
AbSciCon
2019

1
00:00:00,790 --> 00:00:07,320
[Music]

2
00:00:11,440 --> 00:00:09,120
[Applause]

3
00:00:13,959 --> 00:00:11,450
thank you very much for this invitation

4
00:00:16,800 --> 00:00:13,969
here this is my first EPS icon and I'm

5
00:00:20,800 --> 00:00:16,810
enjoying it very much

6
00:00:22,480 --> 00:00:20,810
before I'm gonna tell you about the

7
00:00:25,450 --> 00:00:22,490
research we have been doing in the lab I

8
00:00:28,210 --> 00:00:25,460
first want to mention two thoughts on

9
00:00:30,070 --> 00:00:28,220
the RNA world hypothesis because over

10
00:00:35,590 --> 00:00:30,080
the last days a number of people have

11
00:00:37,390 --> 00:00:35,600
expressed disappointment or negative

12
00:00:39,460 --> 00:00:37,400
feelings against the RNA world

13
00:00:44,110 --> 00:00:39,470

hypothesis and I want to clarify two

14
00:00:46,420 --> 00:00:44,120
things no laughing and I want to clarify

15
00:00:48,520 --> 00:00:46,430
two things so first of all we have

16
00:00:51,070 --> 00:00:48,530
pretty good evidence from a ribosome

17
00:00:54,670 --> 00:00:51,080
tRNA or an ASP in nucleotide cofactors

18
00:00:57,750 --> 00:00:54,680
that something like an RNA world existed

19
00:01:01,990 --> 00:00:57,760
as an earlier stage in evolution it

20
00:01:05,800 --> 00:01:02,000
probably was not the first stage of life

21
00:01:07,630 --> 00:01:05,810
and probably or most people I think

22
00:01:10,870 --> 00:01:07,640
agree with me that there probably was

23
00:01:13,060 --> 00:01:10,880
not a pure RNA world but something like

24
00:01:15,490 --> 00:01:13,070
in our any world existed as an earlier

25
00:01:18,970 --> 00:01:15,500
stage in life and the other point I want

26
00:01:22,150 --> 00:01:18,980
to make in is the worth of a hypothesis

27
00:01:25,420 --> 00:01:22,160
is not only how well it describes

28
00:01:29,230 --> 00:01:25,430
reality how accurate it is it also is in

29
00:01:31,120 --> 00:01:29,240
- what kind of experiments and tests as

30
00:01:33,310 --> 00:01:31,130
it give rise to to forward our

31
00:01:35,440 --> 00:01:33,320
understanding and I think in this

32
00:01:37,840 --> 00:01:35,450
respect the RNA world hypothesis has

33
00:01:41,080 --> 00:01:37,850
been done except has been exceptionally

34
00:01:43,270 --> 00:01:41,090
fruitful now this this that we can

35
00:01:45,580 --> 00:01:43,280
discuss this I'm happy to discuss this I

36
00:01:48,250 --> 00:01:45,590
want to also mention that this is one

37
00:01:50,620 --> 00:01:48,260
example of a conceptual question that is

38
00:01:52,719 --> 00:01:50,630

very important to address and that we

39
00:01:55,240 --> 00:01:52,729
are addressing in these research

40
00:01:56,640 --> 00:01:55,250
coordination networks I'm part of this

41
00:02:00,880 --> 00:01:56,650
pc3

42
00:02:02,920 --> 00:02:00,890
prebiotic chemistry and early invite all

43
00:02:05,020 --> 00:02:02,930
the earth environments and Lauren

44
00:02:08,949 --> 00:02:05,030
Williams sitting here he is one of the

45
00:02:11,229 --> 00:02:08,959
of the head of that groups and I

46
00:02:13,539 --> 00:02:11,239
encourage you to get in contact with

47
00:02:15,149 --> 00:02:13,549
that group because NASA is stimulating

48
00:02:18,459 --> 00:02:15,159
that Network and we may have a lot of

49
00:02:20,290 --> 00:02:18,469
fruitful outcomes from that so enough

50
00:02:23,350 --> 00:02:20,300
for that I want to now

51
00:02:26,610 --> 00:02:23,360
go to the research that I have been

52
00:02:30,070 --> 00:02:26,620
doing which is focused on the RNA world

53
00:02:33,130 --> 00:02:30,080
so when I think about the RNA world I'm

54
00:02:35,890 --> 00:02:33,140
thinking about this overall picture here

