A publication of
The Williamsburg BioProcessing Foundation

November/December 2004

BioProcessing™ JOURNAL

Advances & Trends In Biological Product Development

Vol. 3 No. 6

www.bioprocessingjournal.com

The Intraocular Delivery of Neuroprotective Factors to the Retina Using Encapsulated Cell Technology

By CAHIL McGOVERN, SANDY SHERMAN, SUE MATEUS, WILLIAM TENTE, MARY JANE DOLD, ANNE MARIE BISHOP, and WENG TAO

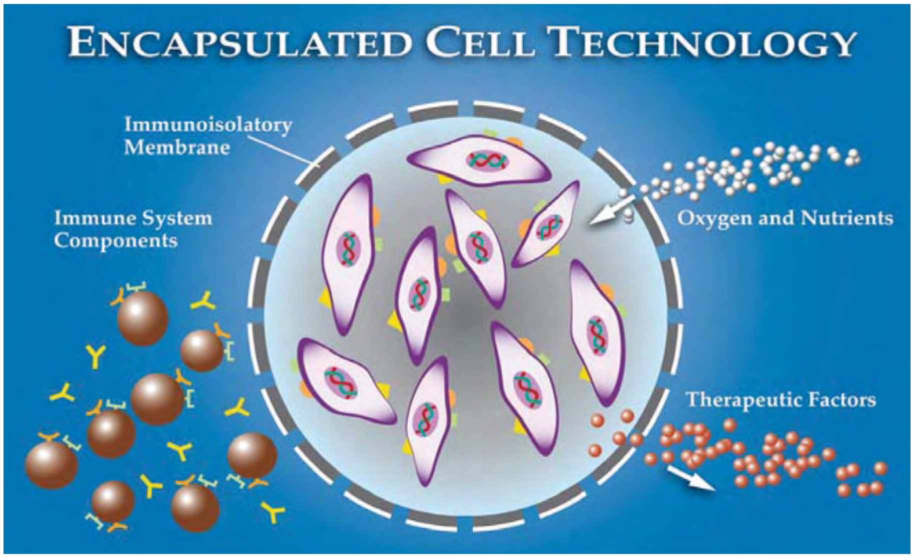


Figure 1. Encapsulated Cell Technology facilitates the long-term delivery of therapeutic molecules secreted by genetically engineered cells in the absence of an immune response. The structure of the HFM allows for the inward diffusion of oxygen and nutrients and the release of a specific therapeutic protein factor to the implantation site.

Ophthalmic disorders are a group of diseases with a rapidly increasing frequency associated with an increase in the aged population.[1–3] Patients with potentially blinding diseases have become one of the largest segments of the healthcare field, with more than 50 million patients in the United States alone. Their sight is threatened by diseases such as age-related macular degeneration (AMD), diabetic retinopathy (DR), glaucoma, or retinitis pigmentosa (RP).[4–12]

Until recently, there were essentially no effective treatment options to halt the progression of chronic, potentially blinding diseases. Biotechnological advances have resulted in the development of a variety of promising new protein factors that, if delivered to diseased cells of the retina, hold promise for treatment by interrupting or reversing disease processes.

Many studies have demonstrated the promise of neurotrophic factors as therapeutics for RP in a variety of animal models of the disease. Among them, ciliary neurotrophic factor (CNTF) is reported to be the most effective in reducing retinal degeneration.[13] CNTF is a member of the IL-6 family of neurotrophic cytokines. Its biological activities are mediated through a heterotrimeric complex consisting of CNTF receptor alpha, gp130, and LIF receptor beta, which activates the JAK/STAT signal transduction pathways. Although its intrinsic function in adult animals is not fully understood, exogenous CNTF affects the survival and differentiation of a variety of cells in the nervous system, including retinal cells. In spite of the consistent demonstration of CNTF to provide a protective effect on photoreceptors in various animal models, the local adverse effects associated with its intraocular injection, its short half-life following intravitreal administration, and the blood-retina barrier, which precludes useful systemic administration of CNTF, have prevented the further clinical development and therapeutic practicality of CNTF as a treatment option for RP. In this regard, the application of ECT-mediated delivery of CNTF is being pursued to determine if the progression of retinal degeneration can be halted or slowed in patients with RP.

Cahil McGovern, Ph.D. *(c.mcgovern@neurotechUSA.com) is senior scientist, process development; Sandy Sherman is manager, clinical manufacturing; Sue Mateus is scientist, manufacturing; William Tente, M.S. is vice president, manufacturing and product development; Mary Jane Dold is manager, quality assurance; Anne Marie Bishop is in quality assurance; and Weng Tao, M.D., Ph.D. is vice president, research and development; Neurotech USA, Inc., Lincoln, RI.*

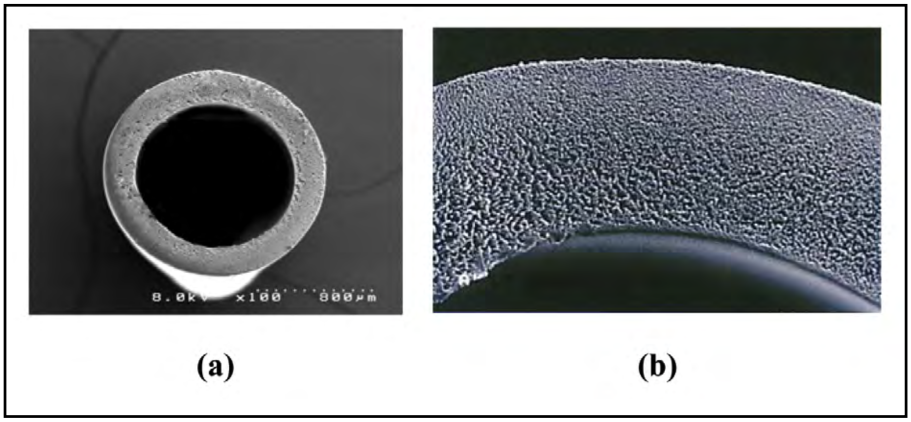


Figure 2. The Hollow Fiber Membrane (HFM) employed in ECT: (a) Cross section of the HFM. (b) Magnified image of the HFM demonstrating the foam pore structure.

