UNIVERSITY OF HELSINKI

mRNA transfection of antigen presenting cells

Initial steps towards *in vitro* immunization

Master's Programme in Microbiology and Microbial Biotechnology

Master's thesis

Author:
Esko Jaatinen

Supervisors:
Docent Jussi Hepojoki
Docent Tomas Strandin

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Author: Esko Jaatinen

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Abstract:

Antibodies are useful in research and medicine. Traditionally, they have been produced *in vivo* using animals, which is expensive, and the production process takes long. In contrast, there is interest for producing antibodies *in vitro*, which is cheaper and faster. *In vitro* immunization (IVI) can also be done against antigens that would be lethal to an animal host. This study focuses on transfection of antigen presenting cells with antigen encoding RNA using lipofection and electroporation with focus on developing an IVI protocol that is fast and cheap to implement to be accessible for research groups without hefty financing.

Monocytes separated from peripheral blood mononuclear cells (PBMC) were successfully transfected with RNA encoding fluorescent protein, although the observed transfection rate was significantly lower than in THP-1 cells. DOTMA and DOPE, which are among the oldest lipofection agents, performed even better than the thousandfold more expensive DOSPA based products in terms of transfection rate on THP-1 cells. However, the DOSPA based products performed far better in terms of observable protein amounts. Electroporation was able to transfect THP-1 cells with typical transfection rate around 80%. The downside with electroporation versus lipofection is that it requires higher RNA amounts.

The differences in observed fluorescent protein amounts between monocytes separated from PBMC and THP-1 cells cannot be attributed to membrane properties alone. There are several possible other reasons, and one of them is that the immune defence is processing the protein, which aligns with the goals of IVI.

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Vasta-aineita käytetään sekä tutkimuksessa että lääketieteessä. Perinteisesti vasta-aineita on tuotettu *in vivo*-menetelmillä, mikä on kallis ja pitkäkestoinen prosessi. Sen sijaan *in vitro* vasta-ainetuotanto on herättänyt kiinnostusta, koska se on halvempaa ja nopeampaa. *In vitro*-immunisaatiossa (IVI) voidaan myös tuottaa vasta-aineita antigeeneille, jotka tappaisivat tuotantoon käytettävän eläimen. Tämä työ keskittyy antigeenejä esittelevien solujen transfektioon antigeenejä koodaavalla RNA:lla käyttäen lipofektiota ja elektroporaatiota. Erityisenä mielenkiinnon kohteena on nopean ja halvan IVI protokollan kehittäminen, jotta sen käyttö olisi mahdollista tutkimusryhmille ilman erityisen paljoa rahoitusta.

Fluoresoivaa proteiinia koodaavan RNA:n transfektio onnistui perifeerisen veren mononukleaarisista soluista eroteltuihin monosyytteihin, mutta mitattu transfektiosuhde oli huomattavasti matalampi kuin THP-1-soluissa. DOTMA ja DOPE, jotka ovat vanhimpia transfektioreagensseja, tuottivat korkeamman transfektiosuhteen kuin tuhat kertaa kalliimmat DOSPA:an perustuvat tuotteet. Sen sijaan DOSPA:an perustuvilla tuotteilla transfektoiduissa soluissa mitattiin kymmeniä kertoja enemmän proteiinia. Elektroporaatiolla tyypillinen transfektiosuhde oli noin 80% THP-1-soluissa. Elektroporaation huono puoli lipofektioon verrattuna korkeampi RNA:n kulutus.

Erot mitatuissa proteiinimäärissä monosyyttien ja THP-1-solujen välillä eivät johdu kokonaan soluseinän rakenteista. Yksi mahdollisista muista tekijöistä on se, että immuunijärjestelmä käsittelee transfektoitua RNA:ta ja siitä tuotettua proteiinia. Tämä olisi IVI:n tavoitteiden mukaista.

1 Introduction

Antibodies are proteins produced by B-cells. Each B-cell produces antibodies that recognize a single structure, antigen, and attaches to it on contact. At maturation phase, B-cells that recognise structures belonging to the animal itself undergo apoptosis, ideally leaving only cells that would react to foreign structures. While this system has evolved for protecting the animal against pathogens, antibodies can be used to detect almost any molecular structures as long as the immune system cells used are from different species than the structures to be detected. Because of this characteristic, they are being widely used for research purposes. Purchasing them for widely used antigens is easy, but when the antigen is less common, the antibodies need to be custom made. The cost and time required for their production can hinder research efficiency, which creates a threshold for using antibodies in research. For antibody production process to be available for researchers, it is preferrable not to involve expensive chemicals. Also, the machinery should only include something that is commonly available in laboratories, like centrifuges.

Currently, the main method for producing antibodies is inoculating animals with antigens, which causes them to develop immunity against them. There are many drawbacks to this, the most significant likely being the long timespan from inoculation to antibody extraction. Instead of this *in vivo* immunization, *in vitro* immunization (IVI) would only require blood from the animal, and the process is far shorter and cheaper (Ichikawa et al., 1999; Michelchen et al., 2021; Wand et al., 2011). There are ethical concerns about using animals for research instead of only blood. There are also many species, like human, that are illegal to use for antibody production whereas their blood is obtainable.

Monoclonal antibodies have been produced by inoculating peripheral blood mononuclear cells (PBMC) with the antigen proteins (TAMURA et al., 2007). The process for *in vitro* immunization starts with separating PBMC from blood. Optionally, monocytes can be separated from PBMC and specialized to either macrophages or dendritic cells using cytokines. These three cell types are called antigen presenting cells (APC). For APC to be able to present antigens, it needs to take in the antigens as proteins. If the immune process works correctly, parts of the proteins will be loaded into MHC-II complexes that are transported to the surface of

the APC using vesicular trafficking (ten Broeke et al., 2013). After transfecting the APCs with antigens, B- and T-cells are added for immunization to take place. T-cells recognize the peptides on MHC-II complexes, and the next phase of immunization process starts.

In our approach, we are transfecting PBMC or APC with mRNA encoding the peptide antigens instead of the peptides, which has not been done before. By doing that, we hope to skip the time-consuming process of producing the antigens.

The overarching goal of our work is to create a workflow for creating antibodies for a given peptide antigen. The workflow should be cheap and simple enough that any research group could make their own antibodies. The planned steps for the whole process are listed in Table 1, but the more specific aim of the current study is to optimize the 1st step of the process, to find the best way to transfect APCs with mRNA.