55
00:02:38,860 --> 00:02:35,900
where by definition we must have used

56
00:02:40,660 --> 00:02:38,870
prebiotic aliy available compounds to

57
00:02:42,940 --> 00:02:40,670
generate nuclear sites how they were

58
00:02:45,130 --> 00:02:42,950
generated I don't know and there's a

59
00:02:47,410 --> 00:02:45,140
number of good organic chemists that are

60
00:02:49,870 --> 00:02:47,420
addressing this question what my group

61
00:02:52,170 --> 00:02:49,880
has been working on mostly is the

62
00:02:55,180 --> 00:02:52,180
activation of the generating of

63
00:02:57,940 --> 00:02:55,190

activated nucleotides from these nuclear

64
00:03:00,460 --> 00:02:57,950
sites and after you have chemically

65
00:03:02,950 --> 00:03:00,470
activated nucleotides you can form by

66
00:03:05,650 --> 00:03:02,960
RNA polymerize ation or any polymers and

67
00:03:07,870 --> 00:03:05,660
I want to emphasize this addresses a

68
00:03:09,670 --> 00:03:07,880
mature RNA world this might not have

69
00:03:12,850 --> 00:03:09,680
been the case in an early or any world

70
00:03:14,440 --> 00:03:12,860
please do not take pictures almost

71
00:03:16,180 --> 00:03:14,450
everything I'm showing you is

72
00:03:17,770 --> 00:03:16,190
unpublished so I'm not too concerned

73
00:03:21,850 --> 00:03:17,780
about this picture but about the other

74
00:03:24,900 --> 00:03:21,860
slides so we are focused on this

75
00:03:28,660 --> 00:03:24,910
reaction here and Leslie Orgel showed in

76
00:03:31,479 --> 00:03:28,670
1978 that if you incubate a dinner scene

77
00:03:35,020 --> 00:03:31,489
with trimeter phosphate at a pH of 13

78
00:03:37,990 --> 00:03:35,030
then this will give rise to ATP to a

79
00:03:40,960 --> 00:03:38,000
five-prime triphosphate within a few

80
00:03:43,570 --> 00:03:40,970
hours about 5% you have about 5% I

81
00:03:45,400 --> 00:03:43,580
converted to ATP and what this shows

82
00:03:47,949 --> 00:03:45,410
that in general this reaction is

83
00:03:50,500 --> 00:03:47,959
possible however a pH of 13 is

84
00:03:52,449 --> 00:03:50,510
incompatible with an RNA world because

85
00:03:56,530 --> 00:03:52,459
everything degrades within minutes or

86
00:03:59,670 --> 00:03:56,540
even seconds and what we did in my lab

87
00:04:04,270 --> 00:03:59,680
in previous years is we have generated

88
00:04:06,460 --> 00:04:04,280

RNA catalytic RNAs that we act trimeter

89
00:04:09,370 --> 00:04:06,470
phosphate with their own 5 prime

90
00:04:12,160 --> 00:04:09,380
hydroxyl group so you're making RNA 5

91
00:04:14,410 --> 00:04:12,170
prime triphosphate which shows that in

92
00:04:16,659 --> 00:04:14,420
general the chemistry works and that

93
00:04:19,060 --> 00:04:16,669
this reaction can be catalyzed by RNAs

94
00:04:21,670 --> 00:04:19,070
at neutral pH however what you

95
00:04:24,159 --> 00:04:21,680
eventually want to have is nucleotide 5

96
00:04:26,920 --> 00:04:24,169
front triphosphates because in every

97
00:04:30,149 --> 00:04:26,930
single living organism we know these are

98
00:04:32,839 --> 00:04:30,159
sent the central metabolic

99
00:04:36,689 --> 00:04:32,849
molecules and especially in an RNA world

100
00:04:39,809 --> 00:04:36,699
so how can you or what is the problem

101
00:04:43,259 --> 00:04:39,819
why haven't we done developed a ribozyme

102
00:04:46,559 --> 00:04:43,269
that makes free nucleoside triphosphates

103
00:04:49,499 --> 00:04:46,569
there's two general difficulties one of

104
00:04:51,540 --> 00:04:49,509
them is from the ribozyme site this

105
00:04:54,570 --> 00:04:51,550
ribozyme needs to bind to small

106
00:04:57,479 --> 00:04:54,580
molecules and catalyze the reaction and

107
00:05:01,320 --> 00:04:57,489