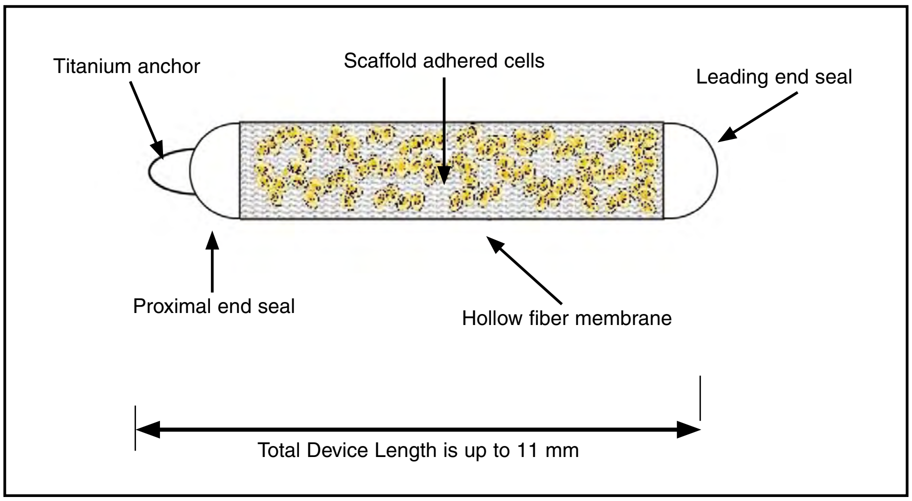


Figure 3. A schematic of the ECT device depicting each component (the titanium anchor, UV light cured adhesive (at the proximal and leading end seals), and the HFM. The sectioned view of the inner lumen shows the yarn scaffolding and scaffold adherent cells.

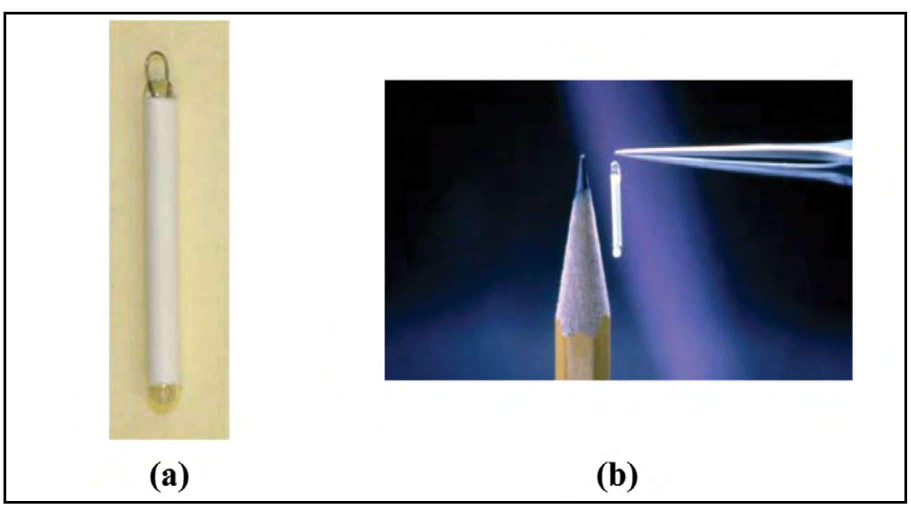


Figure 4. The ECT device. (a) ECT device showing the titanium loop, HFM, and adhesive. (b) The total length of the ECT device is up to 11 mm. The relatively small size of the device makes it easy to implant and retrieve.

Sustained Ocular Delivery of Protein Therapeutic Factors with ECT

The chronic nature of most retinal diseases and the issues associated with other methods of therapeutic protein delivery to the back of the eye served as the basis for ECT product development. ECT relies on the transplantation of genetically engineered cells that stably secrete a specific therapeutic factor at the site of implantation (Fig. 1). The cells are contained within the immunoprotective hollow fiber membrane (HFM) device. The characteristics of the membrane allow for long-term cell survival and continuous protein expression. Pore structure of the HFM allows inward diffusion of oxygen and nutrients and the release of the therapeutic protein to the implantation site (Fig. 2). The HFM structure also prevents the direct contact of the immune system components with the transplanted cells.