Table 1: Steps in our planned workflow for antibody production

Step	Description
Antigen transfection	Getting the mRNA encoding the antigen inside the desired cells
Cell specialization	Specializing monocytes to macrophages or dendritic cells
T- and B-cell challenge	Activation of correct T- and B-cells by antigen presenting
Cell propagation	Propagating B-cells that produce correct antibodies. This could be done using Epstein-Barr Virus (EBV) (Jiang et al., 2025).
Antibody extraction	Extracting antibodies from the cell culture

While most cell types are easy to transfect using lipofection, APCs are very difficult, monocytes being the most difficult (Moradian et al., 2020).

While the goal is to transfect APC, it is faster to find the conditions using immortal monocyte cell line, like THP-1. THP-1 cells can be grown in liquid culture, and unlike regular blood cells, they divide (Tsuchiya et al., 1980). That makes the culture ready to use all the time, and as they do not attach to culture flask surfaces, no scraping is needed. As monocytes are hard to transfect, it can be impossible to see how the results develop when changing parameters. With THP-1, the changes are usually measurable.

As the goal is to be able to transfect cells regardless of the blood donor species, THP-1 cells can only be used for determining the starting conditions for transfection. The final tests need to be done using APC or PBMC.

1.1 Methods of nucleic acid delivery into cultured cells

There are several existing methods for transfecting cells. The most common ones are lipofection (Felgner et al., 1987) and electroporation (Neumann et al., 1982). In lipofection, the RNA is mixed with different kinds of lipids to form lipoplexes. The lipoplex suspension is then added to the cell suspension. There are several pathways for cells to take in lipoplexes, and APCs can also phagocytize them (Means et al., 2022). Lipoplexes smaller than 200nm typically get phagocytized, and the optimal size in terms of protein production is 400nm (Hu et al., 2024). Controlling lipoplex size can be achieved using specialized machinery, but for a method to be viable with limited budget, all should be done using common laboratory methods, like pipetting and vortexing. Also, as there are differences between needs of different research groups, it would be good to offer alternative methods for transfection.

The lipofection methods can be categorized in three groups: old cationic lipids, lipid nanoparticles (LNP), and DOSPA-based products.

1.2 DOTMA, DOTAP, DOPE – old lipofection reagents

N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA) is the oldest chemical utilized for lipofection (Felgner et al., 1987). The DOTMA molecule resembles the phospholipid molecules that are abundant in cell membranes, but instead of the negatively charged phosphate group, the head of the molecule carries a positively charged amino group (Figure 1). The phosphate backbones of RNA strands are negatively charged, which causes them to form lipoplexes with DOTMA molecules.

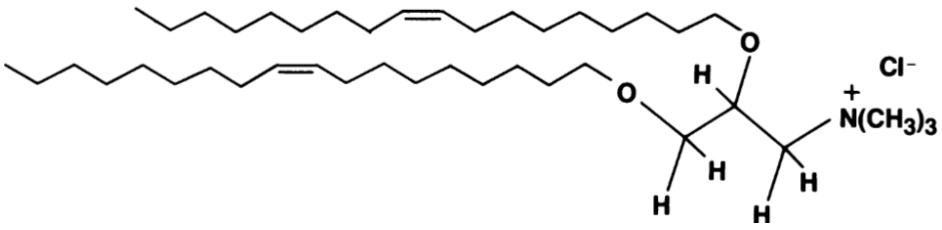


Figure 1: DOTMA molecule. The Cl^- ion does not belong to the lipoplex, but is shipped with the lipid to retain electroneutrality (Felgner et al., 1987).

In addition to DOTMA, adding electroneutral lipids, also called neutral lipids, increases lipoplex transfection efficiency. One such lipid is dioleoylphosphatidylethanolamine (DOPE) (Felgner et al., 1994). Depending on the cell type, the optimal ratio between DOPE and DOTMA varies. Also, the optimal mass ratio between lipofection reagent and RNA is dependent on the cell type.

Instead of DOTMA, lipoplex could be constructed using 1,2-bis(oleoyloxy)-3-(trimethylammonio)propane (DOTAP) (Stamatatos et al., 1988). The difference between DOTMA and DOTAP is that in DOTMA, the long aliphatic chains are connected to propane backbone through ether bonds, but DOTAP has ester bindings instead, like in cell membranes. It depends on the cell type which lipid combination works best.

The main benefits for using cationic lipids are their low price and ease of use. They produce high transfection rates with most cell types.

1.3 Lipid nanoparticles (LNP)

LNPs have been used as drug carriers for a long time, although their composition has changed over time (Binici et al., 2025; Müller et al., 1996). The motivation for using LNP for drug or gene therapy agent delivery is that they can be used for targeting specific tissues. Their intake is cell type specific, and the preferred target cell can be altered by changing the component ratios of the LNP (Cheng et al., 2020). Currently, all FDA approved LNP compositions include four components: 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), cholesterol, cationic/ionizable lipids, and polyethylene glycol (PEG). An example of a commonly used lipid composition is shown in Table 2.

Table 2: Example of component composition of LNP.

Compound	Mass %	Role
DSPC	10	Structure, particle stability, endosomal escape
Cholesterol	38.5	Structure, biocompatibility, cellular intake
Cationic/ionizable lipid	50	Structure, escaping endosome, protection against nucleases
PEG	1.5	Immune defence avoidance, target specification

The structure of LNPs is shown in Figure 2. The PEG molecules typically stick out of the particle, which contributes to the externally observed structure. LNPs typically contain 0.5-1.5% PEG, and varying the amount plays a role in organ targeting (Cheng et al., 2020). The difference between cationic and ionizable lipids is in the pK_a of their amino groups. The pK_a values in cationic lipids are so high, that they remain positively charged in all conditions inside living organisms. For instance, DOTMA and DOTAP are cationic lipids. Ionizable lipids contain amino groups with pK_a values a little below 6. That makes them mostly chargeless in neutral conditions, but they become positively charged when the pH drops, as in endosomes. The change in charge causes the LNP to break, and it contributes to endosomal escape of the cargo nucleic acids.