that is a tall order to ask form from an

108
00:05:04,499 --> 00:05:01,330
RNA and the second one is for in vitro

109
00:05:06,419 --> 00:05:04,509
selections you always need to tag your

110
00:05:08,969 --> 00:05:06,429
pool molecule you're successful pool

111
00:05:11,100 --> 00:05:08,979
molecule with some handle so that you

112
00:05:13,320 --> 00:05:11,110
can later pull on that handle and pull

113
00:05:16,529 --> 00:05:13,330

that molecule out of your population if

114
00:05:19,079 --> 00:05:16,539
you're successful product is a freely

115
00:05:21,239 --> 00:05:19,089
diffusing small molecule and this is

116
00:05:23,040 --> 00:05:21,249
hard to do and in the next slides I'm

117
00:05:26,790 --> 00:05:23,050
going to show you how we were addressing

118
00:05:30,869 --> 00:05:26,800
these challenges first of all this here

119
00:05:32,519 --> 00:05:30,879
is a pool molecule with 150 random

120
00:05:34,559 --> 00:05:32,529
nucleotides that's the pool length we

121
00:05:38,040 --> 00:05:34,569
used and the reaction we want to

122
00:05:40,379 --> 00:05:38,050
catalyze is here six SIA goannas in a

123
00:05:42,749 --> 00:05:40,389
nucleoside that reacts with trimeter

124
00:05:45,209 --> 00:05:42,759
phosphate to form six-sided one OHS in

125
00:05:46,980 --> 00:05:45,219
five-prime triphosphate and i will tell

126
00:05:50,339 --> 00:05:46,990
you in a minute why we have these style

127
00:05:55,199 --> 00:05:50,349
modifications here so this problem how

128
00:05:57,569 --> 00:05:55,209
to tag pool molecules that successfully

129
00:06:01,589 --> 00:05:57,579
carried out a reaction we addressed by

130
00:06:03,509 --> 00:06:01,599
attaching a polymerase ribozyme or by

131
00:06:05,519 --> 00:06:03,519
base pairing a polymerase I was on to

132
00:06:07,049 --> 00:06:05,529
the three prime end of the pool so that

133
00:06:10,499 --> 00:06:07,059
polymerase ribozymes

134
00:06:12,779 --> 00:06:10,509
could take six io GTP that was made by

135
00:06:15,719 --> 00:06:12,789
these ribosomes and tag it to their own

136
00:06:19,139 --> 00:06:15,729
three prime end and because the wild

137
00:06:23,189 --> 00:06:19,149
type polymerase ribozyme is not very

138
00:06:25,559 --> 00:06:23,199

good in using 6io GTP and we performed

139
00:06:29,219 --> 00:06:25,569
an in vitro evolution experiment that

140
00:06:32,040 --> 00:06:29,229
optimized this polymerase ribozyme for

141
00:06:34,230 --> 00:06:32,050
the attachment of six tired GTP and we

142
00:06:36,600 --> 00:06:34,240
found four mutations two of them have a

143
00:06:37,499 --> 00:06:36,610
two-fold effect one of them has a 50

144
00:06:40,889 --> 00:06:37,509
fold effect

145
00:06:43,740 --> 00:06:40,899
so this ribozyme is 200 fold better in

146
00:06:45,390 --> 00:06:43,750
attaching the sixth IO gtp to the

147
00:06:47,280 --> 00:06:45,400
three prime end of the pool and then the

148
00:06:51,900 --> 00:06:47,290
original wild-type ribozyme and that

149
00:06:54,090 --> 00:06:51,910
proved to be good enough for us then we

150
00:06:58,170 --> 00:06:54,100
have these pool molecules with these

151
00:07:01,980 --> 00:06:58,180
three prime tags of vi io GTP or sixth

152
00:07:04,260 --> 00:07:01,990
our guanosine and to separate the

153
00:07:06,510 --> 00:07:04,270
successfully attacked pool molecules

154
00:07:08,280 --> 00:07:06,520
from those but that were not tanked we

155
00:07:10,320 --> 00:07:08,290
used a mean of phenyl mercury

156
00:07:13,590 --> 00:07:10,330
polyacrylamide gel electrophoresis which

157
00:07:16,409 --> 00:07:13,600
was first used by Peter enroll in an in

158
00:07:20,730 --> 00:07:16,419
vitro selection as the Selective step

159