ECT Device Design for Intraocular Delivery

The current ECT product concept for the intraocular delivery of CNTF consists of a HFM surrounding a number of strands of yarn, which serves as a scaffolding for cell adherence and growth. The ends of the HFM are sealed with medical grade, light-cured adhesive

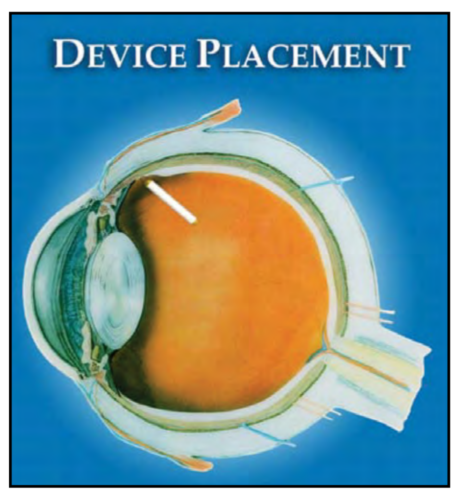


Figure 6. Placement of the ECT device within the human eye. The device is anchored in the scleral tissue using the titanium loop and can deliver therapeutic molecules for up to one year. The device is anchored in a manner so that it sits outside of the visual axis.

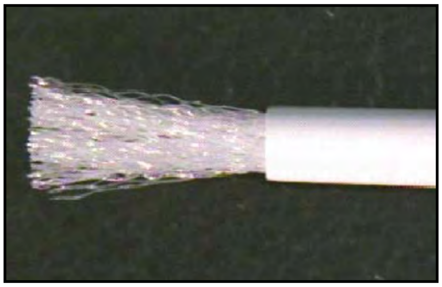


Figure 5. Photomicrograph of the multifilament scaffolding within the membrane of the ECT device.

after the interior has been loaded with cells. A titanium loop, which facilitates surgical implantation of the device and also acts as the suture site, is attached to the proximal end of the device (Figs. 3, 4, & 5). Implantation is achieved by making a small incision in the sclera followed by insertion of the device through the opening. The device is anchored to the pars plana and is secured so that it sits outside of the visual axis (Fig. 6).

The platform cell line employed in ECT-mediated protein factor delivery, NTC-200, was derived from the human adult retinal pigment epithelial cell line, ARPE-19. ARPE-19 was generated from normal donor eyes. This cell line was spontaneously immortalized and retained a number of differentiated properties of retinal pigment epithelia (RPE), including polarized distribution of cellular organelles as well as expression of cellular retinaldehyde binding protein (CRABP) and RPE-65, an RPE cell-specific molecule thought to play a role in the RPE-photoreceptor vitamin A cycle.[14,15] ARPE-19 cells were also found to survive in nutrient poor culture environments, tolerate encapsulation conditions, and could be genetically modified to express a wide variety of recombinant proteins. Neurotech provided the NTC-200 designation to the master stock of these cells after preliminary safety and performance testing had produced acceptable results.

NTC-200 cells were transfected with a proprietary mammalian expression plasmid containing the human CNTF gene to create a number of stable CNTF-secreting cell lines. Constitutive CNTF expression with this plasmid is driven by the mouse metallothionein pro-

Table 1. Testing Performed on CNTF Producing Cell Banks Employed in ECT

Test	Specification	Performed on:		
		MCB[a]	WCB[b]	EOP[c]
Sterility (per USP)	Negative	x	x	x
Mycoplasma (per 1993 Points to Consider method)	Negative	x	x	x
Cell Identity (isoenzyme analysis)	Human origin	x	x	x
Cytogenetic analysis	Chromosomal analysis established for cells at MCB and EOP level	x		x
Detection of adventitious bovine viruses	Negative	x		
Detection of adventitious porcine viruses	Negative	x		
Detection of adventitious viruses via *in vitro* detection	Negative	x		x
Detection of adventitious viruses via *in vivo* detection	Negative	x		x
Detection of adventitious viruses contamination via transmission electron microscopy	Negative	x		x
Detection of retroviral contamination via detection of reverse transcriptase	Negative	x		x
Test for human immunodeficiency virus 1[d]	Negative	x		
Test for human Immunodeficiency virus 2[d]	Negative	x		
Test for hepatitis A virus[d]	Negative	x		
Test for hepatitis B virus[d]	Negative	x		
Test for herpesvirus-6[d]	Negative	x		
Test for hepatitis C virus[d]	Negative	x		
Test for Epstein Barr virus[d]	Negative	x		
Test for human parvovirus B-19[d]	Negative	x		
Test for cytomegalovirus[d]	Negative	x		
Test for human T lymphocytic virus I[d]	Negative	x		
Test for human T lymphocytic virus II[d]	Negative	x		
Tumorigenicity[e]	Negative	x		x

[a]manufacturer's master cell bank
[b]manufacturer's working cell bank
[c]end of production cells
[d]polymerase chain reaction assay
[e]tumorigenicity assessed in nude mice

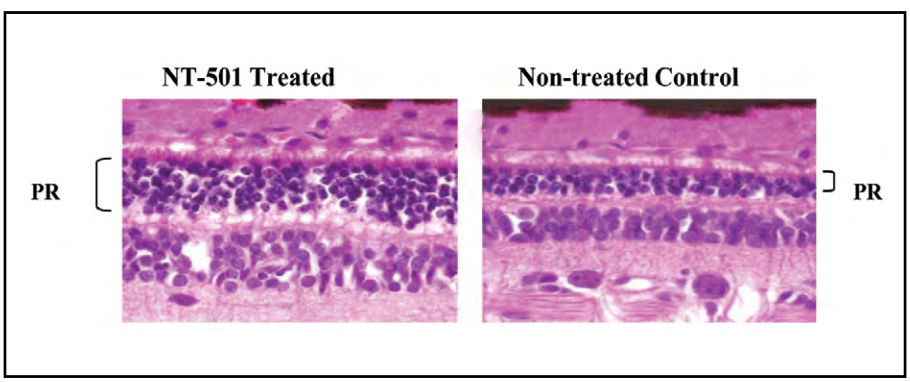


Figure 7. Photoreceptor (PR) protection in *rcd1* canine model of RP. Devices were implanted in one eye of each study animal with the contralateral eye receiving no treatment for a period of seven weeks. The eye implanted with the ECT device had significantly more photoreceptor cells in the ONL than the untreated control (5-6 layers vs. 2-3 layers).