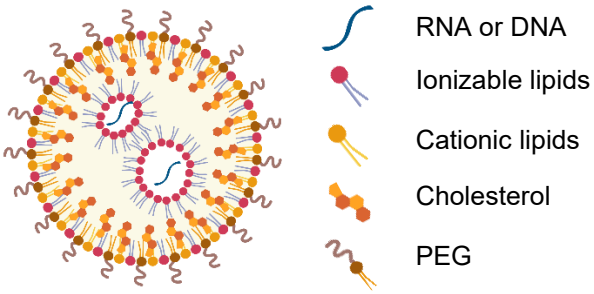


Figure 2: Structure of a lipid nanoparticle

The goals of LNP mediated delivery differ partly from the goals of IVI. They are designed for traveling long distances in living animals while avoiding phagocytizing cells. In IVI, it is desired that the mRNA enters APC in a reaction tube. Also, LNPs are generally produced using expensive equipment not commonly found in research laboratories (Lambart et al., 2025; Lamparelli et al., 2025). If the LNP approach is used, it should be doable with cheap and common laboratory equipment.

1.4 DOSPA based products

There are many other cationic lipids than DOTMA, and the most relevant difference is in the charged branch that starts from the propane backbone. One such molecule is 2,3-dioleyloxy-N-[2-(sperminecarboxamido)ethyl]-N,N-dimethyl-1-propanaminium (DOSPA). It has five amino groups, which gives it higher charge to mass ratio than what DOTMA has. There are products based on DOSPA, like Lipofectamine™ MessengerMax™, which has been able to reach high transfection percentages with monocytes and macrophages (Moradian et al., 2020). The downside is that DOSPA is more expensive than DOTMA by thousandfold. Depending on the needs for IVI, it can be too much, so other alternatives need to be explored.

1.5 Electroporation

Electroporation can reach high transfection rates, but it also reduces cell viability. The parameters observed by the cells are voltage to electrode distance ratio and the voltage decay profile. The distance depends on the used cuvette. When using square wave settings, the voltage and time are entered. Alternatively, some devices take capacitance as input instead of pulse duration. The difference between voltage profiles is shown in Figure 3.

Depending on the device, changing pipetting volume or cuvette size can affect the pulse duration with capacitance setting, which is undesired. With square wave settings, the pipetting volume can be selected according to other factors, like the used cells. To switch between time and capacitance settings, it is good to start by matching time to time constant (TC) value. Some devices display the TC value after pulse has finished running. After initial guess, it is good to experiment with values close to it. If the TC value is hard to obtain, starting from 3ms should also produce good results.

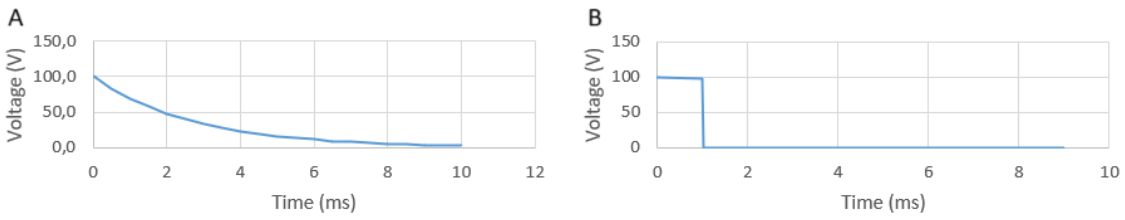


Figure 3: Voltage as function of time when discharging a condenser over constant resistor (A), and with constant voltage as in square wave setting (B).

When switching cuvette size, the voltage needs to be adjusted linearly to retain the voltage to distance ratio. With square wave protocol, that is the only parameter that needs to be changed. With exponential decay protocol, the capacitance should also be adjusted to retain the same discharge profile.

2 Materials and methods

2.1 Cell preparation

PBMC was isolated from human blood by gradient centrifugation using Ficoll-Paque™ PLUS density gradient media (Cytiva). PBMC was either used immediately or frozen in −80°C for later use. Monocytes were separated from PBMC using CD14 MicroBeads, human (Miltenyi Biotec). The media used for growing cells was RPMI-1640 (gibco) supplemented with 10% w/v fetal bovine serum (FBS), 1% w/v streptomycin, penicillin, and L-glutamate.

2.2 Cell specialization to macrophages or dendritic cells

Monocytes were specialized to macrophages by growing them with cell density of 10^6/mL on 96-well plates in 100µL of media with 100ng/mL of Granulocyte-macrophage colony-stimulating factor (GM-CSF) for six days. Media was renewed on days three and six. For specializing monocytes to dendritic cells, also 20ng/mL of IL-4 was added at the same time as GM-CSF.

THP-1 cells were grown on liquid RPMI-1640 culture. For specializing to macrophages, cells were grown on 96-well plate with density of 10^6/mL with 50ng/mL phorbol myristate acetate (PMA) over three days.

2.3 RNA preparation

The RNA should encode the antigen for which the antibodies are desired. However, to test transfection efficiency, it is faster to use RNA that encodes a fluorescent protein, like mCherry or GFP. That way, the results can be seen the following day using either fluorescent microscope or FACS flow cytometry (Cantor et al., 1975).

RNA was *in vitro* translated from sfGFP and mCherry DNA templates that were produced through PCR. The kits used were HiScribe® T7 mRNA Kit with CleanCap® Reagent AG (New England Biolabs) and HiScribe® T7 ARCA mRNA Kit (with tailing) (New England Biolabs), both following manufacturer's protocol. The DNA templates used with the HiScribe® T7 mRNA Kit with CleanCap® Reagent AG (New England Biolabs) contained an internal ribosome entry site (IRES) to enhance translation (Jang and Wimmer, 1990).

2.4 Cell transfection

With lipofection methods, the cells to be transfected were grown on 96-well plate in 100µL of RPMI-1640. When transfecting THP-1, the cell density was $5 \cdot 10^5$/mL and with monocytes 10^6/mL. Amount of RNA/well was 100ng, and the lipid to RNA mass ratios varied.

2.4.1 Transfecting cells with DOTMA or DOTAP

DOTMA or DOTAP was mixed with DOPE using mass ratios 1:1, 1:2 and 2:1. The lipids were stored in 80% ethanol in concentration of 1µg/µL. The mass ratios for lipid mixture to RNA varied from 3:1 to 44:1. The lipid mixture was first incubated in Opti-MEM (gibco) for 10 minutes. At the same time, the RNA was diluted in equal volume of Opti-MEM to concentration of 10ng/µL. The two solutions were combined and incubated for 10 minutes. 20µL of the final solution was added to the cells.