00:07:22,350 --> 00:07:20,740
so once the you have these tagged pool

160
00:07:24,870 --> 00:07:22,360
molecules you run them on these

161
00:07:27,420 --> 00:07:24,880
polyacrylamide gels only those that are

162
00:07:29,670 --> 00:07:27,430
tagged are stuck on this APM interface

163
00:07:32,090 --> 00:07:29,680

and then you can cut out these gel

164
00:07:36,540 --> 00:07:32,100
pieces and use them for your next step

165
00:07:38,760 --> 00:07:36,550
so this is the first challenge that we

166
00:07:41,520 --> 00:07:38,770
overcame how to tag these successful

167
00:07:45,390 --> 00:07:41,530
pool molecules the second challenge is

168
00:07:47,850 --> 00:07:45,400
one next to tagging active pool

169
00:07:50,400 --> 00:07:47,860
molecules how do you avoid tagging

170
00:07:52,560 --> 00:07:50,410
inactive pool molecules because you have

171
00:07:55,650 --> 00:07:52,570
to keep in mind if this here is an

172
00:07:58,800 --> 00:07:55,660
active pool molecule that generates six

173
00:08:00,900 --> 00:07:58,810
style GTP and that hopefully leads to

174
00:08:04,500 --> 00:08:00,910
the tagging of its own three prime end

175
00:08:07,020 --> 00:08:04,510
you can also have the sixth I gtp now

176
00:08:09,210 --> 00:08:07,030
diffusing around to a neighboring pool

177
00:08:11,700 --> 00:08:09,220
molecule and lead to the three prime

178
00:08:13,260 --> 00:08:11,710
tagging of that pool molecule and to

179
00:08:15,960 --> 00:08:13,270
prevent that from happening

180
00:08:18,090 --> 00:08:15,970
you need to compartmentalize the system

181
00:08:21,690 --> 00:08:18,100
and we did this in the form of an

182
00:08:24,630 --> 00:08:21,700
emulsion where each pool molecule was

183
00:08:27,000 --> 00:08:24,640
within its own compartment and I believe

184
00:08:28,860 --> 00:08:27,010
that's the first selection that does

185
00:08:31,409 --> 00:08:28,870
that from completely random sequence

186
00:08:35,940 --> 00:08:31,419
that we compartmentalize each pool

187
00:08:38,550 --> 00:08:35,950
molecule so the system we used is an

188
00:08:41,700 --> 00:08:38,560

emulsion formulation that was shown by

189
00:08:44,550 --> 00:08:41,710
Phil Halle girls lab in 2004 we used the

190
00:08:47,190 --> 00:08:44,560
emulsifier able m90 in heavy mineral oil

191
00:08:49,800 --> 00:08:47,200
and then we used a machine called the

192
00:08:51,990 --> 00:08:49,810
micro fluid either from micro fluidics I

193
00:08:55,290 --> 00:08:52,000
have no association with a company and

194
00:08:57,300 --> 00:08:55,300
and it basically presses the em raw

195
00:08:59,760 --> 00:08:57,310
emulsions through a cell with a

196
00:09:02,610 --> 00:08:59,770
find shearing force so that you end up

197
00:09:05,490 --> 00:09:02,620
with a very homogeneous size

198
00:09:08,210 --> 00:09:05,500
distribution of your droplets and in

199
00:09:12,960 --> 00:09:08,220
collaboration with stark America at UCSD

200
00:09:15,030 --> 00:09:12,970
we measured the diameter of the these

201
00:09:18,060 --> 00:09:15,040
droplets by dynamic light scattering and

202
00:09:19,620 --> 00:09:18,070
you see from the nice single exponential

203
00:09:23,250 --> 00:09:19,630
curves that we got a pretty homogeneous

204
00:09:25,860 --> 00:09:23,260
size distribution with a diameter of 150

205
00:09:28,079 --> 00:09:25,870
nanometers and this diameter is

206
00:09:31,140 --> 00:09:28,089
significant because if you imagine an

207
00:09:34,590 --> 00:09:31,150
aqueous droplet with a diameter of 150

208
00:09:36,710 --> 00:09:34,600
nanometer then if you take a solution of

209
00:09:39,329 --> 00:09:36,720
molecules with a 1 micromolar

210
00:09:43,500 --> 00:09:39,339
concentration then on average each