moter. To target CNTF for secretion, the genomic murine immunoglobulin signal peptide gene was fused in frame to the 5' genomic human CNTF gene. Cultures of NTC-200 cells were exposed to microgram quantities of plasmid DNA in the presence of FuGENE 6 (Boehringer Mannheim, Inc.). Transfectants were selected through exposure of the cultured cells to the selection agent Geneticin (G418). Candidate cell lines derived from different transfections were subjected to a variety of functional tests and selected based on a variety of performance characteristics, including CNTF expression level, stability of expression, and ability to deliver CNTF under *in vitro* and *in vivo* conditions while encapsulated. Once selected, manufacturer's master and working cell banks and end of production cell banks were generated and cryopreserved for the final product candidate cell lines. Safety testing and characterization were performed in accordance with ICH Tripartite Guideline, *Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origin* as well as FDA's 1993 *Points to Consider in the Characterization of Cell Lines used to Produce Biologicals.* Tables 1 and 2 summarize the testing performed on these cell banks.

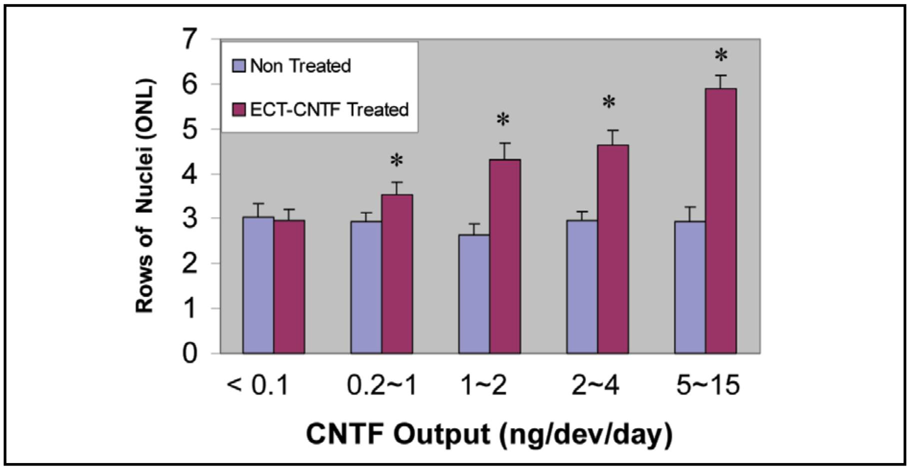


Figure 8. Dose Dependent Protection of the photoreceptors in the ONL from Encapsulated Cell Delivery of CNTF in the *rcd1* Dog Model (*denotes statistically significant effect). Protection of photoreceptors in the ONL required ECT devices with CNTF output > 0.2 ng/device/day. No photoreceptor protection was noted from ECT devices with CNTF output of <0.1 ng/device/day.

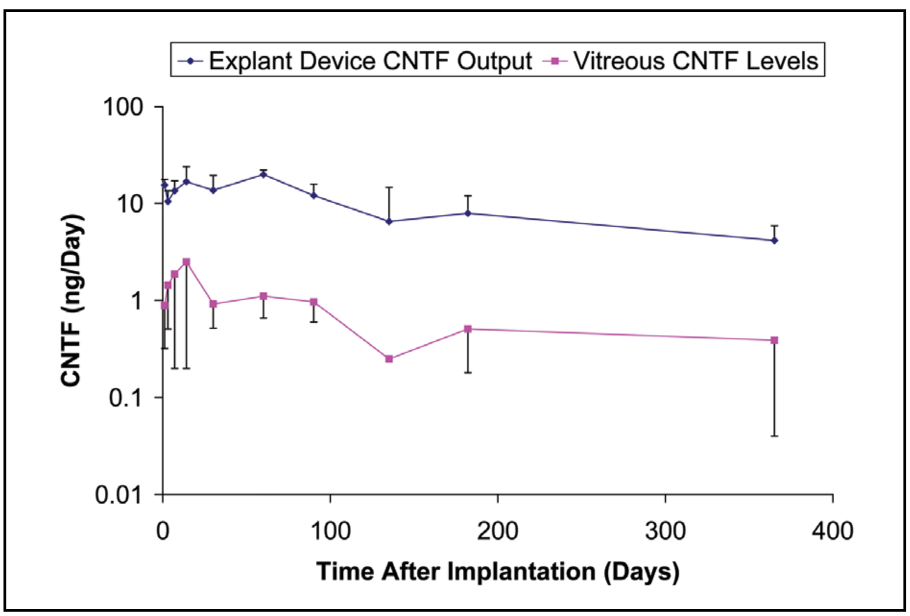


Figure 9. Time course of CNTF device output (after explanting) and vitreous CNTF levels from encapsulated NTC-201-6A cells. Devices were implanted into the eyes of animal subjects, explanted at specific time points, and analyzed for CNTF output. Vitreous samples were collected at the time of explant and analyzed for CNTF.