2.4.2 Transfecting cells with lipid nanoparticles

The LNP were made by mixing the components as in Table 2 using DOTAP as cationic lipid. The lipid mixture was stored in 80% ethanol in concentration of 100ng/µL. The RNA was diluted to concentration of 100ng/µL using 10mM pH 5.1 acetate buffer. The lipid solution was combined with the RNA solution in ratio of 3:1 and mixed vigorously. After 5min incubation in RT, the solution was diluted to RNA concentration of 10ng/µL using the same acetate buffer. The solution was mixed again vigorously and stored in -80°C. 10µL was added to the cells.

2.4.3 Transfecting cells with commercial products

Lipofectamine 2000 (Invitrogen) or Lipofectamine RNAiMAX (Invitrogen) was mixed with Opti-MEM in ratio 6:100. RNA was diluted to concentration of 20 or 10 ng/µL with Opti-MEM. The lipid and RNA solutions were incubated for 10 minutes in RT and combined. After another 10-minute incubation, the solution was added to the cells.

2.4.4 Electroporation

Cells were concentrated to $2.5 \cdot 10^6$/µL in Opti-MEM (Gibco). RNA was added to the suspension, and it was incubated for 10min in RT. 20 or 40µL was pipetted into a

cuvette with 1mm electrode distance. The cuvette was pulsed using either exponential decay protocol with capacitance of 150µF and voltage varying from 50V to 150V. With square wave protocol, the time varied between 1 and 5ms. After pulse, 500µL of 37°C RPMI-1640 was added to the cuvette. The cells were grown on 96-well plate in 100µL of media.

2.4.5 Analysing transfection efficiency

When using mCherry mRNA, the transfection efficiency was measured based on protein fluorescence using Agilent NovoCyte Quanteon flow cytometer. Cell viability was assessed using SYTOX™ Red Dead Cell Stain, SYTOX™ Orange Dead Cell Stain or SYTOX™ Green Dead Cell Stain (Invitrogen).

Measured data was exported to FCS format and analysed using FlowJo software package. The gating strategy was as shown in Figure 4: the cell population of interest was selected using forward and side scatter values (A). A scatter graph was drawn using mCherry and SYTOX specific fluorescences, and the mCherry and dead cell gates were selected from it. The SYTOX stain causes some fluorescence close to mCherry wavelengths, so the gate must be drawn to avoid false positives using a negative control as in Figure 4B.

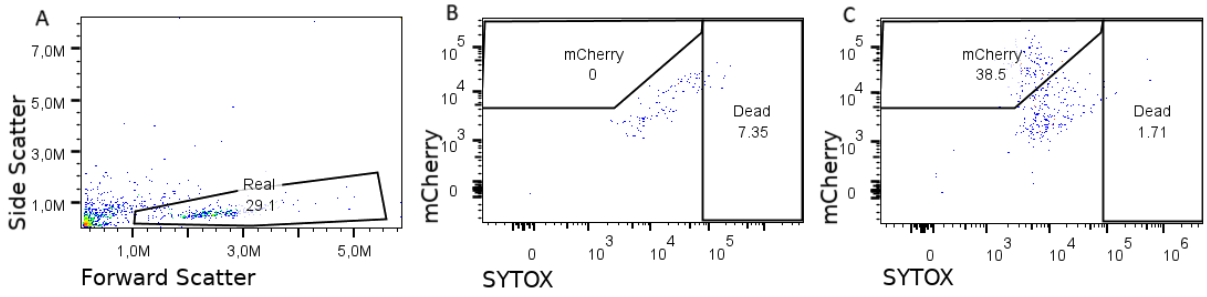


Figure 4: Gating strategy for analysing mCherry fluorescence and cell viability. The cell population is separated from debris using forward and side scatter (A). Using the gate, cells are categorized as dead or mCherry positive. Chart B shows the cell population when using only the transfection lipid, and B shows the population when RNA was added to the lipid.

3 Results

3.1 DOTMA was able to transfect THP-1 efficiently

At first, the strategy was to test transfection with macrophages and dendritic cells. PBMC and monocytes separated from PBMC were placed on 96-well plate with 100µL of media supplemented with GM-CSF and IL-4. On day 3, the media was changed to new similar media. On day 6, the media was changed again, and the lipofection suspension was added to the cells. However, with very low initial success, it turned out to be more feasible to test the conditions with monocytes that are already present in PBMC. But even better than monocytes, the THP-1 cells can be grown in liquid culture, and as dividing monocyte cell line, they do not need to be separated from other cells like monocytes in PBMC.

To find the best transfection conditions, THP-1 cells were grown on 96-well plate, and lipid-RNA suspension was added to the wells. The RNA encoded mCherry fluorescent protein, and the expression was analysed the following day using FACS. The first parameter to iterate was the lipid to RNA mass ratio. The lipids chosen were DOPE:DOTMA in ratio 1:1 and DOPE:DOTAP using the same ratio. With different cells, the most effective lipid to RNA mass ratio in terms of transfection efficiency varies, with 6:1 being probably the most common. The transfection results with ratio 6:1 were so low that they were barely measurable. However, the lipid to RNA charge ratio should be optimally 6:1 to achieve full RNA absorption to lipoplexes (Lamparelli et al., 2025). The 6:1 charge ratio corresponds mass ratio of 25:1, so the test range was extended beyond the already tested 12:1. The Initial test still yielded low transfection rates as seen in Figure 5, but ratio 24:1 was found to be more effective than lower ratios.

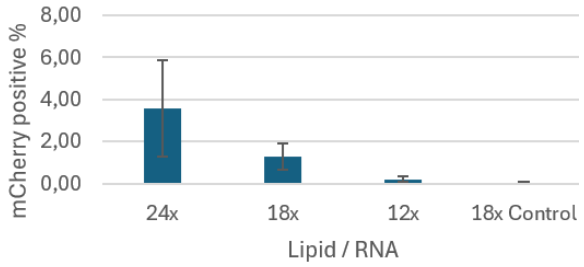


Figure 5: Percentages of mCherry positive THP-1 cells with different lipid to RNA mass ratios. The control has as much lipid as the 18x solution, but no RNA. The lipid used in this comparison was DOPE:DOTMA in ratio 1:1.

Other lipid compositions than DOPE:DOTMA 1:1 and even higher lipid to RNA ratios were tested, but they did not perform as well (Figure 6). Also, DOTMA seemed to perform better than the newer lipid DOTAP.