211
00:09:45,630 --> 00:09:43,510
droplet has one molecule per droplet so

212
00:09:47,850 --> 00:09:45,640
we're going to the molecular scale in

213
00:09:49,620 --> 00:09:47,860

terms of separating out our pool

214
00:09:55,050 --> 00:09:49,630
molecules and that's important that's

215
00:09:57,240 --> 00:09:55,060
essential for the selection so after my

216
00:09:59,910 --> 00:09:57,250
graduate student Arvind a coupie spent

217
00:10:03,090 --> 00:09:59,920
three and a half years of hard work on

218
00:10:05,520 --> 00:10:03,100
establishing this selection system he

219
00:10:07,350 --> 00:10:05,530
was ready to do the selection and the

220
00:10:10,430 --> 00:10:07,360
selection was done in about half a year

221
00:10:13,110 --> 00:10:10,440
and the analysis in another few months

222
00:10:15,720 --> 00:10:13,120
so first I want to quickly show you the

223
00:10:17,850 --> 00:10:15,730
selection system together first we start

224
00:10:19,680 --> 00:10:17,860
with these DNA pool molecules that are

225
00:10:21,810 --> 00:10:19,690
then transcribed in our any pool

226
00:10:24,090 --> 00:10:21,820
molecules and Arvind had an effective

227
00:10:26,760 --> 00:10:24,100
complexity of one point six times ten to

228
00:10:28,740 --> 00:10:26,770
the 14 molecules then they get

229
00:10:30,780 --> 00:10:28,750
compartmentalized in these emulsion

230
00:10:31,590 --> 00:10:30,790
droplets together with the polymerase

231
00:10:33,600 --> 00:10:31,600
ribozymes

232
00:10:36,270 --> 00:10:33,610
with sixth I iguanas in Andromeda

233
00:10:38,640 --> 00:10:36,280
phosphate in the hope that some of them

234
00:10:41,130 --> 00:10:38,650
generate six sire gtp and are then

235
00:10:44,329 --> 00:10:41,140
tagged at their three prime end then the

236
00:10:48,780 --> 00:10:44,339
emulsion is broken open all the aqueous

237
00:10:51,990 --> 00:10:48,790
content is released and thrown on amino

238
00:10:53,940 --> 00:10:52,000

final mercury page and we process these

239
00:10:57,269 --> 00:10:53,950
molecules by reverse transcription PCR

240
00:11:00,510 --> 00:10:57,279
and then complete one cycle arvind went

241
00:11:02,790 --> 00:11:00,520
through 18 cycles and you see that after

242
00:11:05,610 --> 00:11:02,800
10 12 cycles he got an exponential

243
00:11:08,940 --> 00:11:05,620
enrichment he increased the stringency

244
00:11:10,190 --> 00:11:08,950
of the selection to select for the most

245
00:11:12,139 --> 00:11:10,200
efficient ribozyme

246
00:11:14,480 --> 00:11:12,149
and after this was done he didn't high

247
00:11:16,790 --> 00:11:14,490
throughput sequencing analysis we're in

248
00:11:19,730 --> 00:11:16,800
the end and the population was dominated

249
00:11:22,040 --> 00:11:19,740
by five clusters and then Arvind had to

250
00:11:27,139 --> 00:11:22,050
quickly finish his ph.d and go to

251
00:11:28,850 --> 00:11:27,149
biotech industry and Josh aureola my new

252
00:11:31,610 --> 00:11:28,860
grad student he took over for the

253
00:11:33,430 --> 00:11:31,620
biochemical characterization and this is

254
00:11:35,689 --> 00:11:33,440
how we did the biochemical

255
00:11:37,550 --> 00:11:35,699
characterization so the randomized

256
00:11:39,590 --> 00:11:37,560
portion of these poor molecules is

257
00:11:42,139 --> 00:11:39,600
incubated with six side one is intra

258
00:11:44,810 --> 00:11:42,149
metre phosphate to generate six tile gtp

259
00:11:47,750 --> 00:11:44,820
and then the short 10 nucleotide long

260
00:11:49,550 --> 00:11:47,760
radio labeled RNA is attached to the

261
00:11:51,650 --> 00:11:49,560
polymerase I was I'm to serve as the

262
00:11:55,960 --> 00:11:51,660
reporter whether sixth i GT p was

263