Establishment of ECT Performance in Pre-Clinical Studies

The effectiveness of ECT-mediated delivery of CNTF was demonstrated preclinically using an established animal model of RP, the *rcd1* canine model.[16] In this mutant dog strain, retinal degeneration begins at approximately four weeks of age and continues gradually over the course of a year, with 50% of the photoreceptor loss occurring at seven weeks of age and 70-80% loss at 14 weeks of age.[17] ECT devices with different levels of CNTF output were produced by encapsulating different cell lines with inherently different CNTF expressions rates. Devices were implanted in one eye of each study animal with the contralateral eye receiving no treatment. Treatment duration was seven weeks, starting when the animals reached seven weeks of age. For those eyes that had been treated with CNTF releasing ECT devices, significant neuroprotection was observed, as the treated eyes showed significantly more rows of photoreceptors in the outer nuclear layer (ONL) than the untreated contralateral eye (Fig. 7). Additionally, the neuroprotective effect was shown to be dose-dependent. Minimal protection was attained with devices exhibiting post-explant CNTF output levels at 0.2 to 1.0 ng/device/day (Fig. 8). Incrementally greater protection was achieved with higher doses whereas no protective effect was observed when post-explant CNTF output levels were less than 0.1 ng/device/day. Histological examination of explanted devices demonstrated the presence of viable cells within the devices.

The demonstration of neuroprotection in the *rcd1* model with ECT-mediated delivery of CNTF represented an important preclinical finding. However, long-term (approximately one year) CNTF delivery following intraocular implantation still needed to be established because retinal degeneration in humans is a chronic process that occurs over a number of years. To investigate this, ECT devices were implanted into the eyes of normal rabbits for a period of up to one year. Devices were removed at specific time periods and analyzed for CNTF output and encapsulated cell viability by histological analysis. CNTF levels in the vitreous compartment were assessed in the enucleated eyes using an enzyme immunoassay.[18] These results are summarized in Figure 9. The explanted devices continued to produce CNTF up to 12 months. CNTF delivered to the vitreous was also demonstrated in this time period. Histological evaluation indicated that all explanted devices contained viable cells. These time course studies proved that ECT devices are capable of delivering CNTF for up to a year.

Aspects of Product Development and Manufacturing

Device Assembly

Pre-assembled capsules (PAC) are assembled by inserting the yarn scaffold material into the lumen of a length of HFM followed by the attachment of a specially designed hub/tubing assembly to one end of the HFM. The hub/tubing assembly facilitates the injection of cells into the device at the encapsulation stage. An ultraviolet (UV) light-cured adhesive is used to secure this part to the HFM. A titanium anchor loop is joined to the opposite end of the HFM and is also affixed with the adhesive. A specially designed titanium device clip is attached to the loop, which secures the device in the product package and is used to manipulate the device in the surgical field. The completed PAC is flow tested to detect occlusions in the hub/tubing assembly, leak tested to assure seal integrity, and inspected for critical dimensional properties. PAC sterilization is accomplished through exposure to electron beam radiation.

Cell Expansion

Expansion of cells for ECT device manufacturing is a standard procedure that involves cultivation of monolayers in a fetal-bovine-serum-supplemented growth media in tissue culture flasks incubated at 37° C in an atmosphere of 5% CO_2. Production runs are initiated by thawing a vial of WCB cells. Expansion is accomplished by exposing the cells to trypsin and reseeding the cells at split ratios of approximately 1:3. Cells are subcultured three times in a period of two weeks.