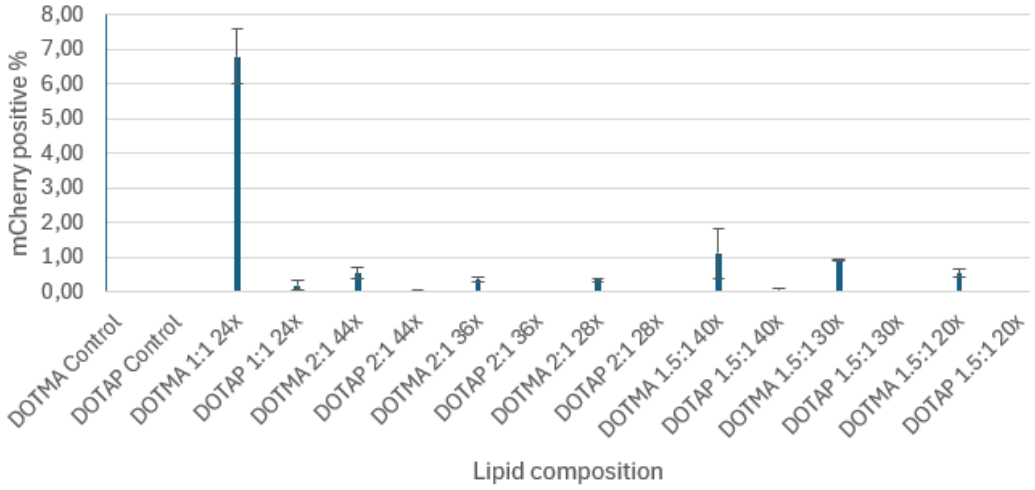
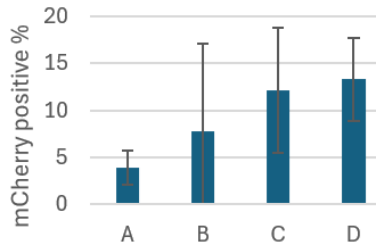


Figure 6: Percentages of mCherry positive THP-1 cells when transfected with different lipid compositions. The ratios under X-axis represent ratios of DOTAP or DOTMA to DOPE, and the figures with “x” represent combined lipid to RNA mass ratio.

Even with the lipid and RNA masses iterated, the transfection rates were low. Inspired by LNP production methods, different common laboratory mixing methods were tested. The special mixing techniques were tested at two different phases of the process: when mixing the lipid to Opti-MEM, and when combining the lipid and RNA suspensions. The tests showed that both phases are important. Commonly, when mixing liquids with pipette, the button is pressed only a few times for the suspension to appear homogenous. However, as lipids are mostly hydrophobic, large aggregates need to be broken down for efficient lipoplex formation with the RNA. At first, we assumed that very thin pipette tip would be good for producing small particles and tested it with capillary tips. That turned out to be wrong. Instead, it was important to produce strong currents in the mixing tube, which could be even done with larger tips. The optimal method was to set the pipet volume a little below half of the whole liquid volume and press 50 to 100 times fast while avoiding bubbles. Vortexing turned out to be less viable method as it spreads the liquid on tube walls. Sonication was also tried, but it produced unpredictable results, which is seen as high variance in Figure 7.



A Mixing with capillary tip, 120 µL liquid
B Mixing with 10 µL tip, 40 µL liquid
C Sonication
D Mixing with 200 µL tip, 40 µL liquid

Figure 7: Percentages of mCherry positive THP-1 cells with different treatments for the transfection mixture.

Pipette handling when adding the lipoplex suspension into well with cells did not seem to have significance in terms of lipoplex destination. Carefully pipetting the suspension into the media produced no different results than thoroughly mixing (Table 3). The lipid used was the same as previously found to be the best: DOPE:DOTMA 1:1 with lipid to RNA mass ratio 24:1 in 20µL of Opti-MEM, and the amount of RNA was 100ng. Earlier, the transfection rates had been 15% at best, but they had risen over 30% with new THP-1 culture and new mRNA batch.

Table 3: Transfection efficiency as mCherry positive cells when either pipetting the lipoplex solution carefully or when mixing it into the media.

Method	mCherry positive %
Pipetted carefully	34
Mixed x10	29

As transfecting to either primary monocytes or PBMC did not seem to produce any detectable mCherry protein, we compared the contents of the medias involved in PBMC separation to the medias involved with THP-1 cultivation and electroporation. The only suspicious compound was ethylenediaminetetraacetic acid (EDTA) used for preventing blood coagulation. EDTA was tested with lipofection on THP-1 cells, but it had no significant effect as seen in Figure 8. EDTA was added in different phases of the lipofection suspension mixing, but the differences are more likely random variation than effects caused by EDTA.

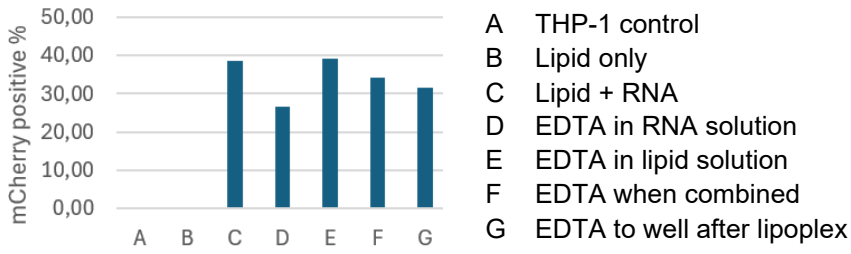


Figure 8: Lipid transfection rates when EDTA was added to concentration of 250µM in different phases of the transfection solution preparation.

Cell death did not increase significantly with DOTMA-based lipofection. It was followed in all tests for noticing major changes, but measuring it is not entirely accurate because dead cells change morphology, which causes some of them to fall outside the first FACS gate (Figure 4A).

3.2 Electroporation can reach very high transfection rates on THP-1

We started determining the best electroporation parameters using THP-1 cells. Our initial guess was 75V voltage, 1mm cuvette electrode distance, and 150µF capacitance. 1µg of RNA and THP-1 cells were suspended in 20µL of Opti-MEM in cell concentration of $2.5 \cdot 10^6$/mL. We also tested which way the results would develop when voltage was changed. 100V produced better results, as seen in Figure 9. We did not test higher voltage as 100v already gave very high transfection rate. 50V did not produce any transfectants, which means that there is a threshold value somewhere between 50 and 75V. We decided to continue tests with 100V.

