growth. This suggests that there are possibilities for fish-based growth factors over bovine based factors; with particular respect to stimulating growth in those cultures which had viable cells but did not exhibit any growth. The requirements of fish cells

vary from those of mammalian cells and formulating media that is specific for fish cell growth needs is an area that is still relatively unexplored.

Unfortunately growth factors will not change the fact that the majority of cell lines developed are finite. Media, growth factors and other supplements will simply optimize the conditions for growth. Conditioned media and feeder layers do provide benefits in keeping a cell monolayer viable which is useful for some types of testing *e.g.* metabolic studies. However none of the primary cultures that were isolated and which developed into confluent cultures gave rise to a continuous cell line. This is not surprising as most normal cells will not. They require transformation to become 'immortal'. This was demonstrated by the confluent cultures produced in this project as the highest passage reached was four. Although the cells appeared to proliferate between sub-culturing, there was a limited number of passages before proliferation ceased and senescence occurred. Freshney (2000) details that most finite cell lines have twenty to eighty possible population doublings before senescence. Kumar *et al.* (2001) achieved only fifteen passages with their ovarian cell line before cell death occurred. As most reported cell lines are not available commercially, it is impossible to determine whether these cell lines are transformed or have a limited number of passages too.

Cells can transform spontaneously *in vitro,* however transformation can also be chemically or virally induced. Hence the attempt to induce transformation by exposing cultures to a mutagen. There is little evidence of this being attempted in fish culture as most transformation appears to be spontaneous. Takarada *et al.* (1989) reported transforming a fin cell line with the mutagen MNNG (N-methyl-N-nitro-N-nitrosoguanidine). After two exposures to MNNG the cells exhibited loss of contact inhibition and

increased growth rate. Guo *et al.* (2003) reported spontaneous transformation of a flounder cell line at around passage 171. The cells became neoplastic and formed spherical cell masses. There was no such success with this project either by using 4NQO as the chemical mutagen or through spontaneous transformation. Further testing is required to fully explore the possibilities of using chemical mutagens to transform cells to bypass senescence and cell death.

There were a few primary cultures produced that although they failed to grow any further survived for an extended period of time (over ten weeks). In order to determine whether these cells had entered a state of senescence or if they were still capable of growth, a Sigma kit test was undertaken to determine if the cells were senescent. The test did not provide any definite answers as to whether the cells were senescent or not. There was a small amount of staining in the brain culture but not enough to draw any definite conclusions. Either the cells were still viable but not proliferating, or the kit was unsuitable for use on fish cell lines. If the cells were still viable this suggests that the conditions for survival were correct but some substance was needed to stimulate growth.

The cultures produced from both cod and halibut in this project were quite varied. More cultures were produced from cod simply because the first attempts at isolating a culture were in halibut. By the time isolation was attempted in cod many of the initial problems encountered with halibut had been improved upon. Most of the successful cultures from both cod and halibut appeared to be epithelial in type rather than fibroblasts. However even cultures developed from the same tissue were often quite different in morphology. Defining cell type through morphology can be subjective as epithelial cell types can appear quite different in shape at a low confluence.

The cells isolated from the spleen appear to be mainly leukocytes. This is because spleen plays an important role in the immune system of a fish. It traps antigens and houses proliferating lymphocytes (Horton and Ratcliffe, 1993). It also holds large numbers of red blood cells which is evident from the large numbers found in culture after isolation (Roberts and Ellis, 2001).

The cultures developed from the optic nerve of the halibut appear to be unique. Fryer and Lannan (1994) mention only one culture isolated from eyeball. Since then there has been one more culture developed from the eye but none specifically from the optic nerve (Lai *et al.,* 2000). The halibut optic nerve is thick (string-like) and very easily removed from the surrounding tissue. This is why it was possible to isolate primary cultures. This is further supported by the difficulties in developing a culture from the cod optic nerve. The fact that the optic nerve was difficult to isolate and remove from the surrounding tissue may be reason for the low numbers of viable cells rather than a failure of the enzymatic digestion. There simply was not enough tissue extracted to obtain a good yield of cells.

The brain cultures isolated from cod were also relatively rare. Fryer and Lannan (1994) mention no brain derived cultures and since their listing there has only been one brain-derived culture from the grouper fish (Lai *et al.,* 2000). It is difficult to say exactly what cell type the brain and optic nerve cultures were composed of. It seems unlikely that it the cells are neurons therefore we can hypothesis that the cells were derived from the supporting glial cells. The same inference could be made for the optic nerve cultures. Further investigation is warranted to determine the exact origin of the cells from both sources.

Liver cultures are also rare (Fryer and Lannan, 1994; Faisal *et al.,* 1995). This is supported by the results of this project. Cells were very difficult to isolate and, even when extracted, failed to grow. The liver assists in digestion by secreting enzymes that break down fats, and also serves as a storage area for fats and carbohydrates. This may indicate why cultures are hard to develop. There was a great deal of fat remaining in the upper layers of the supernatant after centrifugation. Larger pieces of tissue may have been required in order to obtain the same yield of cells isolated from other organs as a high number of cells isolated were fat cells.

The spontaneously contracting heart cells were not anticipated. There has been no report of this phenomenon occurring in fish primary culture previously. Wolf and Quimby (1969) did report a swim-bladder culture which exhibited peristaltic–like movements. It does not appear to be an unusual occurrence for mammalian heart cells to exhibit a contractile ability in culture (Satin *et al.,* 2004). Heart muscle cells are myogenic meaning that they stimulate their own contraction without the need of any neuronal input. A single heart muscle if left without input will contract at a steady rate as well as stimulating any other cells in contact to contract. The contraction of cardiac muscle in vertebrates is activated by an increase in intracellular Ca^{2+} either from the sarcoplasmic reticulum or extracellular fluid (Randall *et al.*, 1997). This may explain how cells within culture may exhibit spontaneous contraction. The media contained enough Ca^{2+} ions to cross the plasma membrane and increase intracellular levels hence stimulating contraction.

In conclusion enzymatic disaggregation seems to be the most efficient technique to isolate cells and develop confluent cultures. Almost seventy attempts were made in total to establish cells and many of these attempts did not produce any primary cultures. Since the method and

reagents were consistent, it must be variability in the fish tissues that explains why the same technique can work on one occasion but not on the next. Although the primary cultures produced here were sub-cultured up to four times, producing a continuous or immortal cell line remained elusive.

E-MEM 10% FBS was the best all-purpose media of use. The use of attachment factors has potential benefits for increasing cell adherence. It would need to be investigated for each species and organ to be cultured as attachment factors have been demonstrated to be inhibitory. The use of growth factors and mitogens still requires further investigation especially in relation to fish specific growth factors. Further investigation into mutagens is also required. There are potential benefits in developing a method that could be applied to cultures to induce transformation. It could allow cell lines to be developed more easily or adapt currently available cell lines.

The main aim of developing a new culture in this instance was for virus replication; however, even short-lived cultures have benefits. The primary cultures developed although not established still provide opportunity for virological studies. The cells in a primary culture vary less from the original fish cells than a transformed cell line therefore providing detail into how viruses infect cells and replicate within the host fish (Freshney, 2000). Further study *e.g.* staining on the cultures that were developed is required to determine the origin of the optic nerve and brain cultures.

Short-lived cultures also have their place for other types of research other than virological requirements. For instance there was interest generated in using the short-term cultures developed for ecotoxicity testing. It was proposed to use a comet assay to detect the amount of DNA damage in cells from a primary cell culture exposed to environmental toxins. This

replaces the need for exposing large numbers of fish to toxins in order to determine what detrimental effects would occur to cellular DNA.

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