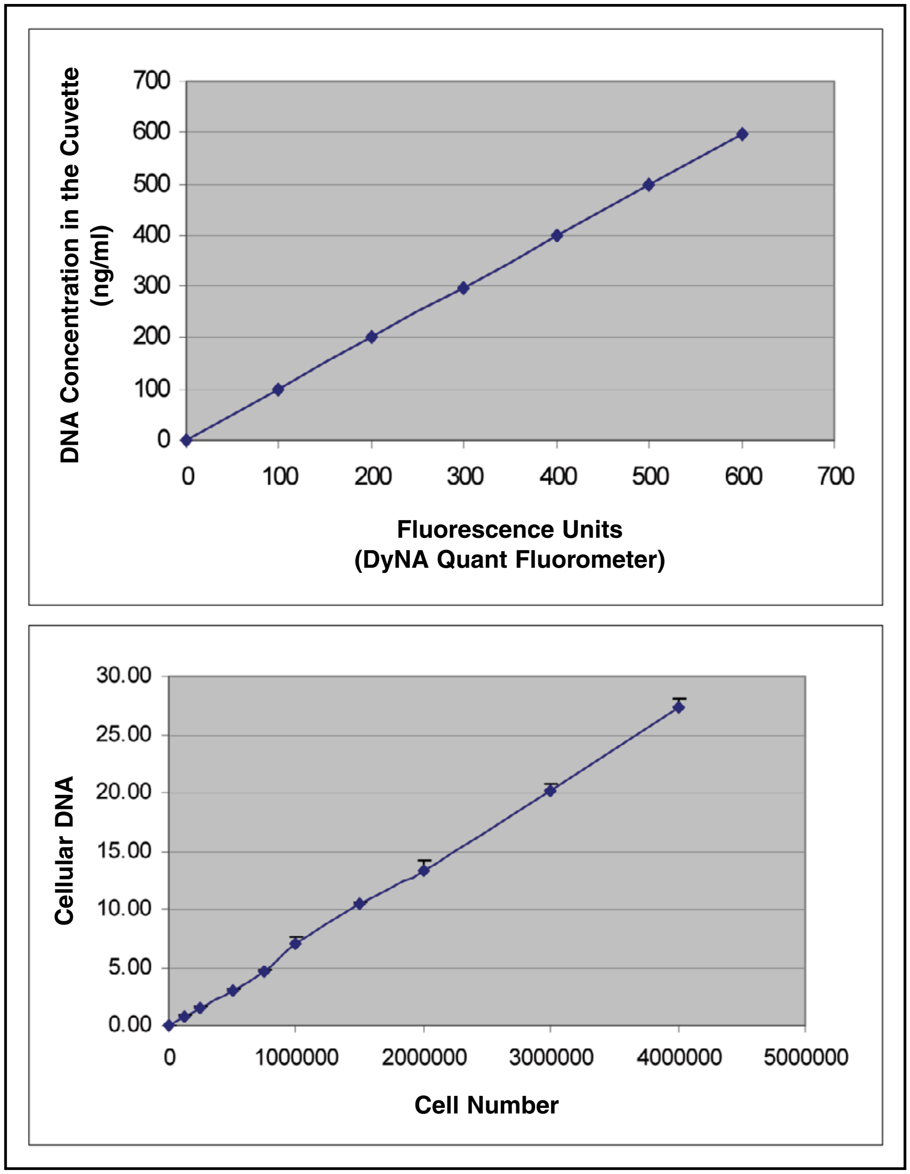


Figure 10. Linear Relationship between Cellular DNA and Cell Number. A standard curve was generated between 100 ng/ml and 600 ng/ml using calf thymus DNA (top). Multiple cell counts were performed using hemacytometer on several cell suspensions. Dilutions of known cell numbers were prepared and analyzed for total DNA content. Results showed a linear relationship between cell number and fluorescence (i.e., cellular DNA concentrations).

Cell Formulation

Immediately before the encapsulation process, the cultured cells are harvested, washed in a serum-free media, and quantified using a cellular DNA measurement system that employs the Hoefer DyNA Quant 200 Fluorometer and Hoechst 33285 dye (Amersham Pharmacia). A standard curve is generated from 100 ng/ml to 600 ng/ml DNA using calf thymus DNA. A sample of the cell suspension is sonicated and an aliquot exposed to the Hoechst binding dye. As the dye binds to the DNA, its spectral profile changes, emitting light at 458 nm. The intensity of the fluorescence is proportional to the cellular DNA concentration in the sample, which is a function of cell number (Fig. 10). Once the cells have been enumer-

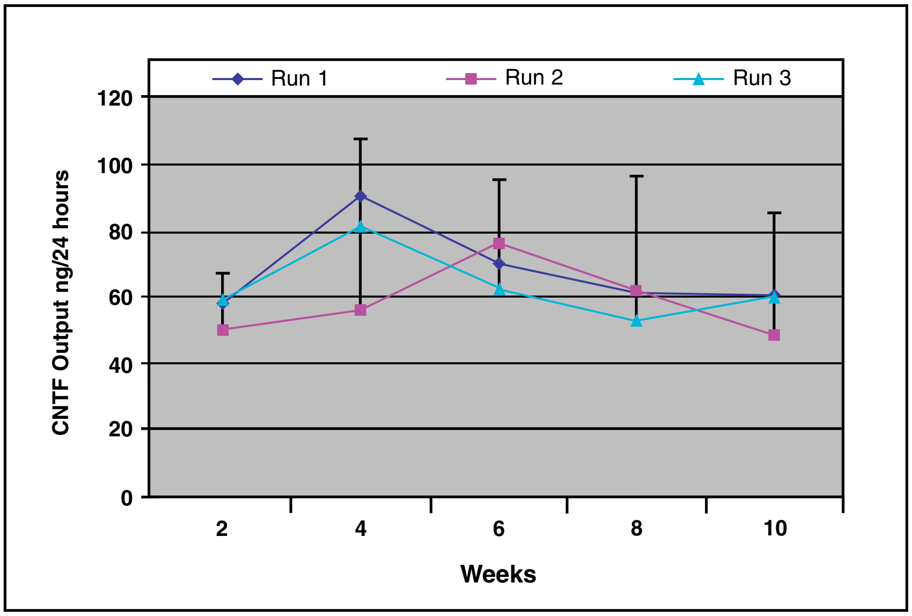


Figure 11. Three lots of ECT devices were manufactured under standard operating procedures. At specified time points the devices were removed from 37° C and analyzed for CNTF output.