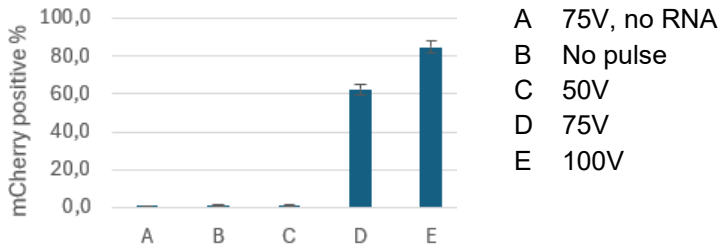


Figure 9: Percentages of mCherry positive cells with different electroporation settings.

As results with PBMC and monocytes had been low, we suspected that EDTA used in separation from blood had some effect on transfection efficiency. Electroporation was done on THP-1 with 250µM EDTA. EDTA was found to marginally reduce transfection efficiency, but not enough that it would explain the lack of observed protein in primary cells (Figure 10).

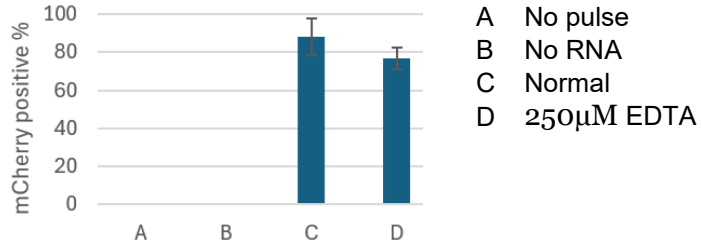


Figure 10: mCherry positive cell percentages when electroporating with or without EDTA

To reduce possible effects from droplet surface shapes in cuvettes, we changed the volume to 40µL instead of the previous 20µL and kept the cell density at $2.5 \cdot 10^6$ cells/mL, thus having 10^5 cells in the cuvette.

When using capacitance setting on electroporation devices, the resulting voltage-time profile depends on the settings and the model of the device. Also, when changing cuvette size, the voltage needs to be adjusted to match the electrode distance, which in turn affects capacitor charge. The cuvette resistance is typically hundreds of kiloohms, and a smaller resistor is connected parallel to it. The resistance of the other resistor is device dependent, which makes capacitor setting badly defined in terms of voltage decay profile. Also, liquid volume affects the decay profile with some devices, as shown in Table 4. The variation is also higher than what is expected from electric circuits. In contrast to that, square wave protocols have well defined voltage profiles.

Table 4: Time constant (TC) values when using exponential protocol with 100V, 150µF, 1mm cuvette and different pipetting volumes.

Volume (µL)	Electrolyte	TC (ms)
20	Opti-MEM	9.5
40	Opti-MEM	3.9
40	Opti-MEM	4.5
80	Opti-MEM	2.0
40	RPMI-1640 with 10% FBS and THP-1 cells	3.8
40	RPMI-1640 with 10% FBS and THP-1 cells	4.1

Square wave protocol with different pulse durations was compared to exponential decay protocol. The RNA amount was only 400ng/cuvette to keep the results in low but measurable percentages (Figure 11). The small variation between square wave protocol results with times 3-5ms comes more likely from randomness than from 4ms being worse than 3 or 5. 2ms is clearly too short.

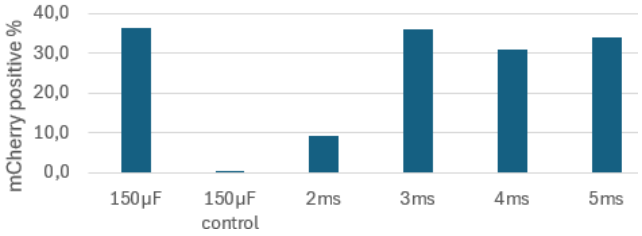


Figure 11: Transfection efficiency with 100V and 400ng mRNA using different settings. 150µF was used with exponential decay protocol, and the times were used with square wave protocol. The control was electroporated without RNA.

As the amount of RNA had been high, we tested the transfection efficiency with different RNA amounts. It turned out that the optimal amount was 1500ng (Figure 12), which means concentration of 37.5 ng/µL in the cuvette.

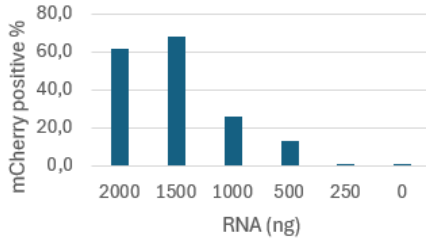


Figure 12: mCherry positive cells with different amounts of mRNA used in electroporation

The cell density used was $2.5 \cdot 10^6$/mL. Halving or doubling it seemed to have no effect on transfection rate, but having more cells would give better yield. The cell death rate did not differ significantly from control.

3.3 Intracellular conditions contribute to low observed transfection in primary APC

Monocytes, macrophages, dendritic cells and PBMC were transfected with mCherry and GFP mRNA several times using DOTMA and electroporation. While the results had been high with THP-1 cells, the protein detection rates were almost non-existent with primary cells.

3.3.1 Differences in lipofection results

Primary monocytes were transfected using same lipofection methods as THP-1 cells. The transfection rates, measured with fluorescent protein, were very low, and the variance was high (Figure 13). Lipofectamine MessengerMAX has been able to reach over 10% rate (Moradian et al., 2020). In our tests, the best transfection rate with

lipofection on primary cells was around 3%, while the best rates on THP-1 had been over 30%.

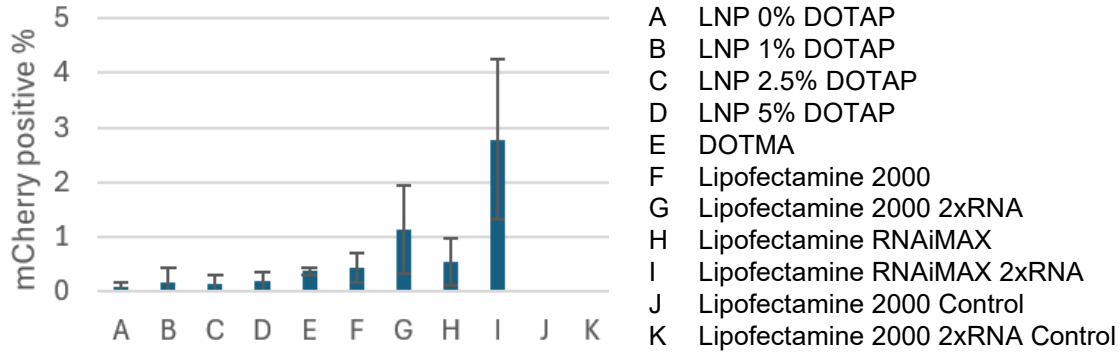


Figure 13: Transfection rates on primary monocytes when using different lipids. The DOTAP % means that DOTAP was added to the master mix in addition to the pre-existing DOTAP. The double RNA samples were made by pipetting double amount of the final suspension on cells. Controls were made without RNA.