00:11:58,759 --> 00:11:55,970

generated and we screened clones a claw

264
00:12:01,750 --> 00:11:58,769
between two and five clones per cluster

265
00:12:04,340 --> 00:12:01,760
and you see that cluster five gave the

266
00:12:08,139 --> 00:12:04,350
strongest signal and here you see

267
00:12:10,970 --> 00:12:08,149
kinetic Alan analysis of this cluster

268
00:12:12,710 --> 00:12:10,980
where this is the negative control no

269
00:12:15,920 --> 00:12:12,720
ribozyme present this is the positive

270
00:12:17,720 --> 00:12:15,930
control with only six thio GDP and here

271
00:12:20,360 --> 00:12:17,730
you see the reaction catalyzed by this

272
00:12:22,160 --> 00:12:20,370
ribozyme and with this I just want to

273
00:12:24,620 --> 00:12:22,170
acknowledge Arvind a coupie who did

274
00:12:25,100 --> 00:12:24,630
establish the selection and that most of

275
00:12:27,350 --> 00:12:25,110
the work

276
00:12:30,620 --> 00:12:27,360
josh Arriola who is currently doing the

277
00:12:31,579 --> 00:12:30,630
biochemical Alice's and Jack mackee who

278
00:12:34,069 --> 00:12:31,589
did the dynamic light scattering

279
00:12:35,990 --> 00:12:34,079
analysis brine Pagal for helpful

280
00:12:38,130 --> 00:12:36,000
discussions on the emulsions and NASA

281
00:12:42,440 --> 00:12:38,140
for funding thank you for your attention

282
00:12:44,580 --> 00:12:42,450
[Applause]

283
00:12:46,710 --> 00:12:44,590
thank you much Julie I can really

284
00:12:48,540 --> 00:12:46,720
appreciate having having one student

285
00:12:50,040 --> 00:12:48,550
work really hard to generate the system

286
00:12:51,330 --> 00:12:50,050
and someone else having to having to

287
00:13:31,110 --> 00:12:51,340
come in and then follow it up to do the

288
00:13:41,230 --> 00:13:39,160

yes so of those clones I showed you you

289
00:13:44,439 --> 00:13:41,240
notice that the most in which cluster

290
00:13:46,420 --> 00:13:44,449
one and two they are not as active in

291
00:13:49,749 --> 00:13:46,430
this specific essay and in this specific

292
00:13:51,790 --> 00:13:49,759
essay we were detaching this randomized

293
00:13:54,660 --> 00:13:51,800
region of the pool from the annealing

294
00:13:58,689 --> 00:13:54,670
region to the polymerase I was I'm so

295
00:14:01,389 --> 00:13:58,699
this cluster five seems to do well the

296
00:14:03,850 --> 00:14:01,399
other clusters are more active in a

297
00:14:07,210 --> 00:14:03,860
different essay so the answer to your

298
00:14:08,800 --> 00:14:07,220
question is yes this many of these pool

299
00:14:11,679 --> 00:14:08,810
molecules seem to have developed a

300
00:14:14,379 --> 00:14:11,689
dependence we will probably go with this

301
00:14:16,509 --> 00:14:14,389
ribozyme because optimizing and cutting

302
00:14:19,090 --> 00:14:16,519
down to the catalytic core of a ribozyme

303
00:14:21,790 --> 00:14:19,100
that is dependent on another 200

304
00:14:23,860 --> 00:14:21,800
nucleotide ribozyme is a pain and we

305
00:14:25,780 --> 00:14:23,870
would like to have a small ribozyme that

306
00:14:28,929 --> 00:14:25,790
hopefully can be optimized to be highly

307
00:14:31,119 --> 00:14:28,939
active and so your follow-up question

308
00:14:33,100 --> 00:14:31,129
was with non canonic with canonical

309
00:14:38,410 --> 00:14:33,110
nucleoside triphosphates is that correct

310
00:14:44,060 --> 00:14:42,079
what kind of and the turnover is

311
00:14:49,010 --> 00:14:44,070
currently quite low we haven't really

312
00:14:54,110 --> 00:14:49,020
characterized that yet it's I think less

313
00:14:56,050 --> 00:14:54,120

than 10 at at this point to stay on time

314
00:14:58,490 --> 00:14:56,060
why don't we move on thank you very much