Table 2. Genotypic and Phenotypic Analysis of CNTF-Producing Cells

Test	Description	Result
CNTF gene sequence analysis	Determination of gene sequence via RT-PCR	Confirmed fidelity of CNTF gene sequence in MCB and EOP cells
Gene copy number analysis	Determination of gene copy via quantitative PCR	Confirmed that there was no change in CNTF copy number from MCB to EOP cells
Assessment of CNTF gene status	Southern blot analysis following exposure of cellular DNA to restriction enzymes	Confirmed there were no major insertions, deletions or rearrangements of CNTF gene in MCB and EOP cells
Morphological examination	Documentation of cell morphology in T flask culture via photo-microscopy	Confirmed that cell morphology was preserved in culture period of MCB to EOP
Cell growth rate	Assess population doubling time in T flask culture	Confirmed that growth rate was unchanged in culture period of MCB to EOP
CNTF expression rate	Assess CNTF expression level over time in T flask culture	Confirmed that CNTF expression rate was unchanged in culture period of MCB to EOP
CNTF characterization	Assess CNTF protein secreted by cells using SDS-PAGE and western blot and in vitro biological assay	Confirmed that CNTF protein secreted by MCB and EOP cells was the proper molecular weight and was bioactive
Characterization of other cell products produced by cells	Quantitation of the following proteins in culture supernatants via enzyme immunoassay (brain-derived neurotrophic factor, basic fibroblast growth factor, vascular endothelial growth factor)	Determine protein expression levels and confirm that the protein expression level rates are unchanged in culture period of MCB to EOP

ated, the cell suspension is adjusted to a cellular DNA concentration of 0.19 µg/µl which corresponds to approximately 29,000 cells/µl in serum-free media.

Cell Encapsulation

The formulated cell suspension is transferred into the reservoir of a specifically designed instrument engineered to inject a precise volume of the suspension into sterilized PAC units. The instrument continuously mixes the cell suspension during the encapsulation process and controls the cell suspension injection rate to a very tight standard. Once the injection is complete, the instrument separates the cell-loaded PAC from the hub/tubing assembly. The device is then transferred to a second instrument where the orifice remaining from the removal of the hub/tubing assembly is sealed with the UV light-cured adhesive. The completed device is transferred into a primary package containing approximately 40 ml of a serum free medium. This primary package is then transferred into a protective package that is hermetically sealed with a heat activated foil. Labeling is placed on the outer seal package, which is maintained at 37° C for a two-month shelf life.

ECT Product Stability

Extensive studies were conducted to demonstrate the ability of ECT devices to consistently deliver CNTF during the defined product shelf-life period. The ability of encapsulated cells to survive in ECT devices over time and continuously secrete CNTF is a function of the unique ability of the NTC-200 cell line to tolerate these culture conditions and the genotypic stability of the transfected cell lines. Stability studies demonstrated that device CNTF output was maintained over a 10-week *in vitro* hold period (Fig. 11).

ECT Quality Control Test Procedures

A number of test methodologies are employed for the assurance of quality of manufactured lots of ECT device units (Table 3). Compendial test methods for sterility and bacterial endotoxins as well as tests for mycoplasma contamination of the cell preparation satisfy the safety test requirements for biological products. Unlike many cell-based products, the eight-week shelf life of ECT devices allows for completion of the USP sterility test as well as the remaining lot-release tests. There is also redundant in-process sterility testing performed upstream at the media supplementation and cell formulation steps to enhance process aseptic assurance. The mycoplasma testing procedure utilizes the established 1993 *Points to Consider* method, which incorporates two forms of mycoplasma detection: a 28-day broth cultivation procedure and an indirect method that uses the fluorescent dye Hoechst 33258 to detect non-cultivable strains in cell monolayers exposed to the test sample. A rapid polymerase chain reaction (PCR) method for mycoplasma detection is also performed to augment the *Points to Consider* methods. Assays to establish the biological status of the encapsulated cells (i.e., potency) include a trypan blue dye exclusion assay for cell viability at the formulation step and an assay that assesses the *in vitro* device CNTF secretion rate. The latter assay represents an important stability indicating assay. Histological analysis, although not employed as a lot-release assay, is another procedure to assess encapsulated cell viability in devices over time in culture (Fig. 12).

Table 3. Release Specification for the ECT Device

Test	Sample Description	Release Specification
Appearance	Final Device Units	Meets criteria
Sterility USP <71>	Final Device Units	No Growth
Sterility USP <71>	Hold Media	No Growth
Sterility USP <71>	Cell Harvest Sample	No Growth
Bacterial Endotoxin USP <85>	Final Device Units	Device contained in packaging unit: ≤ 20 EU/device Hold Media contained in packaging unit: ≤ 0.5 EU/ml
Potency (Device Output)	Final Device Units	Meets criteria
Mycoplasma (PCR)	Cell Harvest Sample	None Detected
Mycoplasma (Direct Cultivation)	Cell Harvest Sample	Negative
Mycoplasma (Non-Cultivable)	Cell Harvest Sample	Negative
Cell Viability	Cell Harvest Sample	≥ 90% viable

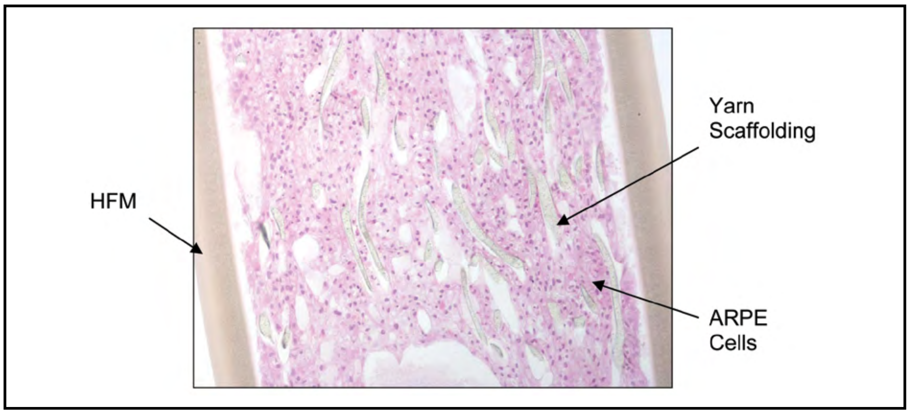


Figure 12. Histological Analysis of ECT Device showing the HFM, scaffolding, and adherent ARPE cells at 2 weeks post manufacturing. Sections of devices from each manufacturing run are stained with hematoxylin and eosin and examined microscopically.