When transfecting THP-1 cells using DOPE:DOTMA or Lipofectamine 2000, the protein detection profiles differ in frequence and intensity (Figure 14 A and B). In terms of transfection rate, DOTMA has over four times higher percentage. However, Lipofectamine 2000 produced stronger signal by tenfold, which can be seen from point positions in the graph.

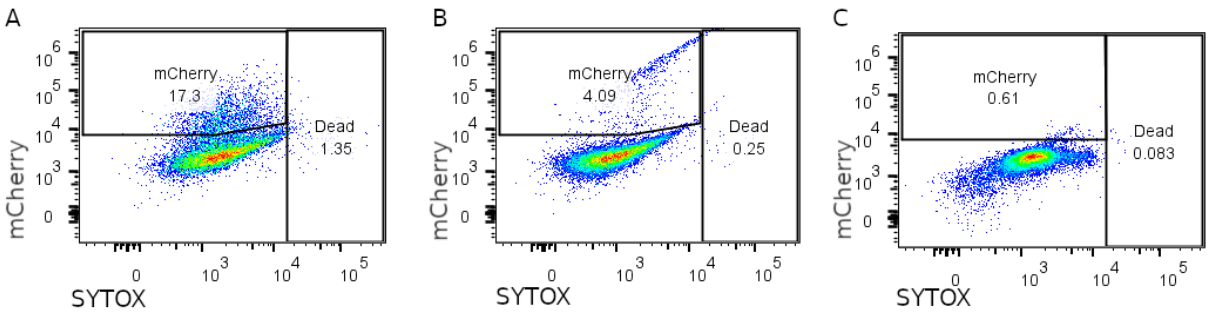


Figure 14: mCherry fluorescence measured from THP-1 cells when using DOTMA (A) or Lipofectamine 2000 (B). Chart C shows mCherry measured from primary monocytes transfected using Lipofectamine 2000.

When using Lipofectamine 2000 on primary monocytes, both the transfection rate and the fluorescence signal were low compared to results with THP-1 (Figure 14 B and C). In the mCherry-gate of primary monocytes, the few hits are either false positives or cells with very low protein amounts.

3.3.2 Differences in electroporation results

mCherry mRNA was electroporated into THP-1 and primary monocyte cells. With same electroporation conditions, most THP-1 cells showed stronger fluorescence signal than monocytes (Figure 15).

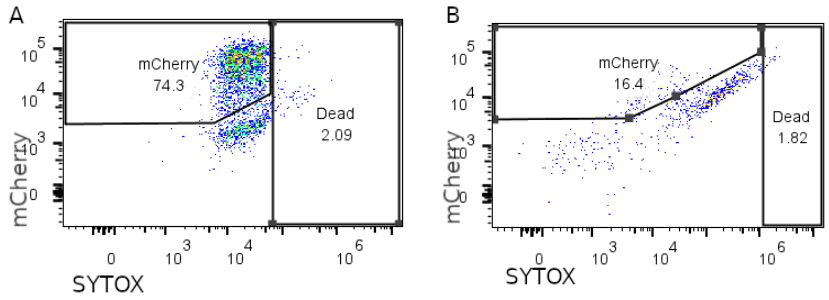


Figure 15: mCherry protein in electroporated THP-1 cells (A) and monocytes (B).

When electroporation experiments started, the mRNA batch was changed. The previous batch had been made using *in vitro* translation kit which uses Anti-Reverse Cap Analog (ARCA), and the new batch with a kit that uses CleanCap AG reagent. The transfection rates were generally above 50%, and that was attributed to electroporation technology alone. However, the earlier tests with DOTMA had given top result of 15%, but with the new mRNA, the result was over 30% following the same protocol. When the batch ran out and mRNA made with ARCA kit was used instead, the transfection rates were low again.

4 Discussion

4.1 DOTMA is a viable choice for low cost lipofection

The optimal lipofection reagent mix turned out to be DOPE and DOTMA in mass ratio of 1:1, and the mass ratio of reagent to RNA 24:1 with final RNA concentration of 5ng/µL. The suspension should be mixed thoroughly by pipetting 50 to 100 times when adding lipid to the media or when combining RNA and lipid suspensions. It would also be possible to add peptides to the mix to target specific receptors (Cui et al., 2023). We considered different viral proteins that could mediate entry, but decided against it because they would require separate testing and they would also make storing the lipid reagent more complicated.

We were able to transfect cells using manually mixed LNPs, but the transfection rates were not as high as with DOPE:DOTMA. As a technique, LNPs have been successful, but they are usually constructed with special hardware. As *in vivo* gene therapy vectors, their requirements also differ from the needs of IVI. In comparison, there is a new nanostraw-mediated technique that has been able to transfect T-cells in vivo (Kumar et al., 2025). Using nanostraws also requires special hardware.

Products like Lipofectamine MessengerMAX can be used instead of DOTMA. Except for being more expensive, they differ from DOTMA with higher protein yield. It is still unknown if that is desirable, as the goal is not to have high amounts of protein inside the cells, but to immunize against it. The price can also be less of an issue, depending on the other needs of IVI.

4.2 Electroporation can reach high transfection rates at the cost of high RNA consumption

Electroporation was found to be effective with 1500ng of RNA and 10^5 cells in 40µL of Opti-MEM, which means concentration of 37.5ng/µL and cell density of $2.5 \cdot 10^6$/mL. The cuvette electrode distance was 1mm, voltage 100V and pulse duration 4ms. That means the electric field strength was 100V/mm, which should be retained when using different kind of cuvette. It might be desirable to change cuvette sizes because 1mm cuvette holds about 80µL of liquid between the electrodes whereas 2mm cuvette holds 500µL.

With DOTMA lipofection, 100ng of RNA was used in a well with $5 \cdot 10^4$ cells. With non-dividing cells, 10^5 cells would be optimal, but THP-1 amount doubles in a day. In contrast, electroporating 10^5 cells required 1500ng of RNA/cuvette, which makes electroporation costly in terms of RNA consumption. The cell density could be increased while retaining the RNA concentration but having more cells does not bring many benefits when exploring the conditions. Even though the parameters listed here can be used as they are, there are still other factors to iterate when proceeding with the immunization process. For instance, the RNA construct used for immunization might require several parallel experiments.

4.3 Observed transfection rate depends on multiple consecutive factors

Transfection efficiency was measured using fluorescence. While it is an easy method to use, it does not completely measure achieving the final goal, which is to activate APC to start immunization process. There are several stages where the efficiency can be hindered: cellular entry, escaping possible endosome, avoiding intracellular defence against foreign RNA, translation, and the protein's ability to stay in fluorescent state.

There are many differences between THP-1 and regular monocytes. The obvious difference is that THP-1 divides, but considering this study, the most important difference is probably that the THP-1 cell donor was 1 year old (Tsuchiya et al., 1980). There are differences between THP-1 and regular monocyte cell membranes, but it is unlikely that the differences between lipofection and electroporation transfection rates would attribute to only that. As infant cell, the THP-1 is likely to have less developed defence mechanisms against foreign RNA or particles. The cells have also been passaged over 150 times, which can have accumulated many mutations to the line used.

4.3.1 Cellular entry

With the relatively high transfection rates in THP-1 cells, it is unlikely that the RNA could not get into primary monocytes. Also, Lipofectamine produced higher protein amounts while having lower transfection rate than DOTMA, which means that there is something else involved than breaching the membrane.

While successful lipofection relies on cell membrane properties, electroporation is mostly a mechanical method where the nucleic acids enter the cell through broken membrane, ending up in cytoplasm. However, with lipofection, the cellular entry can happen through different pathways: phagocytosis, receptor mediated endocytosis, micropinocytosis or macropinocytosis (Means et al., 2022). Lipoplex size plays a role in which pathway is used: particles smaller than 200nm enter the cell using clathrin- or caveolae-mediated pathways, and larger ones through phagocytosis or macropinocytosis.

4.3.2 Endosomal escape

If a lipoplex enters a cell through phagocytosis, the intention is to destroy it in the endosome. The endosomes in phagocytosis pathway have low pH, which is used in LNP technology by adding lipids that change their charge in low pH. The endosomes in different endocytosis pathways have different conditions, and the lipoplexes could get stuck in them. Ability to escape endosomes could be the reason why products like Lipofectamine can induce higher protein amounts than DOTMA. However, the DOTMA lipoplexes are not necessarily destroyed in the endosomes. The low protein amounts can also result from slower release.

4.3.3 Avoiding RNA degradation

Regular phagocytizing cells are specialized in destroying foreign material. As THP-1 is an infant cell line, it can have weaker defence against foreign RNA. The differences in RNA produced with different kits can also play a role in intracellular RNA degradation. The CleanCap AG reagent which is added to the 5’ end of the RNA has AG sequence after the cap structure whereas ARCA reagent has G. Both kits should add poly-A tail, but they might have differences in length and attachment success rate.

4.3.4 Translation

When the mRNA has entered cytoplasm, it needs to be translated. The mRNA used has IRES structure, which has been found in viruses, but not in human RNA. Ribosome mutations can cause ribosomes to have lower affinity to IRES sequences (Chen and Jin, 2025), but the opposite could also be possible.

4.3.5 Intracellular protein processing

Translation alone is not enough for the protein to accumulate and stay in fluorescent state. In early phase of the study, it was found that looking at a well with fluorescence microscope causes photobleaching. But besides getting accidentally destroyed, the protein can be degraded by the immune system functionality. MHC-II complexes display only parts of the protein of roughly 15AA length. If the protein ends up in the immune system pipeline, the transfection results align with the goals of IVI. This needs to be assessed further with mRNA encoding the real antigens, which is longer process than detecting fluorescence.

4.4 Medical use instead of vaccine

Antibodies are produced by specialized B-cells. Besides using the antibodies for research, there is the prospect of injecting the B-cells back to the animal, thus giving it immunity to the antigen. The main benefit to vaccination would be that it could be done on individual who is already infected with the pathogen. For instance, vaccinating populations against rabies is often considered too costly or difficult (Zha et al., 2025), but B-cells could be given only to the infected. There are also viral diseases without existing medicines, and recovering from them works through developing immunity against the pathogen. The medical use would require permits, but the risks are not entirely new, as injecting foreign blood is already a common practise in medicine. Blood is generally donated by adults, who have already developed resistances to multiple pathogens during their life, and their blood contains similar specialized B-cells. With *in vitro* immunized B-cells, the risk for cancer should be assessed. Unlike using antibodies for research, developing treatments for pathogens have better resources, and the production would be done using automation instead of manual laboratory equipment.

4.5 Possible error sources

The measurement of transfection rate by fluorescence is problematic in several ways. The main issue with it is that it measures accumulation of protein instead of immune system activation. It is also difficult to select the gates correctly in some cases because the cell population of interest does not always show as distinct point group on the graph. The cell death fluorescence marker also needs to be selected so that it

interferes with the measured signal wavelength as little as possible. The used equipment and analysis program have compensation system for overlapping fluorescence, but compensation is only a calculational method for curating data.

The cells used can have differences. With PBMC, the tests were always done with at least two different donors. With THP-1, the cultures were sometimes grown longer periods. Initially, the cell counts in our cultures increased by 3% in an hour. When the culture had been grown longer, the increase could be over 5% in an hour. This tells about mutations in the frozen THP-1 cell aliquots, which cause faster dividing cell populations to become more abundant.

The ethanol amounts in lipid stocks can have affected transfection efficiency. It is also known that too much ethanol will reduce cell viability. We observed batch effects with the mRNA, and it is possible that there were also differences between lipid batches. Going forward, lowering the ethanol amount in the lipoplex suspension should be explored as well as adding more suspension to the cells.

Gradients can form in both solutions and suspensions. That makes concentrations unreliable and dependant on where a sample is taken. Also, measuring concentrations with NanoDrop is inaccurate. Cell counting uses a small volume sample to represent the whole culture, making the method vulnerable to gradients.

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