Current Clinical Development Status

A Phase I trial of ECT-mediated delivery of CNTF in subjects with RP is currently in progress at the National Eye Institute in Bethesda, Maryland. ECT devices secreting two different levels of CNTF are being evaluated in ten subjects with RP. The primary purpose of the study is to evaluate product tolerability and safety.

REFERENCES

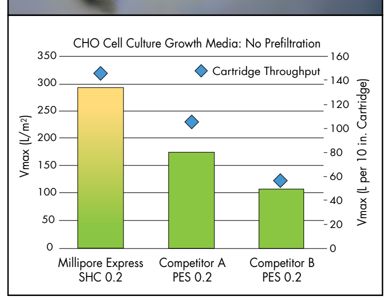
1. Leibowitz HM et al. The Framingham eye study monograph: An ophthalmological and epidemiological study of cataract, glaucoma, diabetic retinopathy, macular degeneration, and visual acuity in a general population of 2,631 adults, 1973-1975. *Surv Ophthalmol* 1980;24:335–610.
2. Martinez GS, Campbell AJ, Reinken J et al. Prevalence of ocular disease in a population study of subjects 65 years old and older. *Am J Ophthalmol* 1982;94:181–189.
3. Gibson JM, Rosenthal AR, Lavery J. A study of the prevalence of eye disease in the elderly in an English community. *Trans Ophthalmol Soc UK* 1985;104:196–203.
4. Vingerling JR et al. The prevalence of age-related maculopathy in the Rotterdam study. *Ophthalmology* 1995;102:205–210.
5. Hawkins BS, Bird A, Klein R et al. Epidemiology of age-related macular degeneration. *Mol Vis* 1999;5:26.
6. Podgor MJ, Cassel GH, Kannel WB. Lens changes and survival in a population-based study. *N Engl J Med* 1985;313:1438–1444.
7. Klein R, Klein BE, Moss SE. Age-related eye disease and survival: The Beaver Dam eye study. *Arch Ophthalmol* 1995;113:333–339.
8. Coleman AL. Glaucoma. *Lancet* 1999;354:1803–1810.
9. West SK. Looking forward to 20/20: A focus on the epidemiology of eye diseases. *Epidemiol Rev* 2000; 22:64–70.
10. Hoyng PF, van Beek LM. Pharmacological therapy for glaucoma: A review. *Drugs* 2000;59:411–434.
11. Dryja TP, Li T. Molecular genetics of retinitis pigmentosa. *Hum Mol Genet* 1995;4:1739–1743.
12. Meindl A et al. A gene (RPGR) with homology to the RCC1 guanine nucleotide exchange factor is mutated in x-linked retinitis pigmentosa (RP3). *Nat Genet* 1996;13:35–42.
13. McDonald NQ, Panayotatos N, Hendrickson WA. Crystal structure of dimeric human ciliary neurotrophic factor determined by MAD phasing. *Embo J* 1995; 14:2689–2699.
14. Dunn KC, Aotaki-Keen AE, Putkey FR et al. ARPE-19, A human retinal pigment epithelial cell line with differentiated properties. *Exp Eye Res* 1996;62:155–169.
15. Kanuga N, Winton HL, Beauchene L et al. Characterization of genetically modified human retinal pigment epithelial cells developed for *in vitro* and transplantation studies. *IVOS* 2002;43:546–555.
16. Tao, W, Wen, R, Goddard MB, Sherman SD, O'Rourke PJ, Stabila PF, Bell WJ, Dean BJ, Kauper KA, Budz VA, Tsiaras WG, Acland GM, Pearce-Kelling S, Laties AM, and Aguirre GD. Encapsulated Cell-Based Delivery of CNTF Reduces Photoreceptor Degeneration in Animal Models of Retinitis Pigmentosa. *Invest Opthalmol Vis Sci.* 2002; 43(10):3292–98.
17. Schmidt SY, Aguirre GD. Reductions in taurine secondary to photoreceptor loss in Irish Setters with rod-cone dysplasia. *Invest Ophthalmol Vis Sci* 1985;26:679–683.
18. Thanos CG, Bell WJ, O'Rourke P et al. Sustained secretion of CNTF to the vitreous using encapsulated cell therapy-based intraocular devices. *Tissue Eng* In Press;10:1617–1622.

MILLIPORE

turn up your volume

Twice the capacity to double your filtration output.

Pump up your filtration volume with Millipore Express® SHC sterilizing-grade high capacity PES filters. Millipore Express SHC devices significantly increase throughput across a range of moderate- to high-fouling process solutions, so you can reduce filter change-outs, minimize your filtration area requirements, and lower filtration costs. Tune in to the high performance of Millipore Express SHC filters. Visit www.millipore.com/shc, and **Discover the More in Millipore.™**



Higher throughput for cell culture media, media additives, process intermediates and protein-containing solutions.

Call 1-800-MILLIPORE today! Outside the U.S., www.millipore.com/offices